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ABSTRACT OF THESIS

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ame of Candidate EBRAHIM KHALIL NADERALI

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tle of Thesis INVESTIGATIONS INTO THE INTRACELLULAR MECHANISMS INVOLVED IN PROSTAGLANDIN SYNTHESIS BY AND RELEASE FROM THE GUINEA-PIG UTERUS.

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Caffeine (10 mM) stimulated the outputs of prostaglandin (PG) $F_{2\alpha}$, PGE_2 and PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) from the day 7 and day 15 guinea-pig uterus superfused *in vitro*. Caffeine-induced PG production was unaffected by the removal of extracellular calcium, ryanodine (RY) and ruthenium red (inhibitors of calcium release from ryanodine receptor (RYR) channel), and calmodulin inhibitors (W-7 and trifluoperazine (TFP)). In fact, W-7 greatly potentiated the caffeine effect on $PGF_{2\alpha}$ output. TMB-8, an intracellular calcium antagonist, inhibited the increase in $PGF_{2\alpha}$ output produced by caffeine without preventing the increases in outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$. Caffeine (1 mM but not 0.1 mM) and theophylline (Theo; 10 mM) also stimulated PG outputs from the day 7 guinea-pig uterus superfused *in vitro*. Caffeine and RY both stimulated prostaglandin production from the perfused mesenteric vascular bed of the rat.

Caffeine (10 mM) stimulated the output of $PGF_{2\alpha}$ after 8 h, and the output of 6-keto-$PGF_{1\alpha}$ after 2, 8 and 24 h from the day 7 guinea-pig endometrium in culture. Caffeine (10 mM) inhibited $PGF_{2\alpha}$ output after 2 h, but stimulated $PGF_{2\alpha}$ output after 24 h from the day 15 guinea-pig endometrium in culture.

The amounts of PG released from cultured epithelial cells were between 100- to 200-fold and 300- to 1000-fold higher than those from cultured stromal cells from the day 7 and day 15 endometrium, respectively. Outputs of all three PGs from epithelial cells, but not from stromal cells, were 4.4- to 5.5-fold higher from cells obtained on day 15 than on day 7. $PGF_{2\alpha}$ was the major PG released from the epithelial cells obtained from day 15 guinea-pig uterus. Caffeine had both a stimulatory and an inhibitory effect on PG output from endometrial cells cultured from the day 7 and day 15 guinea-pig uterus. It inhibited the output of $PGF_{2\alpha}$ from the epithelial cells after 8 and 24 h of culture, and stimulated PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the stromal cells after 2 h of cell culture. Ryanodine had no effect on the actions of caffeine, whereas dantrolene (a modulator of calcium release from RYR-channel) inhibited the caffeine-induced decrease in $PGF_{2\alpha}$ output from the epithelial cells and potentiated the stimulatory effects of caffeine on PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from cultured stromal cells. When changes in intracellular calcium levels of the epithelial and stromal cells were measured, caffeine did not increase the intracellular calcium concentrations ($[Ca^{2+}]_i$). In fact it caused a reduction in the $[Ca^{2+}]_i$ of both the epithelial and stromal cells. All of these findings indicate that caffeine stimulates PG output from the guinea-pig uterus in a time-dependent manner, and this stimulatory effect of caffeine is not dependent on the presence of extracellular calcium and is not mediated via calmodulin. It also appears that the stimulatory effect of caffeine on PG release does not involve mobilisation of calcium from an internal calcium store.

Calcium ionophore (A23187) stimulated $PGF_{2\alpha}$ output from the cultured epithelial and stromal cells after 2 h and inhibited $PGF_{2\alpha}$ output after 8 and 24 h culture. It also inhibited 6-keto-$PGF_{1\alpha}$ output from the stromal cells after 8 and 24 h culture. Phospholipase A_2 (PLA_2) stimulated the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from eipthelial and stromal cells after 24 h of cell culture. PLA_2 also stimulated the output of PGE_2 from epithelial and stromal cells after 2 h and 8 h of cell culture. Melittin (a PLA_2 stimulator) stimulated the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ after 2 h and $PGF_{2\alpha}$ output after 2 and 24 h of epithelial cells culture. Aristolochic acid (a PLA_2 inhibitor) inhibited $PGF_{2\alpha}$ output from the epithelial cells after 8 and 24 h of culture. It also inhibited 6-keto-$PGF_{1\alpha}$ output from the stromal cells after 8 h culture. Interestingly aristolochic acid significantly stimulated PGE_2 output from the stromal cells after 8 h culture. Oestradiol-17β (3.7 μM) and progesterone (3.7 μM) both caused an inhibition of $PGF_{2\alpha}$ output from the epithelial cells after 8 and 24 h culture and to a much lesser extent PGE_2 output from the stromal cells after 8 h culture. These findings indicate that prostaglandin production by the guinea-pig uterus is controlled by different mechanisms.

A novel cyclooxygenase II (COX-2) inhibitor, NS-398, inhibited PG production by day 7 and day 15 guinea-pig endometrium homogenates, by day 7 endometrium and myometrium in culture, and by cultured epithelial and stromal cells obtained from day 7 guinea-pig endometrium. Western blotting analyses showed that the prostaglandin H synthase-2 (Mr 72 KDa) was present in the soluble extracts of guinea-pig endometrium obtained on both days 6 and 17 of the oestrous cycle.

Investigation into the intracellular mechanisms involved in prostaglandin synthesis by and release from the guinea-pig uterus.

By

Ebrahim Khalil Naderali

Thesis presented for the degree of

Doctor of Philosophy

University of Edinburgh

1995

DEDICATED TO BABA, MAMA, BUJI AND DADASH WHOSE HARD WORK AND UNSELFISH SACRIFICES MADE IT ALL POSSIBLE.

In accordance with the requirements of regulation 3.4.7 this thesis has been composed by myself and the work presented herein is my own.

Ebrahim Khalil Naderali

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CONTENTS

SECTION TWO

SECTION ONE

GENERAL INTRODUCTION

1.1 PROSTAGLANDINS

1.1.1 History and Discovery

Prostaglandins attracted the attention of scientists long before their structures were identified. In 1930, American gynaecologists, Kurzrok and Lieb, reported that fresh human semen caused contraction and relaxation of strips of human uterus *in vitro*. Almost five years later von Euler (1935) reported a smooth muscle contractile action of extracts obtained from the prostatic and related glands of human, monkey, sheep and goat, and of extracts from sheep seminal vesicles. This action was also reported independently by another scientist (Goldblatt, 1935). All these extracts were reported to be acidic and lipid soluble (von Euler, 1936). Since von Euler (1935) first identified this substance from the prostate gland, he termed it "prostaglandin" (von Euler, 1939). He described prostaglandin as a lipid-soluble, unsaturated, hydroxy fatty acid.

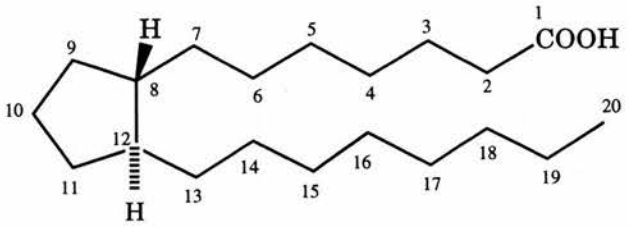
A decade later, a Swedish scientist (Bergström, 1949) confirmed the chemical nature of prostaglandin proposed by von Euler. Bergström (1949) also reported that seminal fluid extract contained more than one prostaglandin. Almost another decade passed before prostaglandin was actually isolated. Bergström and Sjovall (1957) finally succeeded in isolating two prostaglandins from sheep vesicular

glands, which these prostaglandins are known today as prostaglandin (PG) E_1 and $F_{1\alpha}$. These structures were confirmed by the use of mass spectrometry by Bergström *et al.* in 1963. PGE_1 and $PGF_{1\alpha}$ were also identified in extracts from sheep prostate gland (Bergström & Sjovall, 1960a, b). Eliasson (1959) showed that the seminal fluid prostaglandins originate from the seminal vesicles and not from the prostate gland. Since these initial studies, many prostaglandins have been isolated and identified, including the related compounds, namely the thromboxanes (TXs; Hamberg *et al.*, 1975).

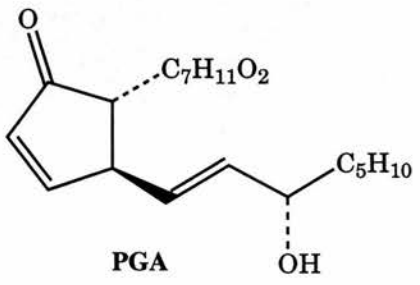
1.1.2 Structure and Chemistry of Prostaglandins

Prostaglandins were found to be a family of structurally related compounds but with a wide range of biological activities ranging, even within one tissue, from contraction to relaxation. From the family of prostaglandins, the structures of PGE_1 and $PGF_{1\alpha}$ were the first to be identified (Bergström *et al.*, 1963). Prostaglandins are 20 carbon-containing, unsaturated fatty acids consisting of a cyclopentane ring and two side chains of 7 and 8 carbons (prostanoid skeleton), respectively (Figure 1.1.2.1). Prostaglandins are classified into nine categories with respect to groups attached to the cyclopentane ring and to the side chain, namely PGA, PGB, PGC, PGD, PGE, PGF, PGG, PGH, and PGI. Thromboxane, a prostaglandin related compound, possesses a different ring structure in that an additional oxygen atom is enclosed in the ring.

The nine categories of prostaglandin and thromboxane mentioned above are further classified into three sub-groups according to the number of double bonds present in



PROSTANOIC ACID



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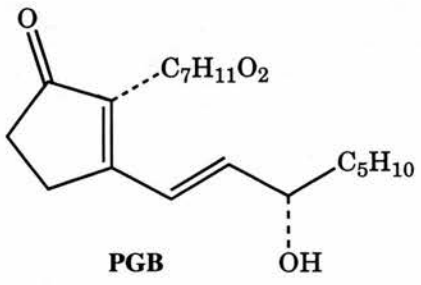

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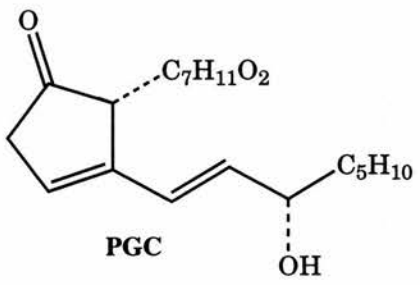

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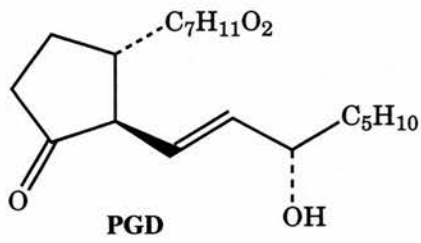
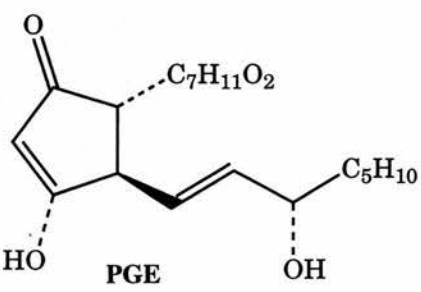
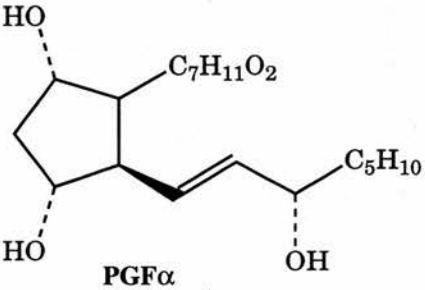


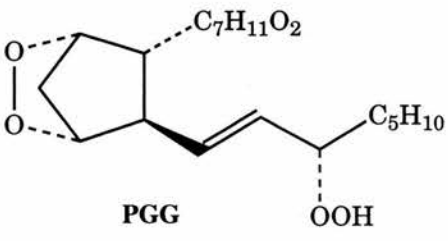
PGD



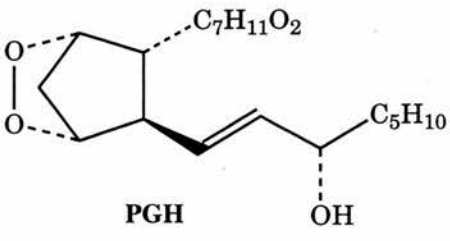
PGE



PGFα



PGG



PGH

Figure 1.1.2.1 Structure of prostanoic acid and structural differences between prostaglandin A to H. The structure for prostaglandin (PGI_2) is shown in Figure 1.1.2.2.

the prostanoid skeleton, and the series classification of any given prostaglandin is denoted by the subscript numbers 1, 2 or 3 placed after the prostaglandin category letter. Prostaglandins of " 2-series", or the dienoic prostaglandins, are the most important biologically, since they are usually the most abundant series formed. They have double bonds between carbons 5 and 6 and between carbons 13 and 14 (Figure 1.1.2.2).

1.1.3 Prostaglandin Biosynthesis and Metabolism

Prostaglandins differ from all the known locally produced mediators in many ways. Firstly, almost all cell types, except erythrocytes, have the capability of synthesising prostaglandins (Christ & Van Dorp, 1972). Secondly, with the exception of the reserve found in seminal fluid, prostaglandins are not normally stored but their release is *de novo*, that is they are synthesised immediately prior to their release from the cell (Piper & Vane, 1971). Thirdly, prostaglandins have a very short half-life since they are rapidly metabolised by most tissues, particularly the lungs, liver and kidney.

All prostaglandins are synthesised from three essential unsaturated fatty acids which, respectively, give rise to the three series of prostaglandins named earlier (see structure and chemistry of prostaglandins). The precursor for the 2-series prostaglandins is 5,8,11,14-all-cis-eicosatetraenoic acid (better known as arachidonic acid (AA)) which can be obtained from the diet or synthesised from the essential fatty acid, linoleate, by chain elongation and desaturation. Arachidonic acid is released by the action of phospholipase A_2 (PLA_2) from its phospholipid

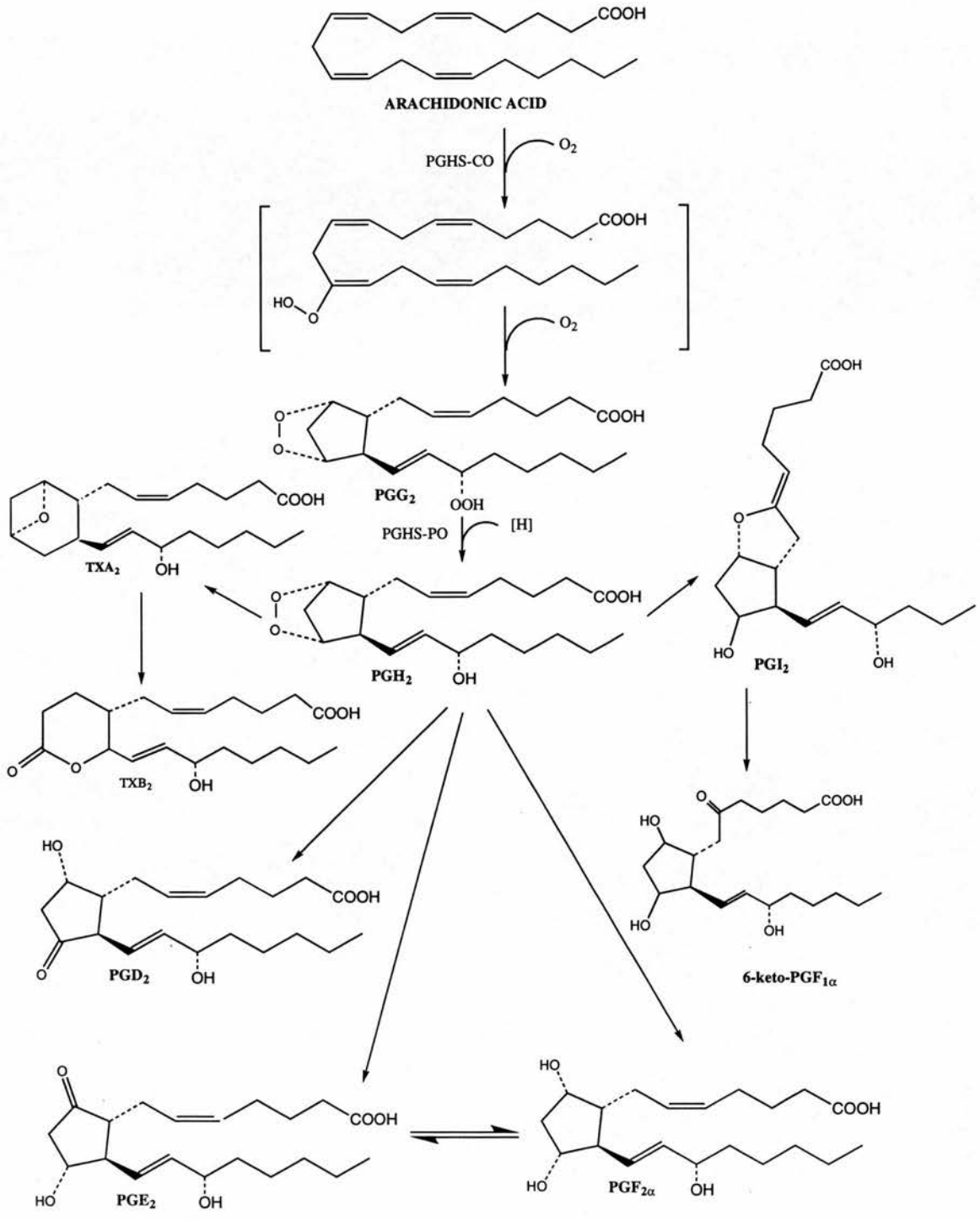


Figure 1.1.2.2. Major pathways of biosynthesis of prostaglandins (PG) and thromboxanes of the '2' series. PGHS-CO: cyclooxygenase component of prostaglandin H synthase enzyme complex. PGHS-PO: peroxidase componenet of prostaglandin H synthase enzyme complex. 12-L-hydroxy-5,8,10-heptadecatrienoic (HHT) and malondialdehyde (MDA) may also be formed from PGH_2.

conjugates. Arachidonic acid is then converted to PGH_2 by the action of prostaglandin H synthase (PGHS). This enzyme (i.e. PGHS) is a holoenzyme which contains two different forms of enzymatic activity, namely a cyclooxygenase (COX) component and a hydroperoxidase component (Miyamoto *et al.*, 1974). The cyclooxygenase component, which requires both tryptophan and haemoglobin, catalyses the bis-dioxygenation of arachidonate to the cyclic endoperoxide and 15-hydroperoxide derivative, namely PGG_2. PGG_2 is then rapidly converted to PGH_2 by the hydroperoxidase component (Miyamoto *et al.*, 1974). By 1976, the isolation and purification of prostaglandin endoperoxide synthetase complex was achieved. The prostaglandin synthetase system of bovine vesicular gland microsomes was solubilized and separated into Fractions I and II. Incubation of Fraction I with 8,11,14-eicosatrienoic acid, in the presence of hematin, resulted in the formation of PGG_1. The enzyme in Fraction I further catalysed PGG_1 to PGH_1 when heme and tryptophan were supplied. Fraction II catalysed PGH_1 to PGE_1 in the presence of glutathione (Miyamoto *et al.*, 1976). PGH_2 is the key intermediate in the biosynthesis of the prostaglandins and thromboxanes, and the final form of prostaglandin produced is dependent upon the cell type (Samuelsson *et al.*, 1978). More recently, it has been shown that there is more than one type of PGHS, namely PGHS-1 (a constitutive enzyme) and PGHS-2 (an inducible enzyme) (Hedin *et al.*, 1987; Wong & Richards, 1991). PGHS-1 from ram seminal vesicles is encoded by a 2.8-kb mRNA and PGHS-2 by a 4-kb mRNA (Lee *et al.*, 1992). Selective expression of mitogen-inducible prostaglandin H synthase-2 (PGHS-2) in macrophages stimulated by lipopolysaccharide (LPS) has shown by Lee *et al.*

(1992). They reported that the LPS-selective induction of PGHS-2 was completely inhibited by dexamethasone, whereas PGHS-1 was unaffected. Prostaglandin formation can be prevented by the glucocorticoids and by the non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin. Steroids inhibit the release of AA by stimulating the formation and release of lipocortin which reduces PLA_2 activity (Raz *et al.*, 1989), whereas NSAIDs inhibit the cyclooxygenase component of PGHS (Miyamoto *et al.*, 1976). PLA_2 activity in the plasma membrane and microsomes (i.e. membrane bound) is calcium-dependent (Newkirk & Wait, 1973) and is inhibited by ethylene diamine tetra-acetic acid (EDTA; McMurray & Magee, 1972).

Whilst most tissues are capable of metabolising PGs, the major site for inactivation of PGs in the blood is the lungs. Almost all of the $PGF_{2\alpha}$ and PGE_2 in the circulation are metabolised following just one passage of the blood through the lungs (Ferriera & Vane, 1967). In the case of prostacyclin (PGI_2) and thromboxane A_2 (TXA_2), the highly labile nature of these eicosanoids in water results in a rapid spontaneous formation of 6-keto-$PGF_{1\alpha}$ and TXB_2, respectively (Hamberg *et al.*, 1975; Johnson *et al.*, 1976).

From the initial research carried out by von Euler in 1935, the physiological importance of PGs in many tissues has attracted the attention of many scientists. One place where PGs play an important role is the female reproductive system (see Lange, 1984; see Poyser, 1981). In many non-primate mammalian species, the life-span of the corpus luteum, and consequently the length of oestrous cycle, is

dictated by the uterus. As a consequence the uterus has been studied extensively in this respect.

1.1.4 Uterine Prostaglandins

Loeb (1923) was the first to demonstrate the importance of the uterus on the life-span of the corpus luteum and hence on oestrous cycle length. He reported that the excision of this organ from the guinea-pig prevented the regression of the corpus luteum, and markedly increased the length of the oestrous cycle. A few years later, Loeb (1927) observed that the removal of the unregressed corpus luteum but not of the uterus resulted in a new ovulation. This observation led Loeb (1927) to hypothesis that the guinea-pig uterus secretes a luteolytic factor which effects the corpus luteum.

The prolongation of corpus luteum life-span after hysterectomy was demonstrated in other species, including the pseudopregnant rabbit (Asdell & Hammond, 1933), pseudopregnant rat (Bradbury, 1937), sheep, cow (Wiltbank & Casida, 1956) and pig (Spies *et al.*, 1958). Moreover, destruction of the endometrial layer of the guinea-pig uterus was shown to result in luteal maintenance and a prolonged oestrous cycle (Butcher *et al.*, 1962). However, the corpus luteum life-span after hysterectomy was not affected in the dog (Cheval, 1934) and human (Jones & Telinde, 1961; Beling *et al.*, 1970; Doyle *et al.*, 1971), indicating that in these species the uterus is not involved in the maintenance of the corpus luteum so does not dictate the length of the oestrous or menstrual cycle.

The involvement in luteolysis of a vasoconstrictor substance secreted from the uterus such as $PGF_{2\alpha}$ (Ducharme *et al.*, 1968) was suggested by Pharriss and Wyndgarden (1969). They demonstrated that infusion of $PGF_{2\alpha}$ into pseudopregnant rats causes luteolysis. At the same time, other researchers independently reported that $PGF_{2\alpha}$ also shortened the life-span of the corpus luteum in many other animals, including the guinea-pig (Blatchley & Donovan, 1969), rabbit (Gutknecht *et al.*, 1969), ewe (McCracken *et al.*, 1970), monkey (Kirton, *et al.*, 1972), mare (Douglas & Ginther, 1972) and goat (Ott, *et al.*, 1980), but not in the ferret (Blatchley & Donovan, 1973). Studies regarding the effect of $PGF_{2\alpha}$ on luteolysis in the human have proved to be somewhat equivocal. Lehmann *et al.* (1972) reported that the intravenous infusion of $PGF_{2\alpha}$ on day 21 of the menstrual cycle led to a sharp decline in the plasma progesterone concentration, but other studies reported that the infusion of $PGF_{2\alpha}$ did not affect the length of the luteal phase, or the pattern of steroid secretion in cycling women or in pregnant women prior to abortion (Le Maire & Shapiro, 1972; Speroff *et al.*, 1972).

In those animals in which $PGF_{2\alpha}$ administration mimics the effect of the uterine luteolytic hormone on the ovaries, it was shown that $PGF_{2\alpha}$, at a time when the corpora lutea had become fully established and functional, not only caused structural regression of the corpus luteum (morphological luteolysis), but also resulted in a drop in ovarian progesterone output (functional luteolysis). Both of these effects (i.e. morphological and functional luteolysis) were considered as the preconditions for any substance to be a candidate for the "luteolytic hormone". In addition, it was shown that the $PGF_{2\alpha}$ concentration increases in uterine venous

blood prior to the time of normal luteal regression in sheep (Bland *et al.*, 1971), pig (Gleeson & Thorburn, 1973), cow (Nancarrow *et al.*, 1973), and guinea-pig (Blatchley *et al.*, 1972), and that this measured concentration was capable of reducing plasma progesterone levels (McCracken *et al.*, 1970). In the guinea-pig, the increase in uterine venous plasma $PGF_{2\alpha}$ concentration takes place from about day 11 of the oestrous cycle, and by day 15 of the cycle the plasma $PGF_{2\alpha}$ concentration has increased significantly and the plasma concentration of progesterone has decreased (Blatchley *et al.*, 1972). Elevated levels of $PGF_{2\alpha}$ were maintained throughout the period of luteal regression. The accelerated decrease in peripheral plasma progesterone levels on day 17 in pseudopregnant rabbits was found to be associated with an increase in $PGF_{2\alpha}$ levels in uterine venous plasma (Lytton & Poyser, 1982).

In many species, the uterine horn effects the corpora lutea in the ipsilateral ovary but not the corpora lutea in the contralateral ovary. It is reported that in hemi-hysterectomised sheep (Inskeep & Butcher, 1966), guinea-pig (Bland & Donovan, 1966) and rat (Barley *et al.*, 1966), the luteolytic factor affects the corpora lutea only in the ovary adjacent to the retained uterine horn and that normal luteal regression occurs in the ovary contralateral to the hemi-hysterectomy.

McCracken (1971) reported a local action for $PGF_{2\alpha}$. He reported that a dose of $PGF_{2\alpha}$, which was luteolytic when administered directly into the ovary, had no effect on progesterone secretion when administered systemically. Different effects of $PGF_{2\alpha}$ infusion were also demonstrated by Liehr *et al.* (1972). They showed that the infusion of $PGF_{2\alpha}$ into the uterine horn ipsilateral to the ovary bearing the

corpus luteum has a different effect when compared to the infusion of $PGF_{2\alpha}$ into the uterine horn contralateral to the ovary bearing the corpus luteum. $PGF_{2\alpha}$ administered to cyclic heifers on day 9 resulted in an average cycle length of 11.4 days when infused into the uterine horn ipsilateral to the ovary bearing the corpus luteum, but 15.2 days when infused into the uterine horn contralateral to the ovary bearing the corpus luteum (Liehr *et al.*, 1972). Immunisation against $PGF_{2\alpha}$ prevented luteal regression in sheep (Scaramuzzi *et al.*, 1973) and lengthened oestrous cycle in guinea-pigs (Horton & Poyser, 1974). Poyser and Horton (1975) also showed that the intrauterine administration of a NSAID (indomethacin) to guinea-pigs resulted in increased progesterone concentrations being maintained for much longer than normal, suggesting that luteal regression had been prevented. All of the above findings identified $PGF_{2\alpha}$ as the " uterine luteolytic hormone".

1.1.5 Transfer of Prostaglandin $F_{2\alpha}$ From The Uterus to The Ovary

It is logical to assume that, for uterine $PGF_{2\alpha}$ to exert a local luteolytic effect on the corpus luteum, there must be a local mechanism by which $PGF_{2\alpha}$ is transferred to the ovary. Bland and Donovan (1969) reported that, in the guinea-pig, ligation of the vascular tissues to the uterus and ovaries, but not the ligation of the oviduct, prevents regression of the corpus luteum. A similar effect was found in the rat (Clemens *et al.*, 1968), hamster (Orsini, 1968) and sheep (Kiracofe *et al.*, 1966). These results suggested that the vascular system is the means by which $PGF_{2\alpha}$ travels from the uterus to the ovaries. A study in sheep showed that the structural arrangement of the vascular system, in particular the position of the ovarian artery

which runs over the surface of the utero-ovarian vein, is of prime importance in the transport of $PGF_{2\alpha}$, since separation of these vessels resulted in luteal maintenance (Barrett *et al.*, 1971). Infusion of $PGF_{2\alpha}$ labelled with radioactivity into the uterine vein of sheep at a point before its merger with the utero-ovarian resulted in the appearance of radioactive $PGF_{2\alpha}$ in the blood passing through the ovarian artery, but not in the blood passing through the iliac artery (McCracken *et al.*, 1972). As a result, a counter-current transfer mechanism between the utero-ovarian vein and ovarian artery was postulated for $PGF_{2\alpha}$ transport from the uterus to the ovary. However, only 5% of the labelled $PGF_{2\alpha}$ infused into the uterine vein was transported directly into the ovarian artery. Horton & Poyser (1976) suggested that, if the efficiency of the counter-current mechanism of transfer of $PGF_{2\alpha}$ from the uterine vein to the ovarian artery was too high, the levels of $PGF_{2\alpha}$ in the system would increase and remain high, since most of the tissues involved are incapable of metabolising prostaglandins. Consequently, if this transfer process were 100% efficient and no prostaglandin metabolism took place, then there would be a continuous passage of increasing amounts of $PGF_{2\alpha}$ through the ovary. Thus, under normal conditions of low $PGF_{2\alpha}$ transfer efficiency, the $PGF_{2\alpha}$ that is not retained by the counter-current mechanism will pass into the systemic circulation and be inactivated in the lungs (Ferreria & Vane, 1967).

Studies in the rabbit (Hunter & Casida, 1967), mare (Del Campo & Ginther, 1973) and guinea-pig (Egund & Carter, 1974) failed to show a counter-current mechanism of transferring $PGF_{2\alpha}$ between the uterus and ovary as proposed for the sheep. Abdel Rahim *et al.* (1984) proposed that, in sheep, as well as the transfer of

$PGF_{2\alpha}$ between the uterine vein and the ovarian artery, the transfer of $PGF_{2\alpha}$ between uterine lymph vessels and the ovarian artery is necessary for complete luteolysis. They showed that luteal function in the ewes was extended when the uterine vein was cannulated and all other direct connections between the uterine horn and the ipsilateral ovary (namely, oviduct and accompanying vessels, broad ligament, nerves, lymphatics and arteries) were surgically separated (Abdel Rahim *et al.*, 1984). The counter-current transfer of $PGF_{2\alpha}$ between the utero-ovarian vein and artery was not impaired. This experiment indicated that uterine venous blood alone cannot account for the luteolytic factor passing from the uterus to the ipsilateral ovary. The presence of $PGF_{2\alpha}$ in the lymph draining from the sheep uterus at concentrations comparable to those on the uterine venous blood has also been shown (Abdel Rahim *et al.*, 1983). There is a significant increase in $PGF_{2\alpha}$ concentrations in uterine lymph towards the end of the oestrous cycle at the time when luteal regression is taking place (Abdel Rahim *et al.*, 1983). Labelled $PGF_{2\alpha}$ infused into the uterine lymph vessel is recovered in the adjacent ovarian artery (Heap *et al.*, 1985). The efficiency of this transfer system was reported to be similar to the efficiency of transfer of $PGF_{2\alpha}$ from the uterine vein into the ovarian artery in sheep. These results clearly demonstrated that, in sheep, the transport of uterine $PGF_{2\alpha}$ to the ovary takes place via both the uterine lymphatic system and the uterine vascular system.

The precise means of transferring uterine $PGF_{2\alpha}$ to the ovary in the guinea-pig, rabbit, hamster, rat and mare still is not fully understood. In these species, extremely limited contact is found between the uterine vein and the ovarian artery

(Del Campo & Ginther, 1972, 1973). In the pig, the ovarian artery is located between two of the three channels of the utero-ovarian vein (Ginther, 1974), indicating that the vascular anatomy of the pig is compatible with a local veno-arterial pathway. However, studies by Harrison and Heap (1972) suggested that $PGF_{2\alpha}$ acts systemically in pig. They demonstrated that autotransplantation of the left ovary to the right side of the abdominal cavity and removal of the right ovary had no effect on the length of the oestrous cycle in the pig. Furthermore, infusion of $PGF_{2\alpha}$ into one anterior uterine vein on day 14 of the pig oestrous cycle caused a decrease in progesterone concentrations in both the ipsilateral and contralateral utero-ovarian veins. Nevertheless, the infusion of $PGF_{2\alpha}$ into the jugular vein of the pig did not cause luteolysis (Kotwica, 1980). This suggested that the lymphatic system is possibly involved in the transfer of $PGF_{2\alpha}$ within the sow's reproductive system, since there is a rich lymphatic system in the sow uterus (Kotwica, 1980).

1.1.6 Mode of Release of Prostaglandin $F_{2\alpha}$ From The Uterus

Interestingly, the release of $PGF_{2\alpha}$ from the uterus of several species is pulsatile in nature (see Poyser, 1981). This effect was best demonstrated by Kindhal *et al.* (1981) on $PGF_{2\alpha}$ release from the uterus in heifers. They measured the plasma concentration of the main metabolite of the $PGF_{2\alpha}$ (13,14-dihydro-15-keto-$PGF_{2\alpha}$; PGFM) at hourly intervals in heifers over a period of two to three days. They observed a pulsatile appearance of PGFM with a duration of 1-5 h, together with a corresponding decrease in plasma progesterone concentrations. The reason for this pulsatile nature of uterine $PGF_{2\alpha}$ release is not fully established yet. However,

Poyser (1991) has proposed that there is a pool of arachidonic acid (AA) in the endometrial cells of the uterus which may readily be released by the enzymatic action of PLA_2 resulting in depletion of this AA pool. This AA released is then rapidly converted to $PGF_{2\alpha}$. Poyser (1991) demonstrated that arachidonic acid (AA) and PLA_2 increased the output of $PGF_{2\alpha}$ from the guinea-pig uterus superfused *in vitro*. Similar increase in $PGF_{2\alpha}$ output was observed when AA treatment was repeated after an interval of 1, 3 or 5 h, but repeating the PLA2 treatment 1 h later failed to stimulate $PGF_{2\alpha}$ output. Furthermore, the uterine responsiveness to PLA_2 was partially restored after 3 h and was fully restored after 5 h. These finding led Poyser (1991) to propose that there is no failure with time in the mechanism which converts AA into $PGF_{2\alpha}$ in the guinea-pig uterus and that the refractoriness of uterine $PGF_{2\alpha}$ production following repeated stimulation by PLA_2 occurs at the level of AA release. The time which is required (3-5 h) to refill this releasable pool of arachidonic acid may be the factor responsible for the pulsatile release of uterine $PGF_{2\alpha}$.

1.1.7 Mechanism of Luteal Regression

It is clear that $PGF_{2\alpha}$ is the uterine luteolytic hormone in many non-primate mammalian species, and that $PGF_{2\alpha}$ is released from the uterus and then transported to its site of action (i.e. the ovary). The question then arises how precisely $PGF_{2\alpha}$ causes the regression of the corpus luteum? Based on the report by Ducharme *et al.* (1968) that $PGF_{2\alpha}$ is a vasoconstrictor, Pharriss *et al.* (1970)

suggested that $PGF_{2\alpha}$ causes vasoconstriction in blood vessels supplying the corpus luteum. This brings about luteal anoxia which in turn leads to luteal regression. However, infusion of $PGF_{2\alpha}$ directly into the ovary of the sheep did not cause a drop in total ovarian blood flow despite causing a rapid decline in progesterone output (McCracken *et al.*, 1970; Baird, 1974). Nevertheless, a selective restriction of the blood flow to the corpus luteum without effecting the total ovarian blood-flow by $PGF_{2\alpha}$ around the time of luteolysis in the sheep has been reported (Thorburn & Hales, 1972; Niswender *et al.*, 1975). However, this "vasoconstriction" hypothesis in not shared among all researchers (O'Shea *et al.*, 1977), especially as a fall in progesterone output from the corpus luteum prior to the reduction in luteal blood flow was observed in sheep (Einer-Jensen and McCracken 1977) and guinea-pig (Hossain *et al.*, 1979). Other mechanisms of action for $PGF_{2\alpha}$ have therefore been reported with respect to its luteolytic effect.

In many non-primate mammalian species, the corpus luteum is resistant to the luteolytic effect of $PGF_{2\alpha}$ during the early stage of the oestrous cycle. It is thought that during this refractory period, the corpus luteum develops 'susceptibility' to the luteolytic hormone (i.e. $PGF_{2\alpha}$). In the cow, binding of $PGF_{2\alpha}$ to the receptors on luteal cell membrane increases as the oestrous cycle progresses (Rao *et al.*, 1979). Existence of luteal $PGF_{2\alpha}$ receptors have also been reported in the sheep (Powell *et al.*, 1974a), mare (Kimball & Wyngarden, 1977), and rat (Wright *et al.*, 1979). It is postulated that an increase in the number and/or affinity of $PGF_{2\alpha}$ receptors may play a role in luteolysis brought about by the action of uterine $PGF_{2\alpha}$. Interestingly, $PGF_{2\alpha}$ receptors on human corpus luteum have also been observed (Powell *et al.*,

1974b). However, these human luteal receptors have only very low affinity for $PGF_{2\alpha}$, which may be the reason why this prostaglandin is not luteolytic in women (see Poyser, 1981).

Secretion of progesterone is brought about by the action of luteinizing hormone (LH) on luteal granulosa cells. The binding of LH to its receptor on luteal granulosa cells activates adenyl cyclase enzyme which in turn causes an increase in the amount of cyclic adenosine 3´-5´-monophosphate (cyclic-AMP; cAMP). Cyclic-AMP acts as a second messenger and stimulates the synthesis and release of progesterone. Previous experiments on rat and sheep have shown that $PGF_{2\alpha}$ rapidly blocks the LH-induced increase in cAMP levels of corpora lutea (Lahav *et al.*, 1976; Fletcher & Niswender, 1982). Luborsky *et al.* (1984a) reported that the LH-bound receptors aggregate, and that this receptor aggregation appears necessary for the activation of adenylate cyclase and cAMP production. $PGF_{2\alpha}$ inhibits LH-bound receptor aggregation and the stimulation of adenylate cyclase (Luborsky *et al.*, 1984a). Luborsky *et al.* (1984b) proposed that $PGF_{2\alpha}$ prevents "up-regulation" of the LH-induced LH receptors. He also reported that $PGF_{2\alpha}$ may effect luteolysis by reducing the binding of LH to its receptors, since $PGF_{2\alpha}$ has shown to reduce the affinity of LH for its receptors in rat (Behrman *et al.*, 1978) and sheep (Diekman *et al.*, 1978).

Prostaglandin $F_{2\alpha}$ inhibits the activities of several enzymes involved in the synthesis of progesterone from cholesterol (Dwyer & Church, 1979a, b). It has also been shown that $PGF_{2\alpha}$ inhibits progesterone production by cultured luteal cells (see Abayasekara *et al.*, 1993; Pitzel *et al.*, 1993). Dorflinger *et al.* (1984) reported

that the treatment of rat luteal cells with the calcium ionophore, A23187, inhibits LH-sensitive adenylate cyclase and subsequent LH-induced cAMP production. In rat luteal cells, $PGF_{2\alpha}$ stimulates the phosphatidylinositol (PI) cycle (Raymond *et al.*, 1983). Since inositol-1,4,5-trisphosphate (IP_3) mobilises intracellular calcium from an internal store (Berridge, 1984), the studies by Raymond *et al.* (1983) suggest that in luteal cells $PGF_{2\alpha}$ can release calcium from an intracellular store by stimulating the formation of IP_3. In the rat, it has been shown that $PGF_{2\alpha}$-induced luteal regression is associated with increased membrane rigidification which is dependent on protein, calcium and calmodulin (Riley and Carlson, 1985). Riley and Carlson (1985) speculated that a calcium-dependent, calmodulin-modulated mechanism causes phosphorylation of the membrane proteins and subsequent membrane rigidification which, in turn, prevents the movement and appearance of LH receptors and hence LH binding to its receptors in the luteal membrane.

1.1.8 Physiological Control of $PGF_{2\alpha}$ Synthesis by The Uterus

(a) Involvement of Oestrogen and Progesterone in Uterine $PGF_{2\alpha}$ Synthesis

Since uterine $PGF_{2\alpha}$ output changes during the oestrous cycle, it was thought that the ovarian steroids may have a regulatory effect on uterine prostaglandin production. Choudary and Greenwald (1968) demonstrated a luteolytic effect of oestrogen. A single subcutaneous injection of 10 or 50 μg of oestradiol cyclopentylpropionate (OECP) but not of progesterone or testosterone, on day 1 of the oestrous cycle induced premature luteolysis in cyclic guinea-pigs with

regression of the corpora lutea being complete by day 10 (Choudary & Greenwald, 1968). Thus the life-span of the corpora lutea was reduced by about 5 days, and the length of the oestrous cycle was decreased. Subcutaneous administration of oestrogen to guinea-pigs on day 7 of the cycle also causes premature luteolysis. This effect was abolished by hysterectomy (Bland & Donovan, 1970). In ewes, the injection of oestradiol on day 10 and day 11 of the oestrous cycle caused early luteal regression, but failed to affect corpus luteum function in hysterectomised ewes (Stormshak *et al.*, 1969). Oestrogen treatment reduced the weight of the corpus luteum in cycling, pregnant and hysterectomised heifers, but progesterone concentrations were consistently higher in the hysterectomised heifers compared to the cycling and pregnant animals (Kaltenbach *et al.*, 1964). Therefore, the luteolytic effect of oestrogen is mediated via the uterus. Oestrogen treatment of guinea-pigs on days 4 to 6 of the oestrous cycle resulted in increased $PGF_{2\alpha}$ concentrations in the utero-ovarian venous blood on day 7 (Blatchley *et al.*, 1972), which accounts for the shortening of the luteal phase and of the oestrous cycle reported previously (Choudary & Greenwald, 1968; Bland & Donovan, 1968). These studies suggested that endogenous oestradiol released by the developing ovarian follicles after day 10 of the oestrous cycle is the physiological stimulus for the uterine $PGF_{2\alpha}$ synthesis.

It has been shown that progesterone treatment of ovariectomised guinea-pigs (Blatchley & Poyser, 1974) results in a $PGF_{2\alpha}$ concentration in the utero-ovarian venous plasma which is similar to the values observed between days 3 and 10 of the cycle (Blatchley *et al.*, 1972). Oestradiol treatment of ovariectomised guinea-

pigs alone resulted in a mean utero-ovarian venous plasma concentration of $PGF_{2\alpha}$ which was higher than that observed for progesterone treatment. However, this increase occurred only in two of the six animals treated with oestradiol. On the other hand, ovariectomised guinea-pigs treated with a progesterone for several days followed by 3 days of oestradiol and progesterone injections were shown to have the greatest concentration of $PGF_{2\alpha}$ in the utero-ovarian venous blood when compared to the ovariectomised guinea-pigs treated with progesterone or oestradiol alone (Blatchley & Poyser, 1974). Therefore, it was proposed that oestradiol acting on a progesterone-primed uterus was the physiological stimulus for the $PGF_{2\alpha}$ synthesis by and release from the guinea-pig uterus. Furthermore, it was shown that the output of oestradiol from the guinea-pig ovary increased after day 10 of the oestrous cycle (Joshi *et al.*, 1973), immediately preceding the rise in uterine $PGF_{2\alpha}$ output on day 11 (Blatchley *et al.*, 1972). In sheep, the plasma oestradiol concentration showed a short-lived rise between day 11 to 12 before the first peak of $PGF_{2\alpha}$ on day 13 (Cox *et al.*, 1974). The oestradiol concentrations then declined and the next major rise in oestradiol-17β occurred at pro-oestrus. A similar relationship between the release of ovarian oestradiol and subsequent increase in $PGF_{2\alpha}$ output from the uterus has also been shown in the pig (Henricks *et al.*, 1972), cow (Shemesh *et al.*, 1972) and rat (Welschen *et al.*, 1975).

Antonini *et al.* (1976) reported that $PGF_{2\alpha}$ output from the guinea-pig uterus *in vivo* increased without any changes in the outputs of PGE_2. The increased output of $PGF_{2\alpha}$ preceded a drop in progesterone levels in the non-pregnant animal (Antonini *et al.*, 1976). Furthermore, the output of $PGF_{2\alpha}$, but not the outputs of PGE_2 and

6-keto-$PGF_{1\alpha}$, from ovariectomised guinea-pig uteri superfused *in vitro* significantly increased in those guinea-pigs which were treated with progesterone for 7 days followed by 3 days of oestradiol plus progesterone when compared to ovariectomised guinea-pigs which had received progesterone or oestradiol alone (Poyser, 1983c). These findings suggest that the ovarian steroid hormones have a preferential stimulatory effect on $PGF_{2\alpha}$ synthesis by and release from the uterus.

In ovariectomised ewes, plasma $PGF_{2\alpha}$ concentration increased after oestradiol treatment following a sequence of progesterone injections (Caldwell *et al.*, 1972). It has also been reported that, in ovariectomised sheep, exogenous progesterone treatment for seven days significantly increased uterine venous plasma $PGF_{2\alpha}$ concentrations (Scaramuzzi *et al.*, 1977), whereas 14 day progesterone treatment caused a decrease in $PGF_{2\alpha}$ output from the sheep uterus compared to the output after 7 days of treatment. When ewes were infused with progesterone for ten days starting immediately after oestrus, stimulation of uterine $PGF_{2\alpha}$ output was seen as an increase in the number of $PGF_{2\alpha}$ peaks of low magnitude on days 9 or 10 of the treatment. The maximal stimulation of $PGF_{2\alpha}$ secretion from the sheep uterus occurred following the infusion of oestradiol after pre-treatment with progesterone (Scaramuzzi *et al.*, 1977).

The combined effect of progesterone and oestradiol treatment was also reported in mice. Saksena and Lau (1973) showed that there was a significant increase in the concentration of $PGF_{2\alpha}$ in uterine tissue of ovariectomised mice after 3 days of treatment with progesterone followed by 3 days of oestrogen treatment when compared with either treatment alone. All of these findings indicate that

progesterone-priming followed by oestradiol treatment is the optimum stimulus for increasing the output of $PGF_{2\alpha}$ from the uterus of several species.

It has been shown that the destruction of ovarian follicles by X-ray irradiation (Karsch *et al.*, 1970) or mechanically (Ginther, 1970) prevents normal luteal regression in the sheep and cow (Villa-Godoy *et al.*, 1985). These findings further support the theory that the release of ovarian oestradiol acting on a progesterone-primed uterus is the actual physiological stimulus for increased uterine $PGF_{2\alpha}$ synthesis at the end of the oestrous cycle. However, there is some controversy regarding the role of ovarian steroids in the stimulation of the uterine $PGF_{2\alpha}$ output from the sheep, since luteal regression was not delayed following destruction of ovarian follicles in another study (Warren *et al.*, 1973), or by the injection of an antisera to oestradiol (Fairclough *et al.*, 1976). Nevertheless, more recent studies using progesterone and oestrogen receptor antagonists have indicated the importance of progesterone and oestradiol in the stimulation of $PGF_{2\alpha}$ production by the uterus. The administration of onapristone (a progesterone receptor antagonist) or ICI 182780 (an oestrogen receptor antagonist) to guinea-pigs on days 11-14 of the cycle significantly reduced $PGF_{2\alpha}$ output on day 15 (Poyser, 1993). Concentrations of progesterone in the plasma of onapristone- and ICI 182780-treated guinea-pigs were still high on day 15 indicating that luteal regression had been prevented. In another study, the progesterone antagonist, mifepristone (RU 486), when injected i.m. into ewes during the early luteal phase of the oestrous cycle (i.e. on days 5, 6, 7 and 8) resulted in the failure of luteolysis and the maintenance of a functional corpus luteum to at least day 24 after oestrus (Morgan

et al., 1993). Furthermore, RU 486 treatment of sheep inhibited the plasma profile of $PGF_{2\alpha}$ (measured as PGFM) normally seen on days 12, 13, 14 and 15 (Morgan *et al.*, 1993).

Although $PGF_{2\alpha}$ is not a luteolytic hormone in human, prostaglandin may have a role in the female reproductive tract. A very recent study has reported the cloning of $PGF_{2\alpha}$ receptors from the human uterus (Lake *et al.*, 1994). In women, there is an increase in the amounts of $PGF_{2\alpha}$ and, to a lesser extent, PGE_2 produced by the endometrium at menstruation (Downie *et al.*, 1974; see Poyser, 1981). There is an increase in the concentration of $PGF_{2\alpha}$ in the human endometrium from the proliferative phase to the secretory phase, with peak levels being reached during menstruation (Downie *et al.*, 1974; see Lundström, 1986). PGE_2 concentrations increase only at menstruation. The ratio between the $PGF_{2\alpha}$ and PGE_2 concentrations is higher in the secretory and menstrual phases compared with the proliferative phase (Downie *et al.*, 1974). Since prostaglandins, in particular $PGF_{2\alpha}$, contract the uterus (see Lundström, 1986) and affect the vasculature, it is assumed that they have a role in the menstrual process. The plasma concentration of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) varies between 50-100 pg/ml in women with dysmenorrhoea. The oral administration of indomethacin decreased the plasma concentration of PGFM to less than 15 pg/ml (Lundström *et al.*, 1976). They also showed that the intravenous infusion of $PGF_{2\alpha}$ at a rate of 18 μg/ml induced severe dysmenorrhoeic pain and marked uterine hypertonicity. Lundström *et al.* (1976) speculated that an overproduction of $PGF_{2\alpha}$ by the endometrium is the probable cause of dysmenorrhoea. The concentrations of $PGF_{2\alpha}$ and PGE_2 in the menstrual

fluid of women suffering from dysmenorrhoea are much higher than normal (Lumsden *et al.*, 1983). Lumsden *et al.* (1983) have reported that the amounts of $PGF_{2\alpha}$ and PGE_2 in the menstrual fluid of women suffering from dysmenorrhoea are 710 ± 131 and 105 ± 23 ng/ml, respectively while the amounts of $PGF_{2\alpha}$ and PGE_2 in the menstrual fluid of control women are 153 ± 27 and 38 ± 5 ng/ml respectively. On the other hand, an excess output of prostacyclin (PGI_2) may be the cause of menorrhagia (Smith *et al.*, 1981). Smith *et al.* (1981) reported that production of $PGF_{2\alpha}$, PGE_2 and PGD_2 by cultured endometrium from women with excessive menstrual blood loss (57-186 ml) was similar to that observed for the endometrium of women with normal blood loss (5-50 ml). However, the endometrium from women with excessive menstrual blood loss was more effective than endometrium from women with normal menstrual blood loss at enhancing the production by the myometrium of 6-oxo-prostaglandin $F_{1\alpha}$, the stable metabolite of prostacyclin. The reasons for abnormal PG production in these disorders of menstruation are not yet known. However, non-steroidal anti-inflammatory drugs such as mefenamic acid, flufenamic acid (Anderson *et al.*, 1976; Haynes *et al.*, 1980) and indomethacin (Lundström *et al.*, 1976) reduce significantly the blood loss in patients with menorrhagia and the pain associated with dysmenorrhoea.

Progesterone (1 μM) suppressed the release of PGE from cells obtained from proliferative endometrium during the first 24 h in culture (Mitchell & Smith, 1992). After 48 h in culture, progesterone at concentrations of 100 nM and 1 μM suppressed both the release of $PGF_{2\alpha}$ and PGE_2 from the cells, an effect which lasted for a further 2 days. It has also been shown that progesterone treatment *in*

vitro inhibits $PGF_{2\alpha}$ output from both proliferative and secretory endometrium in culture (Cane & Villee, 1975; Abel & Baird, 1980; Schatz *et al.*, 1985). Oestradiol treatment, however, stimulates $PGF_{2\alpha}$ output from both proliferative and secretory human endometrium in culture and this stimulatory effect of oestradiol is overcome by progesterone treatment (Abel & Baird, 1980; Schatz *et al.*, 1985).

In the guinea-pig, $PGF_{2\alpha}$ output from the endometrium in culture was stimulated by oestradiol 17-β and inhibited by progesterone treatment *in vitro* (Leaver & Seawright, 1982). However, Riley and Poyser (1987a) reported that oestradiol does not stimulate endometrial $PGF_{2\alpha}$ output in culture. In fact, oestradiol at a high concentration (3.7 μM) *in vitro* inhibited $PGF_{2\alpha}$ output. An inhibitory effect of progesterone *in vitro* on $PGF_{2\alpha}$ production by cultured guinea-pig endometrium was also reported by Riley and Poyser (1987a). It is clear that, while treatment *in vivo* with oestradiol following progesterone priming stimulates $PGF_{2\alpha}$ production by the endometrium, the effect of oestradiol *in vitro* is variable and the effect of progesterone *in vitro* is inhibitory. Riley and Poyser (1987a) reported that hydrocortisone had no effect on endometrial PG production. These result led Riley and Poyser (1987a) to speculate that the inhibitory effect of progesterone on endometrial PG synthesis and release in the guinea-pig is not due to progesterone having a glucocorticoid-like action.

(b) The Role of Oxytocin in The Regulation of Uterine $PGF_{2\alpha}$ Synthesis

In some species, oxytocin is also involved in the control of the uterine $PGF_{2\alpha}$ production. In sheep, the infusion of oxytocin into the uterine artery after day 14

of the oestrous cycle caused a marked rise in uterine $PGF_{2\alpha}$ output (Roberts *et al.*, 1975). Flint and Sheldrick (1985) have also reported that the continuous infusion of oxytocin blocks luteal regression in cyclic ewes. This stimulatory effect of oxytocin on uterine $PGF_{2\alpha}$ output has also been shown in the rabbit (Small *et al.*, 1978), rat (Campos *et al.*, 1980), cow (Wathes *et al.*, 1983) and goat (Cooke & Homeida, 1985). It has been reported that, in sheep, the immunisation against oxytocin delays luteal regression (Flint *et al.*, 1979; Sheldrick *et al.*, 1980). In ewes, the stimulatory effect of oxytocin on uterine $PGF_{2\alpha}$ output is potentiated by oestradiol and progesterone (Sharma & Fitzpatrick, 1974; Homanics & Silvia, 1988). Therefore, in the sheep, oestradiol acting on a progesterone-primed uterus causes an initial increase in endometrial $PGF_{2\alpha}$ output which is subsequently maintained and increased further by oxytocin. However, the maximum responsiveness of the endometrium to oxytocin occurs after plasma progesterone levels have started to decline (Sheldrick & Flint, 1985), indicating that progesterone may also have an inhibitory effect on the action of oxytocin.

A similar result has been shown in the cow *in vivo*. Mann & Lamming (1995) studied the effects of oestradiol-17β on oxytocin-induced $PGF_{2\alpha}$ release using steroid-treated, ovariectomised cows. Long-term ovariectomised cows were pre-treated with progesterone for 14 days followed by oestradiol-17β for 2 days to induce oestrus (Day zero), and were then treated for further 16 days with physiological doses of progesterone and oestradiol-17β to simulate a luteal phase. On day 11, oestradiol was either withdrawn (low group) or maintained (normal group). Oxytocin was administered daily by the i.v. injection of 50 IU oxytocin

from days 12-16, and oxytocin-stimulated $PGF_{2\alpha}$ secretion was monitored by measuring the concentration of the principal metabolite of $PGF_{2\alpha}$ (PGFM), in plasma samples collected before and after the daily injections of oxytocin. In the "normal group", oxytocin significantly raised plasma PGFM concentrations following administration on days 12-16. In the "low group", significant elevations in plasma PGFM concentrations were only seen on days 15 and 16, and were significantly smaller than those seen in the normal group (Mann & Lamming, 1995). Thus, oxytocin causes greater production of $PGF_{2\alpha}$ by the cow uterus after treatment with physiological concentrations of both progesterone and oestradiol.

There are conflicting reports regarding the stimulatory effect of oxytocin on $PGF_{2\alpha}$ outputs from the guinea-pig uterus. It was reported that oxytocin stimulated the output of $PGF_{2\alpha}$ from the guinea-pig uterus at the end of the oestrous cycle (when basal $PGF_{2\alpha}$ output is high), but not at mid-cycle (when basal $PGF_{2\alpha}$ output is low) (Leaver & Seawright, 1982). They also reported that, in the presence of oestradiol-17β, oxytocin stimulated $PGF_{2\alpha}$ output at mid-cycle. However, Riley and Poyser (1987a) reported that oxytocin had no effect on the endometrial $PGF_{2\alpha}$ output at any stage of the cycle. Oxytocin was also shown to have no effect on the basal output of $PGF_{2\alpha}$ from the day 7 and day 15 guinea-pig uterus superfused *in vitro* (Poyser & Brydon, 1983). Thus the initial findings of Leaver and Seawright (1982) have not been confirmed. Furthermore, the injection of oxytocin into the guinea-pigs failed to affect the length of the oestrous cycle (Donovan, 1961). These finding suggest that oxytocin is not involved in the physiological control of uterine

$PGF_{2\alpha}$ output from the guinea-pig uterus, and that the role of oxytocin in controlling uterine $PGF_{2\alpha}$ output is species-dependent.

(c) Mechanism of Action of Steroids

In many tissues, including the uterus, steroids have been shown to exert their effect by stimulating protein synthesis (see Brenner & West, 1975). Oestradiol stimulates protein synthesis in the rat uterus (Kumar *et al.*, 1983; Komm *et al.*, 1986) and mouse uterus (Henrikson *et al.*, 1987). The importance of fresh protein synthesis in the uterus in the control of uterine $PGF_{2\alpha}$ output has been demonstrated in the sheep (French & Casida, 1973) and guinea-pig (Poyser, 1979; Poyser & Riley, 1987; Riley & Poyser, 1989). In the sheep, administration of actinomycin D (an inhibitor of DNA-dependent RNA synthesis) into the uterine lumen on day 11 of the oestrous cycle prevented luteal regression (French & Casida, 1973). In the guinea-pig, the intra-uterine administration of actinomycin D on day 10 of the oestrous cycle lengthened the oestrous cycle by two-fold and progesterone concentrations remained elevated throughout the duration of these cycles (Poyser, 1979). In another study, the intrauterine administration of actinomycin D on day 10 of the oestrous cycle reduced the output of $PGF_{2\alpha}$ from the day 15 uterine horn superfused *in vitro* (Poyser & Riley, 1987). Oestradiol treatment *in vivo* did not reverse the inhibitory effects of actinomycin D on uterine PG production. It has also been shown that actinomycin D inhibits $PGF_{2\alpha}$ output from guinea-pig endometrial tissue in culture, but not from guinea-pig myometrial tissue in culture (Riley & Poyser, 1989). Cycloheximide (an inhibitor of the elongation step of

protein transcription) and puromycin (a releaser of nascent polypeptide chains before their synthesis is complete) also reduced PG output from the day 7 and day 15 guinea-pig endometrium in culture (Riley & Poyser, 1989). All these findings suggest that $PGF_{2\alpha}$ synthesis by the endometrium is dependent upon the synthesis of fresh protein. These findings led Poyser and Riley (1987) to conclude that increased protein synthesis is involved in stimulating endometrial $PGF_{2\alpha}$ synthesis and release. As a result, it was proposed that oestradiol induces the synthesis of a protein ('lipostimulin') which, when acting on a progesterone-primed uterus, "switches on" endometrial $PGF_{2\alpha}$ synthesis and release by causing the activation of endometrial PLA_2 (Poyser & Riley, 1987).

Although it is clear that the endometrium is the source of increased $PGF_{2\alpha}$ output from the guinea-pig uterus, it is not known which cell type is involved. One aim of this study is to investigate whether the epithelial or stromal cells obtained from the guinea-pig endometrium are the main site of $PGF_{2\alpha}$ synthesis.

1.2. INVOLVEMENT OF PHOSPHOLIPASE ENZYMES, CALCIUM, CALMODULIN, PROTEIN KINASE C, cAMP, G-PROTEIN AND INOSITOL TRISPHOSPHATE IN UTERINE PROSTAGLANDIN PRODUCTION.

1.2.1 Phospholipase Enzymes and Calcium

The release of the bound arachidonic acid (AA) from the phospholipids is the rate limiting step in the synthesis of prostaglandins in tissues (Vogt, 1978). The major phospholipids include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin. In the guinea-pig uterus, it has been shown that 90% of the total AA is bound to phospholipids, and that of this proportion 80% is esterified as phosphatidylcholine and phosphatidylethanolamine (Leaver & Poyser, 1981). A greater uptake of AA into the phospholipids of the guinea-pig endometrium on day 15 compared to day 7 of the oestrous cycle (i.e. at the end of the oestrous cycle) was clearly demonstrated by Ning *et al.* (1983). This mechanism may be responsible for the compensation of the higher AA release from phospholipids at the end of the oestrous cycle.

Release of AA from phospholipids is brought about by the action of phospholipase enzymes. So far, four major phospholipase enzymes have been identified, namely phospholipase (PL) A_1, PLA_2, PLC and PLD (Dennis, 1983). It has been reported in the rat (Dey *et al.*, 1982), guinea-pig (Downing & Poyser, 1983) and human (Bonney, 1985) that an increase in endometrial prostaglandin synthesis is associated with an increase in PLA_2 activity. It has been shown that phospholipase A_2 activity

is a calcium-dependent, and has an optimum pH of 8-9 in women (Bonney, 1985), of 8.0 in guinea-pig (Downing & Poyser, 1983) and of 6.0 in the rat (Pakrasi *et al.*, 1983).

In the guinea-pig, the concentration of PLA_2 in the endometrium increases significantly by 1.4- to 1.8-fold between day 7 and day 16 of the cycle (Downing & Poyser, 1983). In women, the enzyme activity was low in the early proliferative phase tissue but rose significantly during the late proliferative phase (Bonney, 1985). Bonney (1985) also reported a 10-fold increase in PLA activity 2-4 days after ovulation which thereafter declined to reach values which at menstruation were not significantly different from those of the proliferative phase. These finding led Bonney (1985) to suggest that PLA_2 activity in human endometrium is related to the stage of the menstrual cycle and that arachidonic acid release may be influenced by oestradiol and progesterone. Later it was reported that oestradiol sulphate and testosterone sulphate, but not progesterone, stimulated PLA_2 activity *in vitro* in human endometrium by 2- to 13-fold. The effect was dose related, linear with the time, and independent of the stage of the menstrual cycle (Bonney & Frank, 1987b). Cultured human endometrium (Bonney *et al.*, 1987) treated with progesterone followed by oestradiol caused a stimulation of PLA_2 activity, which was greater than the increase in PLA_2 activity caused by oestradiol alone. These finding indicate a direct action of steroids on PLA_2 activity *in vitro*.

Stimulation of uterine PLA_2 activity by oestradiol has also been reported in the rat (Pakrasi *et al.*, 1983; Dey *et al.*, 1982). Uterine PLA_2 activity showed a 167-fold increase in ovariectomized animals treated with oestradiol-17β as compared to

those treated with saline-vehicle (Pakrasi *et al.*, 1983). Dexamethasone treatment reduced the stimulatory effect of oestradiol-17β on uterine PLA_2 activity. Also, the simultaneous treatment of rats with oestradiol-17β and progesterone significantly reduced the rise in uterine PLA_2 activity caused by oestradiol alone. Progesterone alone had no effect on uterine PLA_2 activity in ovariectomised rats (Pakrasi *et al.*, 1983).

Bonney *et al.* (1987) reported that there are two isoenzymes of PLA_2, namely PLA_2 type 1 and PLA_2 type 2. Phospholipase A_2 type 1 is a calcium-dependent enzyme located mainly in the glandular component of the endometrium and its activity increases in the secretory phase of the menstrual cycle. PLA_2 type 2 is a calcium-independent enzyme found in the stromal cells. These PLA_2 isoenzymes also differ with respect to their pH optimum, and their regulation by steroid hormones. PLA_2 type 1 is optimally active at pH 7.5 to 9, whereas PLA_2 type 2 is optimally active at pH 7.0. (Bonney *et al.*, 1987). Progesterone priming followed by treatment with oestradiol caused a 2-fold stimulation of PLA_2 type 1 but not PLA_2 type 2 (Bonney *et al.*, 1987). Melittin, a PLA_2 activator, stimulated the release of $PGF_{2\alpha}$ from ovine endometrial tissue in culture. Aristolochic acid, an inhibitor of PLA_2 activity, blocked melittin-induced $PGF_{2\alpha}$ production (Lee & Silvia, 1994).

Another important phospholipase is PLC. In platelets, a source of AA for PG synthesis was postulated to be phosphatidylinositol. The AA is released by the action of PLC and diacylglyceride lipase (Bell *et al.*, 1979). It has been shown that PLC is a calcium-dependent enzyme and has a pH optimum of 5.5 (Bonney &

Franks, 1987a). In human endometrium, PLC activity was independent of the stage of the menstrual cycle (Bonney & Franks, 1987a).

In the guinea-pig, Poyser (1987) reported that PLC as well as PLA_2 increased uterine prostaglandin output, in particular the output of PGE_2 from the day 7 and day 15 guinea-pig uterus superfused *in vitro*. What is important to note that the PLC used by Poyser (1987) was of bacterial origin which uses phosphatidylcholine as substrate rather than PLC of mammalian origin, which uses phosphatidylinositol (PI) as substrate. These data suggest that, in human and guinea-pig, PLC activity is not associated with increased uterine $PGF_{2\alpha}$ synthesis and release. However, Bonney & Franks (1987a) suggested that, in human, the PLC concentration may rise during certain pathological conditions associated with abnormally high PG output from the endometrium. A role for PLC in oxytocin-induced release of $PGF_{2\alpha}$ from ovine endometrial tissue was suggested by Flint *et al.* (1986). Silvia and Homanics (1988) also reported that oxytocin stimulates endometrial $PGF_{2\alpha}$ output and activates PLC. A similar finding was reported in another study (Silvia & Raw, 1993). Silvia and Raw (1993) reported, that in non-pregnant ewes, oxytocin stimulates the release of $PGF_{2\alpha}$ from endometrial tissue collected on days 14 and 16, but not on days 4 to 7, 10 or 12 after oestrus. The days on which oxytocin stimulated $PGF_{2\alpha}$ output also coincided with the days on which oxytocin stimulated the activity of PLC (Silvia & Raw, 1993). In a more recent study, it was shown that U-73122, a PLC inhibitor, blocked the ability of oxytocin to stimulate $PGF_{2\alpha}$ output from ovine endometrial tissue in culture but surprisingly failed to inhibit oxytocin-induced stimulation of PLC activity (Silvia *et al.*, 1994).

Aristolochic acid (an inhibitor of PLA_2) prevented the increased output of $PGF_{2\alpha}$ from ovine endometrium produced by oxytocin (Lee & Silvia, 1994). These findings indicate that oxytocin-induced increase in uterine $PGF_{2\alpha}$ release from ovine endometrium is mediated by PLA_2 and not by PLC.

Although the concentration of PLA_2 in guinea-pig endometrium increases up to 1.8-fold between days 7 and 16 of the oestrous cycle (Downing & Poyser, 1983), uterine $PGF_{2\alpha}$ output increases 22-fold between days 7 and 15 (Poyser & Brydon, 1983). This difference indicated that an increase in the activity of PLA_2 is not the sole factor controlling $PGF_{2\alpha}$ synthesis by the endometrium. Since PLA_2 is a calcium-requiring enzyme it was postulated that the activation of PLA_2 by calcium "switches on" $PGF_{2\alpha}$ synthesis in the uterus (Downing & Poyser, 1983). This activation of PLA_2 leads to the release of AA from phosphatidylcholine and phosphatidylethanolamine (Ning & Poyser, 1984) which, in turn, results in increased prostaglandin production, in particular $PGF_{2\alpha}$ synthesis by and release from the endometrium. The calcium ionophore (A23187) increases the output of prostaglandins from the guinea-pig uterus superfused *in vitro* (Poyser & Brydon, 1983) presumably by activating PLA_2, a calcium-requiring enzyme. Poyser (1984) reported that the presence of extracellular calcium is necessary for this stimulatory effect of A23187. However, the high basal output of $PGF_{2\alpha}$ from the day 15 guinea-pig uterus superfused *in vitro* was not dependent upon extracellular calcium for at least 150 min. This suggests that the release of calcium from an internal store may be important in PG synthesis towards the end of the oestrous cycle. The intracellular calcium antagonist, 8-(N,N,-diethylamino)octyl-3,4,5-trimethoxy-

benzoate hydrochloride, (TMB-8) inhibited the A23187-induced rise in uterine PG output (Poyser, 1985a). The output of $PGF_{2\alpha}$ from day 15 guinea-pig endometrium tissue cultured up to 3 days was reduced by the lack of extracellular calcium, EGTA (a Ca^{2+} chelator) and TMB-8 (Riley & Poyser, 1987b). These findings led Riley and Poyser (1987b) to suggest that both intracellular and extracellular calcium were necessary for the $PGF_{2\alpha}$ synthesis by and release from the guinea-pig endometrium. The extracellular calcium may just be necessary to refill the intracellular calcium pool.

1.2.2 Calmodulin, Protein Kinase C and cAMP

Calmodulin is a 17 kDa acidic protein which binds calcium at a ratio of 1:4. It has been shown that calmodulin increases the stimulatory effect of calcium on the activity of PLA_2 from the platelet membrane (Wong & Cheung, 1979), the renal medulla (Craven & De Rubertis, 1983) and the amnion (Olson *et al.*, 1985). Trifluoperazine (TFP), a calmodulin inhibitor, prevented both the release of AA and PG synthesis induced by A23187, vasopressin, NaCl and mannitol in rat renal medulla (Craven & De Rubertis, 1983). It has also been shown that TFP inhibits PLA_2 activity in platelets (Walenga *et al.*, 1981) and, A23187-induced PGE_2 output from human amnion cells (Olson *et al.*, 1985).

The involvement of calmodulin in mediating the calcium-induced increase in $PGF_{2\alpha}$ output from guinea-pig uterine horns superfused *in vitro* (Poyser, 1985a, b) and from guinea-pig endometrium in culture (Riley and Poyser, 1987b) has been investigated. It was reported that N-(6-aminohexyl)-5-chloro-1-naphtalene-

sulphonamide (W-7) (Poyser, 1985a) and TFP (Poyser, 1985b), both calmodulin antagonists, inhibited A23187-induced PG output from the day 7 and day 15 guinea-pig uterus superfused *in vitro*. Furthermore, these calmodulin antagonists (i.e. W-7 & TFP) were reported to inhibit basal $PGF_{2\alpha}$ output from day 15 guinea-pig endometrium cultured in the absence or presence of extracellular calcium (Riley & Poyser, 1987b). These result suggest that calmodulin may have a role in mediating the action of calcium in the activation of PLA_2 for the subsequent release of AA and consequent increase in $PGF_{2\alpha}$ synthesis by and release from the guinea-pig endometrium. As nifedipine and verapamil, both calcium channel blockers, were ineffective in inhibiting $PGF_{2\alpha}$ output from the cultured guinea-pig endometrium (Riley & Poyser, 1987b), calcium entry through voltage-dependent calcium channels appears not to be involved in increased $PGF_{2\alpha}$ production by the guinea-pig uterus. Poyser (1987a) reported that the depolarisation of the guinea-pig uterus with K^+ caused the uterus to contract, but there was no increase in $PGF_{2\alpha}$ production. This lead him to conclude that an intermittent synchronous depolarisation is therefore not responsible for the pulsatile nature of endometrial $PGF_{2\alpha}$ production.

Kinases are known to regulate arachidonic acid release, and hence PLA_2 activation. Protein kinase C (PKC) has long been implicated in the activation of AA release due to the potent stimulatory effects of phorbol esters on macrophages, and the effects of selective inhibitors of PKC (see Glaser *et al.*, 1993). Wightman *et al.* (1982) suggested that PLA_2 activity in the murine peritoneal macrophage was also regulated by the level of cAMP-dependent kinase activity. In the platelet, PKC has

been shown to be involved in thromboxane synthesis (Mobley & Tai, 1985). Touqui *et al.* (1986) reported that PKC prevents PLA_2 deactivation in platelets. In the guinea-pig the PKC activator, phorbol 12-myristate 13-acetate (TPA), had no effect on the outputs of prostaglandins from cultured day 7 or day 15 endometrium (Riley & Poyser, 1987b) or from uterine horns superfused *in vitro* (Poyser, 1987a). These data indicate that in the guinea-pig PKC activation is not involved in PG output from the guinea-pig uterus. However, TPA has been shown to stimulate $PGF_{2\alpha}$ output from cultured human endometrial cells (Skinner *et al.*, 1984), and to stimulate PLA_2 activity in human neutrophils (Stocker & Richter, 1982) and in human amnion and decidual cells (Schrey & Read, 1986), indicating that species differences may therefore exist.

It has been reported that there are no differences in the basal levels of cAMP in guinea-pig endometrium and myometrium between day 7 and day 15 of the oestrous cycle (Poyser, 1987a). Poyser (1987a) also reported that basal cAMP levels in the myometrium on day 7 and day 15 were about 8- to 9-fold greater than cAMP levels in the endometrium on the same day. Forskolin (an activator of adenyl cyclase) increased cAMP levels in guinea-pig endometrium and myometrium by 50- and 7-fold, respectively, on both days 7 and 15 of the oestrous cycle (Poyser, 1987a), but there were no increases in the outputs of prostaglandins. This study clearly indicated that cAMP has no role in the process of increased prostaglandin production by the guinea-pig uterus.

1.2.3 G-proteins

The presence of a G-protein in sheep uterine endometrium has been shown by Flint (1988). PLA_2 has been reported to be activated by a G-protein which is inhibited by pertussis toxin; hence, pertussis toxin inhibits PG synthesis in several tissues (Burch *et al.*, 1986; Burgoyne *et al.*, 1987; Axelrod *et al.*, 1988; Wang *et al.*, 1988; Nakashima *et al.*, 1988). However, pertussis toxin and cholera toxin stimulate PGI_2 synthesis by aortic endothelial cells and PGE_2 synthesis by a murine macrophage cell line via activating a G-protein (Pirotton *et al.*, 1987; Burch *et al.*, 1988). In guinea-pig neutrophils, A23187 and N-formyl peptide receptor stimulation cause AA release. Pertussis toxin inhibits AA release caused by the stimulation of the N-formyl peptide receptor but not by A23187 (Bokoch & Gilman, 1984). Pertussis toxin has also been reported to inhibit noradrenaline-stimulated AA release but not noradrenaline-stimulated inositol phosphate formation (Burch *et al.*, 1986). Fluoride, another activator of G-proteins, stimulates PGI_2 synthesis by rat aorta (Jeremy & Dandona, 1988). Sodium fluoride (NaF) stimulated the outputs of prostaglandin from the day 7 and day 15 guinea-pig uterine horn superfused *in vitro* suggesting that a G-protein is involved in uterine PG synthesis (Leckie & Poyser, 1990a). The action of NaF was not inhibited by the lack of extracellular calcium nor by the actions of calmodulin inhibitors (W-7 & TFP) or neomycin (an inhibitor of PLC). However, TMB-8 inhibited NaF-induced $PGF_{2\alpha}$ output, but not PGE_2 or 6-keto-$PGF_{1\alpha}$, outputs from the day 7 guinea-pig uterus superfused *in vitro* (Leckie & Poyser, 1990a). These finding suggest that the action of NaF on PG synthesis is not dependent upon

extracellular calcium, calmodulin or PLC, but that intracellular calcium may be involved.

Cholera toxin and pertussis toxin had no effect on the outputs of prostaglandins from both day 7 and day 15 cultured guinea-pig endometrium indicating that toxin-sensitive G-proteins are not involved in the processes which control $PGF_{2\alpha}$ release from the guinea-pig endometrium (Leckie & Poyser, 1990b). Interestingly, 10 mM NaF induced $PGF_{2\alpha}$ output from the guinea-pig endometrial tissue cultured for 1 h, but inhibited $PGF_{2\alpha}$ output and protein synthesis from the endometrium cultured for 6, 12, 18 and 24 h (Leckie & Poyser, 1990b). These reports suggest that NaF has two effects, i.e. a stimulatory (short term) effect and an inhibitory (long term) effect. Leckie & Poyser (1990b) suggested that the long term effect of NaF is probably due to its inhibitory effect on endometrial protein synthesis. The short term effect may involve a G-protein which is not regulated by toxins. A similar type of conclusion was also drawn by Burch & Axelrod (1987). They reported that a pertussis toxin-insensitive G-protein is involved in the activation of PLA_2 by bradykinin in Swiss 3T3 fibroblast cells. In cultured human endometrial epithelial and stromal cells, NaF stimulated AA release from both cell types when incubated for periods of up to 90 min (Bonney *et al.*, 1991). This finding raises the question whether the NaF effect reported on human endometrial cells in culture is the 'short term effect', as shown by Leckie & Poyser (1990a, b) and whether a long-term inhibitory effect also exist in human endometrial cells.

1.2.4 Inositol trisphosphate

In sheep, oxytocin stimulates the turnover of endometrial inositol trisphosphate (IP_3) and total inositol phosphate (IP) (Mirando *et al.*, 1990). This IP_3 turnover is initiated between days 12 and 16 of the oestrous cycle (Mirando *et al.*, 1990). Furthermore, when slices of caruncular endometrium from steroid-treated ovariectomised sheep were incubated with labelled inositol, oxytocin caused a 2.8-fold increase in the rate of incorporation of labelled inositol into phosphatidylinositol, and IP_3 was the major triphosphate formed (Flint *et al.*, 1986). The action of oxytocin on phosphoinositide hydrolysis was dose- and time-dependent, occurring at concentrations within the range observed in plasma during episodes of secretion *in vivo*, and with a time course comparable with that of the action of oxytocin on uterine prostaglandin production (Flint *et al.*, 1986). They also reported that the effect of oxytocin on incorporation of radioactivity into inositol phosphate was not affected by inhibitors of prostaglandin synthesis. It was proposed that the stimulation of endometrial prostaglandin synthesis by oxytocin is accounted for by hydrolysis of phosphoinositides to diacylglycerol and IP_3 with subsequent release of AA from diacylglycerol (Flint *et al.*, 1986). In another study, oxytocin stimulated total IP_3 by endometrial tissue in culture from day 5 and day 15 post-partum ewes, and from cycling ewes on day 10 and day 15 (Wallace *et al.*, 1993). Oxytocin also stimulated $PGF_{2\alpha}$ release to varying levels at all stages of the cycle in post-partum ewes but only on day 15 of cycling ewes. Thus, stimulation of endometrial $PGF_{2\alpha}$ synthesis in ewes is associated with increased IP_3 levels (Wallace *et al.*, 1993). However, as described in Section 1.2.1, oxytocin-

induced $PGF_{2\alpha}$ release from sheep endometrium is dependent upon the activation of PLA_2 and not PLC. Thus $PGF_{2\alpha}$ production in the sheep endometrium appears to be independent of IP_3 generation.

Ning & Poyser (1984) reported that phosphatidylcholine is the major phospholipid in guinea-pig endometrium and phosphatidylinositol is present at a much lower concentration. There is no increase in inositol turnover in the guinea-pig endometrium at the end of the oestrous cycle when $PGF_{2\alpha}$ synthesis is high (Ning & Poyser, 1984). These findings indicate that the stimulation of endometrial $PGF_{2\alpha}$ synthesis in the guinea-pig is not dependent on the generation of IP_3.

It was discussed in Section 1.2.1 that the stimulation of endometrial $PGF_{2\alpha}$ synthesis is due to an increase in the intracellular calcium concentration which, in turn, activates PLA_2. However, endometrial $PGF_{2\alpha}$ synthesis is dependent upon intracellular calcium but this calcium is not derived from an IP_3-sensitive pool. The question is then raised from what other pool is this intracellular calcium derived?

1.3. INTRACELLULAR CALCIUM

In the early 1960s it became apparent that, although in narrow smooth muscle cells the time required for Ca^{2+} to diffuse from the surface to the interior presents no kinetic limitation on the rate of tension development, the release of intracellular Ca^{2+} is nevertheless involved in agonist induced contraction (Bohr, 1963; Daniel, 1965). The release of intracellular Ca^{2+} induced by high doses of agonist is transient and, in the absence of extracellular calcium, exhausts the Ca^{2+} store (Van Breemen, 1969; Van Breemen *et al.*, 1972). Subsequently IP_3-induced release of

calcium from a non-mitochondrial internal store in permeabilized pancreatic acinar cells (Sterb *et al.*, 1983) and in skinned single cells of porcine artery (Suematsu *et al.*, 1984) was reported. In cultured and freshly isolated, permeabilized smooth muscle cells, IP_3-induced Ca^{2+} release was sufficiently large and rapid, and a half-maximally effective concentration of approximately 1 μM IP_3 was low enough to account for transient smooth muscle activation (Suematsu *et al.*, 1984; Smith & Smith, 1987). The IP_3-induced calcium release mechanism was also reported to be activated during the maturation of hamster oocytes (Fujiwara *et al.*, 1993). The mechanism of action of IP_3 is such that its interaction with its receptor (Baukal *et al.*, 1985; Ferris & Snyder, 1992) opens cation channels, which allows the rapid discharge of calcium from the sarcoplasmic reticulum (SR) into the cytosol. Saida *et al.* (1988) reported that there is a specific GTP requirement for IP_3-induced calcium release from skinned vascular smooth muscle, and that this IP_3-induced calcium release is inhibited by pertussis toxin. Whereas Ghosh *et al.* (1989) postulated that there are two intracellular Ca^{2+} pools distinguishable by IP_3-sensitivity and oxalate-permeability, and that IP_3-induced and GTP-activated Ca^{2+} translocation processes are different. Ross *et al.* (1992) reported that at least four different IP_3 receptors exist. IP_3-evoked channel opening is enhanced by ATP (Maeda *et al.*, 1991; Ferris & Snyder, 1992). IP_3-induced release of calcium from an internal store is also reported in cat adrenal chromaffin cells (Sorimachi *et al.*, 1992), bovine chromaffin cells (Stauderman *et al.*, 1991), intact GH4C1 cells (Tanaka & Tashjian, 1993) and saponian-skinned smooth muscle cells from guinea-pig portal vein (Tsukioka *et al.*, 1994). However, only part of the intracellular

calcium store is IP_3-sensitive. This was shown in cat adrenal chromaffin cells (Sorimachi *et al.*, 1992), bovine chromaffin cells (Stauderman *et al.*, 1991), intact GH4C1 cells (Tanaka & Tashjian, 1993), rat brain (Smith & Nahorski, 1993) and, at a single cell level, in the rat pancreas (Schmid *et al.*, 1990). They all reported that, apart from an IP_3-sensitive calcium store in the SR, there is at least one IP_3-insensitive calcium store in the SR which is activated by caffeine and ryanodine (RY).

It is well-documented that caffeine-induced contractions of skeletal (Weber & Herz, 1988) and cardiac (Blinks *et al.*, 1972) muscles are due to the release of calcium from a storage pool in the sarcoplasmic reticulum which is distinct from the IP_3-sensitive pool (Vigne *et al.*, 1990; Stauderman *et al.*, 1991). This caffeine-sensitive, IP_3-insensitive store of calcium is known to be released through stimulation of the "ryanodine" receptor (RYR). Ryanodine is an alkaloid derived from South American plant, *Ryania speciosa Vahl.* Ryanodine has been shown to cause an irreversible contracture in mammalian skeletal muscle (Protica, 1956), cardiac muscle (Furchgot & De Gubareff, 1956; Hillyard & Procita, 1956), and smooth muscle (Hillyard & Procita, 1958). Hillyard & Procita (1958) reported that, in isolated rabbit duodenal strips, RY failed to cause contraction in the absence of extracellular calcium. Since then, however, a large number of reports have indicated that RY induces calcium release from an internal store resulting in an increase in the free cytosolic calcium concentration and a subsequent contraction (Hwang & Van Breemen, 1987; Ezzaher *et al.*, 1992; Kojima *et al.*, 1994). The action of RY is due to its interaction with a RYR in the membrane of the

sarcoplasmic reticulum (SR). However, one of the intriguing aspects of the RY is that it stimulates calcium release at low concentrations (nM) and inhibits calcium release at high concentrations (μM).

The RYRs, which are intracellular calcium release channels, were originally described in the sarcoplasmic reticulum of the skeletal muscle (Type 1; RYR-1) or cardiac muscle (Type 2; RYR-2) (Flienscher & Inui, 1989). Ryanodine receptors from skeletal (Lai *et al.*, 1988) and cardiac (Henderson & Meissner, 1987) muscles have been purified and biochemically characterised, and were found to be different. Activation of these receptors directly by RY, and indirectly by extracellular stimuli such as methacholine (a muscarinic receptor agonist; Sorimachi *et al.*, 1992), hormones and neurotransmitters (see Berridge, 1993) have been shown to mobilise calcium from an internal store. Ryanodine receptors are reported to be present in many other tissues such as longitudinal muscle of the rabbit intestine (Kuemmerle *et al.*, 1994), rat femoral artery (Komija *et al.*, 1994), rabbit aortic smooth muscle (Hwang & Van Breemen, 1987), guinea-pig ileal cells (Gagov *et al.*, 1993), sea urchin eggs (Buck *et al.*, 1994) and cat adrenal chromaffin cells (Sorimachi *et al.*, 1992). Ryanodine receptor of the cardiac type have been shown to be present in avian embryos at days 4, 5 and 6 of development (Dutro *et al.*, 1993), and in myocardial fibres of foetal rat (Su & Chang, 1993) and 1-day old neonatal rats (Ostadalova *et al.*, 1993). Ryanodine receptor types 1 and 2 are also modulated by calcium and by caffeine. Caffeine releases calcium from RY-sensitive calcium pools (Sorimachi *et al.*, 1992; Stauderman *et al.*, 1991; Kuemmerle *et al.*, 1994). Caffeine-induced calcium release is potentiated by cyclic

ADP-ribose (Lee, 1993), calcium and ATP (Fliescher & Inui, 1989), and is inhibited by high concentrations of RY (Sorimachi *et al.*, 1992; Chen & Cheung, 1992; Kostyuk & Kirischuk, 1993), dantrolene (Usachev *et al.*, 1993), Mg^{2+} (Su & Chang, 1993), and ruthenium red (Vites & Pappano, 1994).

In cardiac muscle, calcium release from SR via RYR is regulated by Ca^{2+} influx through voltage-gated calcium channels in the surface membrane. Hence, this process is known as calcium-induced calcium release (CICR) (Fabiato & Fabiato, 1979; Sipido & Wier, 1991). The cardiac RYR is apparently more sensitive to lower concentrations of calcium, and less sensitive to inhibition by Mg^{2+} and ruthenium red, than the skeletal muscle form (Henderson & Meissner, 1987). The SR in smooth muscle cells contains a calcium-gated calcium release pathway with properties similar to those of skeletal muscle and cardiac ryanodine receptors (Herrmann-Frank *et al.*, 1991).

Ryanodine receptors in rabbit brain are thought to be mainly of the cardiac type (RYR-2) (McPherson & Campbell, 1993b). [^{3}H]RY binding to the purified brain RYR was stimulated by calcium, ATP, KCl, and phosphorylation, and was inhibited by calmodulin, Mg^{2+} and ruthenium red. It has been reported that, in the rat brain, the RYR is similar to the skeletal form but is not identical, as [^{3}H]RY binding was not inhibited by dantrolene (300 μM) (Smith & Nahorski, 1993). However, binding studies have shown that there are two types of RYR in mouse brain, skeletal muscle type (RYR-1) found exclusively in purkinje cells, and the cardiac muscle type (RYR-2) which is localised mainly at the somata of most neurones (Kuwajima *et al.*, 1992). This finding led to the postulation that calcium-

induced calcium release (CICR), probably mediated by the cardiac muscle ryanodine type receptor, functions generally in various neurones, whereas depolarisation-induced calcium release, probably mediated by the skeletal muscle ryanodine type receptor, functions specifically in purkinje cells. In avian sensory neurones, the current produced by caffeine (10 mM) was inhibited by 10 μM RY (Ivanenko *et al.*, 1993). The caffeine-induced current was not dependent on extracellular calcium, suggesting that caffeine raises intracellular free calcium concentration by activating the release of calcium from an intracellular store, and that this calcium activates the membrane conductance responsible for the current.

Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal RYR (i.e. RYR-1) gene has been shown (Takeshima *et al.*, 1994). Skeletal muscles from mice lacking a functional RYR-1 gene failed to response to electrical stimulation nor did they show a contractile response to depolarisation by application of a high K^+ (80 mM) concentration. However, slow contractions were developed when the same electrical stimulation was given in physiological salt solution containing 12 mM calcium and 10 μM Bay K-8644, an agonist of the voltage-gated L-type calcium channel. The slow contractions were abolished by tetrodotoxin (TTX; 10 μM), indicating that the action potentials are generated in the mutant muscle but the calcium release after depolarisation of sarcolemma is impaired in the mutant muscle. This indicated that the RYR functions as the calcium release channel in the skeletal excitation-coupling (Takeshima *et al.*, 1994). Ryanodine-binding affinity was shown to increase by ATP (Zarka & Shoshan-Barmatz, 1993). However, McGarry and Williams (1994)

reported that adenosine discriminates between caffeine and adenosine nucleotide sites on sheep cardiac SR calcium release channels.

In the vascular endothelial cells of bovine and human, a functional RY-sensitive, intracellular calcium store seems to be present. Ryanodine (5 μM) caused a slow increase in intracellular calcium from human aorta endothelial cells, human umbilical cells and bovine pulmonary artery, but not from rat aorta (Ziegelstein *et al.*, 1994). However, Low *et al.* (1993) have reported that RY causes a dose-dependent increase in tension in the rat aorta. These reports raise the question whether RY-induced tension in the rat aorta is due to some other mechanism rather than increasing free cytosolic calcium by activating SR ryanodine receptor. In another study, it was reported that RY (10 μM) alone had no effect on rat aorta in both calcium-containing and calcium-free physiological salt solution (PSS), whereas caffeine (30 mM) caused rat aorta to contract in both types of PSS (Hisayam *et al.*, 1990). RY (10 μM) treatment before caffeine (30 mM) treatment in calcium-free solution prevents caffeine from causing calcium release. Caffeine (10 mM) elicited a rapid rise of intracellular calcium in phaeochromocytoma (PC12) cells (Muller & Daly, 1993). In rat ventricular myocytes, 10 mM caffeine produced a transient increase in the intracellular calcium concentration and contraction (Donoso *et al.*, 1994), indicating caffeine-induced calcium release from an internal calcium store. However, sea urchin eggs rarely responded to 10 mM caffeine, but with 20 mM caffeine they produced a transient rise in intracellular calcium (Harris, 1994).

Caffeine (1 to 30 mM) causes a concentration-dependent contraction of the guinea-pig taenia caecum in calcium-free solution, but increasing the caffeine concentration produces a decrease in the size of contraction (Hisayama & Tanakayanagi, 1988). When tissues were loaded with calcium responsiveness to caffeine was recovered, suggesting that caffeine initially depleted the calcium stores. Ryanodine (10 μM) treatment of tissues prior to caffeine administration inhibited the caffeine effect (Hisayama & Tanakayanagi, 1988). Interestingly, Hisayama & Tanakayanagi (1988) reported a time-dependent inhibitory effect of RY on caffeine action. The treatment of rat diaphragm strips with 3 nM RY increased baseline tension 360% above the original resting tension but only if the diaphragm was concurrently stimulated electrically, whereas caffeine (10 mM) and 100 μM RY induced contracture in quiescent tissue (Zavecz & Anderson, 1992). Caffeine-induced contractions in calcium-free solution were abolished by 3 nM RY, indicating that the RY effect does not depend upon extracellular calcium (Zavecz & Anderson, 1992). In cat adrenal glands, the caffeine-induced rise in intracellular calcium and catecholamine secretion were strongly inhibited by pre-treatment with ryanodine (Sorimachi *et al.*, 1992). All of these findings indicate that RY- and caffeine-induced intracellular calcium release from a ryanodine-sensitive IP_3-insensitive calcium pool may have a role in some of the cellular mechanisms.

There are two genes encoding the RYR-1 (in skeletal muscle) and RYR-2 (in cardiac muscle) receptors, respectively (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990; Otsu *et al.*, 1990). A third gene encoding a new RYR (i.e. RYR-3) has been

shown in mink lung epithelial cells (Giannini *et al.*, 1992). Calcium release experiments in Mv1Lu cells have shown that this RYR (i.e. RYR-3) is sensitive to RY but not to caffeine. Furthermore, a high affinity binding site for RY has also been shown in liver microsomal fraction (Shohan-Barmatz *et al.*, 1991), where expressions of RYR-1 and RYR-2 have not been observed. More interestingly, the gene for RYR-3 expression is not abundant in liver (Giannini *et al.*, 1992). A novel RYR has been reported in rabbit brain, and this receptor is composed of 4872 amino acids and shares characteristic structural features with the skeletal muscle type and cardiac muscle type RY receptors (Hakamata *et al.*, 1992). They also reported that the gene for the brain RYR is also transcribed in smooth muscle. Considering the above information, it is clear that RYRs (i.e. caffeine-sensitive calcium release channels) located on the sarcoplasmic reticulum on the cells are of great importance in the excitation-contraction coupling of skeletal muscle and cardiac muscle. The presence of RYR in many other tissues suggests that RYRs may have a role in the function of these cells. The finding that basal $PGF_{2\alpha}$ output is not inhibited for at least 150 min in the absence of extracellular calcium (Riley & Poyser, 1987b) suggested that the release of intracellular calcium is necessary for PLA_2 activity and hence PG synthesis in the guinea-pig uterus. As outlined previously, it was reported that, in the guinea-pig, increased $PGF_{2\alpha}$ output from the endometrium is not associated with an increase in inositol turnover (Ning & Poyser, 1984), indicating the lack of involvement of an IP_3-sensitive calcium store in the stimulation of endometrial $PGF_{2\alpha}$ synthesis. Hence, it is likely that another internal calcium pool is involved in the synthesis and release of prostaglandins

from the guinea-pig endometrium. This internal calcium store may be the RY- and caffeine-sensitive calcium pool. Thus one of the main aims of this study is to investigate whether a RYR is involved in prostaglandin production by the guinea-pig uterus, predominantly by the endometrium.

1.4. ISOFORMS OF PROSTAGLANDIN H SYNTHASE

It has been shown that the uterus possesses an increased ability to biosynthesise $PGF_{2\alpha}$ from endogenous precursors on days 14 and 15 of the oestrous cycle (Poyser, 1972, 1983a, b). Poyser (1983a) proposed that the increase in endometrial PG synthesising capacities is probably due to an increase in the amount of prostaglandin H synthase (PGHS) present in the endometrium. Oestradiol, but not progesterone, treatment of ovariectomised guinea-pigs stimulated prostaglandin and thromboxane (TX) synthesis, particularly of $PGF_{2\alpha}$, by homogenates of the whole uterus and endometrium, but not by homogenates of the myometrium (Poyser, 1983a). This stimulatory effect of oestradiol on $PGF_{2\alpha}$ synthesis was greater on the endometrium than on the whole uterus, indicating that endometrium is the major site of $PGF_{2\alpha}$ production. Progesterone did not alter the stimulation of $PGF_{2\alpha}$ synthesis by homogenates of the whole uterus, but it reduced by 50% the stimulatory effect of oestradiol on $PGF_{2\alpha}$ synthesis by homogenates of the endometrium (Poyser, 1983a). This lead Poyser (1983a) to speculate that oestradiol stimulates PGHS synthesis in the endometrium which in turn contributes to the increase in $PGF_{2\alpha}$ synthesis and release. The intra-uterine administration of actinomycin D, a protein synthesis inhibitor, on day 10 reduced the output of

$PGF_{2\alpha}$ from the guinea-pig uterus *in vitro* by 80 to 85%, and plasma progesterone levels were high on day 15 due to the reduction in uterine $PGF_{2\alpha}$ output (Poyser & Riley, 1987). This finding indicates that fresh protein synthesis has a role in the high output of $PGF_{2\alpha}$ from the guinea-pig uterus towards the end of the oestrous cycle. This increase in protein synthesis is thought to contribute to the increase in PGHS concentration.

In sheep, the specific activity of PGHS in uterine microsomes was higher on days 13-15 than on earlier days of the oestrous cycle and reached a maximum on day 14 (Husling *et al.*, 1979). This increase in PGHS activity on days 13-15 resulted from increases in the concentration of PGHS. Furthermore, Husling *et al.*, (1979) reported an increase in the specific activity of PGHS in caruncular (which includes luminal epithelium, vascular endothelium and caruncular stroma) microsomes but no cyclical changes were seen in the activity of PGHS in microsomes from myometrium and intercaruncular endometrium. The authors suggested that the onset of luteolysis depends on an increase in the concentration of caruncular PGHS, and possibly the stromal cells are the site of the cyclical changes in PGHS activity. However, a marked cyclical changes in the concentration of PGHS in the epithelial calls of sheep endometrium has been observed (Salamonsen & Findlay, 1990). Most of the PGHS in sheep endometrium is present in the stromal tissue on day 4, but PGHS is detectable in the luminal epithelium by day 7 and reaches its maximum concentration in the epithelium on day 14 (Salamonsen & Findlay, 1990).

Salamonsen *et al.* (1991) studied the expression of the gene for PGHS in sheep endometrium. They reported that, whilst no significant differences were seen in PGHS mRNA concentrations in endometrial tissue during the oestrous cycle or in early pregnancy, treatment of ovariectomised ewes with oestradiol-17β markedly decreased endometrial PGHS mRNA concentration. There were no differences in PGHS mRNA concentrations in ewes treated with progesterone, either alone or in conjunction with oestrogen when compared to ovariectomised controls (i.e. with no steroid-treatment). In contrast, there was a difference in immunolocalization of PGHS in uterine tissue from ovariectomised-steroid-treated ewes. In ovariectomised ewes and those treated with oestrogen, immunocytochemical staining for PGHS was seen in stromal cells, but little immunoreactive PGHS was located in the endometrial epithelial cells. However, in ewes treated with progesterone alone or with oestrogen plus progesterone, PGHS was found in luminal and glandular epithelial cells and in stromal cells. These findings led Salamonsen *et al.* (1991) to speculate that whilst oestrogen lowers PGHS mRNA concentration in the endometrium, presumably in stromal cells, it may also increase the stability of the enzyme itself in the stromal cells.

It has been shown that there are two isoforms of PGHS, namely PGHS-1, a constitutive enzyme and PGHS-2, an inducible enzyme (Hedin *et al.*, 1987; Wong & Richards, 1991; Takahashi *et al.*, 1994; Miller *et al.*, 1994; Crofford *et al.*, 1994). Hedin *et al.* (1987) demonstrated that, in the ovary, the increase in PGHS concentrations at the time of ovulation is due to an increase in the concentration of PGHS-2 levels. They showed that the content of PGHS-2 (Mr, 72 kDa) was low in

granulosa and thecal cells of small antral and preovulatory rat follicles. PGHS-2 was induced preferentially (about 15-fold) in granulosa cells between 1 and 7 h after human chorionic gonadotrophin (hCG) treatment. This effect was also reported by Sirois and Richards (1992), who found a distinct isoform of PGHS (Mr 70-72 kD) was induced by human chorionic gonadotrophin in granulosa cells of rat preovulatory follicles.

It is not known whether this increase in PGHS concentration in guinea-pig and sheep endometrium after day 10 of the cycle is due to the stimulation of the inducible form of PGHS (i.e. PGHS-2). Another aim of this study is to investigate whether the PGHS-2 isoform is present in guinea-pig endometrium, specifically at the end of the oestrous cycle.

1.5. AIMS

In summary, the aims of this study are:

1. To investigate which cell type in the guinea-pig endometrium is responsible for increased $PGF_{2\alpha}$ synthesis by the endometrium at the end of the oestrous cycle.

2. To investigate which cell type responds to the known stimulators and inhibitors of endometrial PG synthesis in the guinea-pig.

3. Whether the stimulation of endometrial $PGF_{2\alpha}$ synthesis in the guinea-pig is through the stimulation of the ryanodine receptor.

4. Whether a non-uterine tissue (i.e. blood vessels) produces prostaglandins in response to caffeine and/or ryanodine.

5. Whether prostaglandin H synthase-2 is present in the guinea-pig uterus, in particular the endometrium at the end of the oestrous cycle.

SECTION TWO

2. METHODS & MATERIALS

INTRODUCTION

Many general methods have been employed on the work described in this thesis which are common to a number of experiments. These include: guinea-pig uterine superperfusion, prostaglandin extraction, rat mesenteric vascular bed perfusion, endometrium and myometrium tissue culture of guinea-pig uterus, guinea-pig endometrium homogenization, primary isolation and culture of endometrial epithelial and stromal cells, intracellular calcium measurement of endometrial epithelial and stromal cells, SDS-PAGE, Western Blotting, and radioimmunoassay. These general methods which are fully described in detail as follows:

2.1 GENERAL METHODS

2.1.1 GUINEA-PIG UTERINE SUPERPERFUSION

Virgin, Dunkin-Hartley guinea-pigs (600 - 900 g) were examined daily and a vaginal smear was taken when the vagina was open. Day one of the oestrous cycle was taken as the day preceding the post-ovulatory influx of leucocytes when cornification was at a maximum. All guinea-pigs had exhibited at least three cycles of normal length (about 16 - 17 days) before being killed (by stunning followed by exsanguination) on day 7 or 15 of the cycle. Each uterus was removed and

separated into its two uterine horns. The uterine horns were blotted dry and weighed. Each uterine horn was opened by cutting longitudinally down the anti-mesometrial side, and was suspended in an organ bath with one end attached to an isotonic leaver under a load of 2 g. Each uterine horn was superfused at 5 ml/min with Krebs solution (see Section 2.2.7.1 for composition) at 37°C, as described by Poyser and Brydon (1983), and superfused initially for an equilibrium period of 60 min. Samples of superfusate were then collected for 10 min periods over the next 80 (i.e 8 samples per uterine horn) or 100 min (i.e 10 samples per uterine horn) depending on the treatment. After collection, the pH of each sample was lowered to 4.0 with 1 M HCl and the prostaglandins were extracted by shaking twice with 50 ml ethyl acetate, according to the method described by Poyser (1972). The two ethyl acetate fractions were combined and evaporated to dryness on a rotary evaporator. The recoveries of $PGF_{2\alpha}$ and PGE_2 by this method are >90% and that of 6-keto-$PGF_{1\alpha}$ is >80% (Poyser & Scott, 1980; Swan & Poyser, 1983). Each dried extract was redissolved in 10 ml ethyl acetate and stored at -20°C until it was assayed.

2.1.2 RAT MESENTERIC VASCULAR BED PERFUSION

Male Wistar rats (250 - 300 g) were killed by a blow to the head followed by exsanguination. The mesenteric vascular bed was dissected out and cleaned from any connective tissues according to the procedure described by McGregor (1965), and perfused in a hot water heated chamber at 37°C with McEwen's solution (for composition see Section 2.2.7.2) at 4 ml/min at 37°C and aerated with 5% carbon

dioxide and 95% oxygen, as described by Lennon & Poyser (1986). After an initial equilibrium period of 30 min, samples of perfusate were collected for periods of 1 min and stored at -20°C before being assayed.

2.1.3 GUINEA-PIG ENDOMETRIAL & MYOMETRIAL TISSUE CULTURE

Tissue culture was performed according to the method of Baker and Neal (1969) for ovarian tissue, which was later modified for endometrial tissue by Abel and Baird (1980), and Ning *et al.* (1983). Uterine horns were removed from the guinea-pigs as described in Section 2.1.1 except that the whole procedure was carried out under aseptic conditions. Tissues were then transferred into sterile petri dishes containing tissue culture medium (TCM) which consists of modified Medium M199 plus Earle's salts and 2.20 g/l sodium bicarbonate, L-glutamine (1.7 μM), amphotericin B (2.5 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). After "opening up" each uterine horn as described in Section 2.1.1, endometrium was cut away from the myometrium, cut into approximately 1 mm^3 pieces, and then placed onto sterile lens paper lying across a stainless steel grid in a vented sterile dish which contained 4 ml of TCM plus any other compound used according to the nature of the experiment. This set-up allowed the tissues to be held above the medium but the medium was readily available to the tissues by the capillary action of the lens paper (Figure 2.1.3.1). The amount of tissue placed in each petri dish varied between 19 - 35 mg. Petri dishes were then placed into racks which were in turn placed into modified kilner jars. The jars were then pressurised

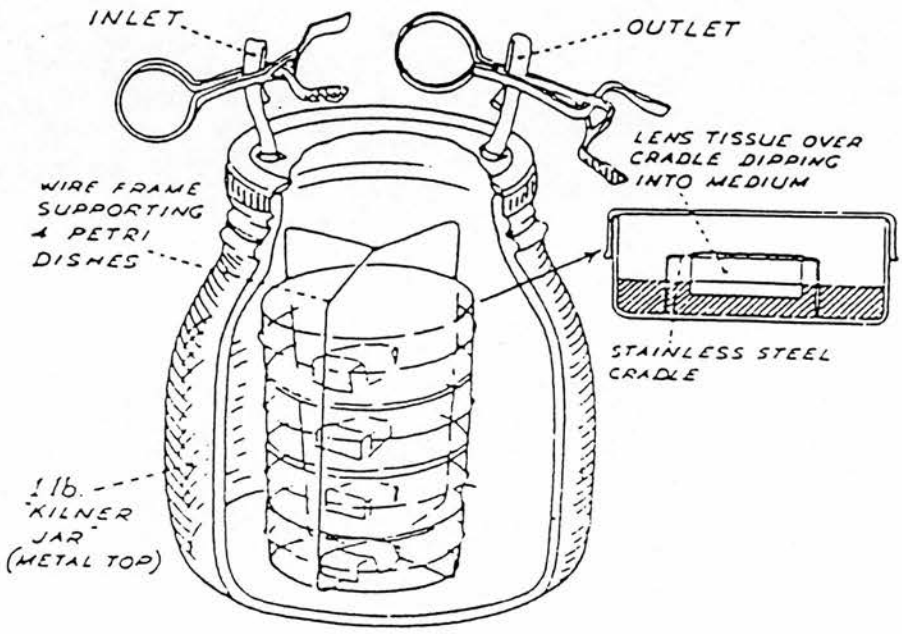


Figure 2.1.3.1 The above diagram shows the arrangements for culturing uterine tissue in a petri dish, and how the petri dishes were stacked and gassed (via the inlet and outlet tubes) in a kilner jar.

to 0.7 kg cm^{-2} (10 lb in^{-2}) with a 1:1 gas mixture of air/CO_2 (95% : 5%) and O_2/CO_2 (95% : 5%) and incubated at 37°C for 24 h. Medium was collected at 2, 8 and 24 h, and fresh medium was added to the dishes. When the medium in the dishes was removed and replaced with fresh medium, it was preheated to 37°C before being used. The jars were then regassed and replaced in the incubator. The samples were stored at -20°C until they were assayed, without extraction, for $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$. At the end of the experiment tissues were blotted dry and weighed. Myometrial tissue was cultured in the same way.

Since diffusion is the only means by which the cultured tissues receive their oxygen, measured pressure of this gas was used to overcome problems of diffusion relative to tissue size. The small amount of CO_2 present is necessary to set up a CO_2/HCO^-_3 buffer system to maintain the pH of the medium at 7.4. Histological examinations previously carried out in this laboratory have shown that tissues remain viable in culture up to 72 h (Leaver & Seawright, 1982). Hence the tissue culture procedure used here is more than adequate for a normal maintenance and survival of both endometrial and myometrial tissues up to 24 h, the only time period used throughout the studies presented in this thesis.

2.1.4 ENDOMETRIUM HOMOGENISATION

Uterine horns were removed from the animals as described in Section 2.1.1. Endometrium was separated from the myometrium, and the myometrium was discarded. Endometrial tissues were then blotted dry, weighed, chopped finely with

scissors and homogenised in a Fisons glass homogeniser with 5 ml of Krebs solution containing appropriate treatments. The homogeniser was then washed with a further 5 ml of the Krebs solution comprising the same treatment. The homogenate and the washings (total volume 10 ml), were then transferred to a 25 ml conical flask, and incubated for 60 min at 37°C in a shaking (230 oscillations/min) Grant Water bath. Prostaglandins were extracted from the homogenates as described in Section 2.1.1 except that 2 volumes of 20 ml ethyl acetate were used. The residue obtained from evaporation of the ethyl acetate fractions was redissolved in 4 ml of ethyl acetate. The samples were then stored at -20°C until assayed.

2.1.5 ISOLATION & CULTURE OF GUINEA-PIG ENDOMETRIAL CELLS

Cells were dissociated according to the method of Satyaswaroop *et al.* (1979) as modified by Chaminadas *et al.* (1986). The uterine horns were prepared as described in Section 2.1.3. All procedures were carried out under aseptic conditions. Endometrium was separated from the myometrium and chopped into fragments of about 0.5 mm^3 in 10 ml Ham's F-10 medium containing HEPES (20 mM), 30% heat treated newborn calf serum (H-NCS), collagenase Type I (2 mg/ml), L-glutamine (10 mM), amphotericin B (2.5 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a sterile conical flask and incubated at 37°C in a shaking (230 oscillations/min) Grant Water bath for 2 h. Tissue fragments were

further disrupted by gentle, repeated pipetting every 15 min. Digestion was periodically monitored by decanting a small volume of the suspension into a sterile tissue culture dish and visualizing the isolated epithelial cells under an inverted microscope. After dissociation, the undigested fragments were collected by sedimentation and discarded. The supernatant cell suspension were centrifuged at 100 *g* for 5 min. The pellet containing epithelial cells was washed twice and then centrifuged at 100 *g* for 5 min. The final pellet containing approximately 95% of epithelial cells and 5% stromal cells was re-suspended in 30 to 40 ml of Cell Culture Medium (CCM) which consisted of Ham's F-10 medium containing HEPES (20 mM), L-glutamine (10 mM), 10% H-NCS, amphotericin B (2.5 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2.5 ml aliquots were dispensed into each of the 12 wells of the 24-well cell culture plate (2.3×10^3 - 4.5×10^3 cells per ml). After epithelial cell isolation, the remaining supernatant was centrifuged at 400 *g* for 10 min. The pellet containing the stromal cells was resuspended in 30 to 40 ml CCM, and 2.5 ml aliquots dispensed into each of the remaining 12 wells of the 24-well cell culture plate (2.2×10^5 - 5.6×10^5 cells per ml). Tissue culture plates were then incubated at 37°C in a humidified atmosphere of 5% CO_2 : 95% air. By 24 h after plating, the epithelial cells had lost their shapes and had become flattened to form a monolayer. Stromal cells were attached to the plastic surface by 24 h, and by day 2 a confluent layer of cells was formed. The medium was changed every 72 h and 2.5 ml of fresh CCM was added into

each well. The cells were cultured for 6 days before being treated with any compound with respect to the nature of the experiment.

Cell viability (at the time of plating and at the end of the experiments) was determined on the base of trypan blue exclusion. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of cell morphology. The method used was that according to the procedure described in the "Sigma catalogue" which is as follows. A cell suspension was prepared in a Hanks Balanced Salts Solution (HBSS). At the end of each experiment, cells were first dispersed by a gentle rubbing of the cells with plastic end of a syringe. The cells were collected and a cell suspension was prepared. Then 0.4 ml of 0.4% trypan blue Solution was added to 0.1 ml of the cell suspension in a test tube. The contents were vortexed and allowed to stand for 10 min. The cells were then counted using a haemocytometer and the percentage cell viability was obtained from the formula:

$$\% \text{ cell viability} = \frac{\text{viable cells (unstained)}}{\text{total cells (stained \& unstained)}} \times 100$$

2.1.6 INTRACELLULAR CALCIUM MEASUREMENT OF EPITHELIAL & STROMAL CELLS OF GUINEA-PIG ENDOMETRIUM

Cell isolation and preparation was as described in Section 2.1.5. Cells were then washed twice in Ca^{2+}-containing or a nominally Ca^{2+}-free Krebs solution (from

which the $CaCl_2$ had been omitted) buffered with 20 mM HEPES pH 7.4, in order to remove phenol red, re-suspended in the appropriate Krebs solution, and loaded with 2 μM fura-2 acetoxymethyl ester (fura-2/AM) by incubating for 30 min at room temperature (25°C). Cells were then washed, resuspended in appropriate fresh Krebs solution and left to stand for a further 30 min to allow hydrolysis of the intracellular fura-2/AM. After 30 min the cells were washed, resuspended in appropriate fresh Krebs solution, and transferred to a quartz cuvette maintained at 37°C. The cells were continuously stirred in the cuvette. Fluorescence was measured in a Shimadzo (type RF-5000) spectrofluorophotometer at 505 nm with alternate excitation at 340 nm and 380 nm. The intracellular free calcium concentration ($[Ca^{2+}]_i$) was then calculated using the equation of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = (k_d) \times (b) \times [R - R_{min}]/[R_{max} - R]$$

and the resultant experimental data of percentage change of $[Ca^{2+}]_i$ versus agonist dose or concentration was obtained, where R is the ratio fluorescence due to excitation at 340 nm to that at 380 nm. R_{max} and R_{min} are the maximal and minimal fluorescence ratio of fura-2 obtained in Krebs solution containing saturated levels of calcium with a calcium ionophore, ionomycin (10 μM), and EGTA (30 mM), respectively. The K_d value for the fura-2-Ca^{2+}-complex was assumed to be 225 nM at 37°C (Buchan & Martin, 1991; Grynkiewicz *et al.*, 1985), and b is the ratio of the 380 nm signals in Ca^{2+}-free and Ca^{2+}-containing Krebs solution.

2.1.7 IDENTIFICATION OF PROSTAGLANDIN H SYNTHASE-2 IN GUINEA-PIG ENDOMETRIUM

2.1.7.1 Preparation of Soluble Cell Extracts

Endometrial tissue from guinea-pigs on days 6 and 17 of the oestrous cycle was prepared as described in Section 2.1.4. All tissues were chopped finely with scissors and homogenised in a Fisons glass homogeniser in PE buffer (10 mM potassium phosphate, pH 6.8, and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane- sulfonate (CHAPS), as described by Hedin *et al.* (1987). The homogenates were sonicated three times for 10 s, and the supernatant (which contained the soluble cell extracts) was obtained by centrifugation for 5 min at 30000 *g* at 4°C. The supernatant was stored at -20°C before electrophoretic analysis.

2.1.7.2 Determination of Total Protein in Soluble Cell Extracts

The protein assay method used to measure the total amount of protein present in soluble cell extracts was that described by Lowry *et al.* (1951). This method based on the ability of protein molecules to combine with tartrate ions and Folin's phenolic reagent to form a complex which has a spectrophotometric absorbance at 750 nm. The optical absorbance of this complex is linearly proportional to the total amount of protein present in solution.

Before the assay, the following stock solutions were prepared as shown below:

solution A	1% (w/v) $CuSO_4.5H_2O$
solution B	2% (w/v) NaK tartrate
solution C	2% (w/v) Na_2CO_3 dissolved in 0.1 M NaOH
solution D	Folin's phenolic reagent diluted 1:1.5 with distilled water
Bovine serum albumin (BSA)	2.5 mg/ml

Six 'standard' BSA solutions of known concentrations were made up as follows: 0, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of stock BSA solution were placed into a separate test tubes and volumes were made up to 5 ml by adding distilled water. From these 'standard' protein solutions, 0.3 ml was used in the assay. Each 0.5 ml aliquot therefore contained 0, 15, 30, 45, 60 and 75 μg BSA, respectively.

A mixture of solution A and solution B (solution A + B) was made by adding 0.5 ml of solution A to 0.5 ml solution B. To this mixture 50 ml of solution C was added (denoted as solution A + B + C). Three ml of solution A + B + C was added to each 'standard' tube and to the samples tubes containing 0.01, 0.025, 0.05 and 0.1 ml of each soluble cell extracts. The tubes were then "whirlimixed" and allowed to incubate for 15 min at room temperature. Solution D (0.3 ml) was added to each tube, then "whirlimixed" and incubated for further 30 min at room temperature. Optical absorbance (OA) was then measured using a Cecil™ spectrophotometer at 750 nm. The data obtained from the 'standard' solutions were used to plot a "Standard Graph". OA values obtained from the sample cell extracts

were subsequently read off the "Standard Graph" to obtain corresponding protein concentration for each sample. A new "Standard Graph" was constructed for each new assay to minimise the possible error due to measurements and incubation periods (Figure 2.1.7.2.1).

2.1.7.3 SDS-PAGE & Western Blotting

One dimensional SDS-PAGE was carried out according to the method which was first described by Laemmli (1970). Western Blotting which is a technique for transferring the separated proteins from gel matrices into membrane surface, where the proteins are readily accessible, was performed according to the procedure described by Hedin *et al.* (1987) which is a modification of the method of Towbin *et al.* (1979).

(a) Chloroform/Methanol Precipitation of Protein & SDS-PAGE

To 100 µl of protein sample (containing 70 µg protein), 400 µl of methanol was added, vortexed and centrifuged for 10 s in an eppendorf microfuge. Chloroform ($CHCl_3$; 100 µl) was then added to the mixture, vortexed and once again centrifuged for 10 s after which 300 µl of deionised distilled water (MQ water) was added and the centrifugation process was repeated. The upper layer was carefully removed, without disturbing the protein layer at the interface, and discarded. Then 300 µl of methanol was added to the protein layer, vortexed vigorously and centrifuged for 4 min in an eppendorf microfuge. The supernatant was discarded and the pellet left to dryness. The pellet was resuspended in 10 µl of sample

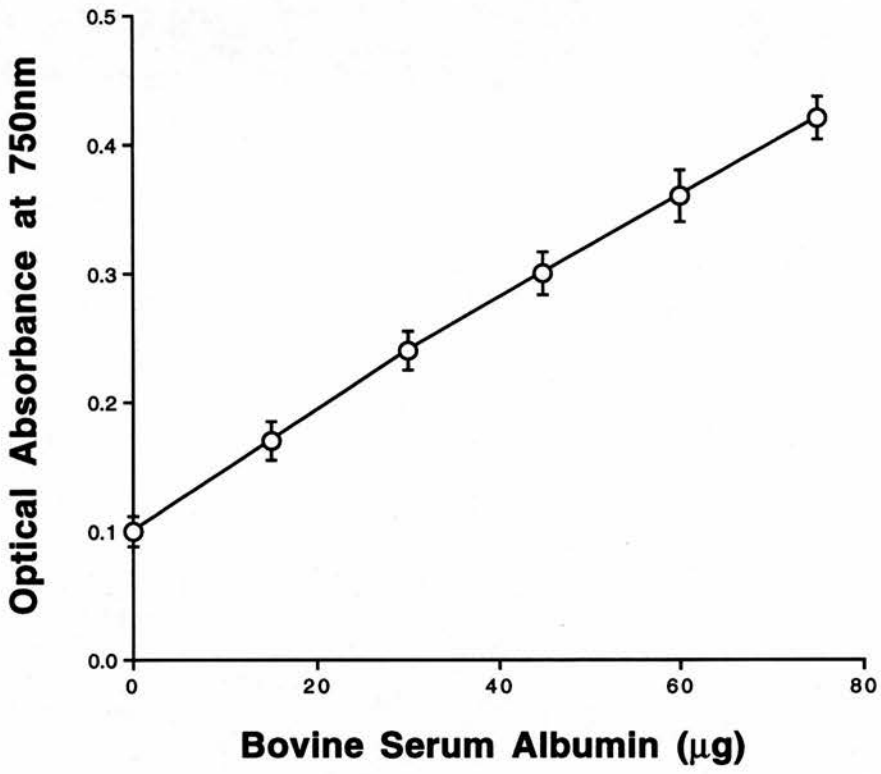


Figure 2.1.7.2.1. Standard curve for Lowery protein assay. Each point indicates the mean (±SEM, n=5).

(Laemmli) buffer (see Section 2.2.7.7.1 for composition) and boiled for 10 min. Molecular weight markers (MWM) were diluted (1:5) with Laemmli buffer and boiled for 10 min. Samples and MWM were allowed to cool and then loaded directly onto a 10%-15% SDS gels via a sample applicator 8/1 as follows. Lanes 1, 3, 5 and 7 were loaded with 1 µl of Molecular Weight Markers (MWM), 2 and 4 with 1 µl of day 6, and 6 and 8 with 1 µl of day 17 protein samples. Molecular Weight Marker contained :

Content	Mr	Content	Mr
Phosphorylase b	97400	Carbonic Anhydrase	31000
BSA	68000	Trypsin Inhibitor	20100
Ovalbumin	46000	Lysozyme	14400

Gels were then run on a Pharmacia PhastSystem (separation & control unit) according to the manufacturers (PhastSystem Owner's Manual) instructions using SDS-PAGE buffer, on 250 V voltage, 10 mA constant current at 15°C for 25 min.

(b) Western Blotting

A "Transfer Membrane" of equal size to SDS gels was prepared by soaking it in methanol for 30 s, washing with MQ water, followed by soaking in Transfer Buffer (see Section 2.2.7.7.2 for composition) at pH 8.2 until use. After the gels were run, they were then transferred on to a "Transfer Membrane" and run on a Pharmacia PhastSystem (separation & control unit) according to the manufacturers

instructions (PhastSystem Owner's Manual) for 15 min. Briefly, gels were taken out, placed on top of the cutter and cut from the second line on the gels. The blotting paper ("Transfer Membrane") was then placed on top of the gels and three filter papers which were soaked in Transfer Buffer were put on top of the blotting paper, and placed in a Pharmacia PhastSystem (separation & control unit). Then three more filter papers were added on top of the membrane. The blotting took 15 min after which it was removed from the machine. The filter papers were discarded; the blotting paper was washed with MQ water 5 times, placed in 10 ml of 5% BSA in Tris Buffer Saline (TBS) (see Section 2.2.7.7.3 for composition) solution at pH 7.6, and was shaken gently for 3 h. The 5% BSA was acting as the blocking agent in order to reduce the background by blocking the site on the Transfer Membrane. After 3 h, this solution was discarded and replaced with primary antibody (PGHS-2) (1:10, PGHS-2:MQ water). The "Transfer Membrane" was incubated with primary antibody at 4°C overnight on a shaking plate. The following morning, the primary antibody was discarded. The "Transfer Membrane" was washed 5 times with 3 ml of TBS containing 1% Tween-20 (TTBS; 1% BSA & 0.1% Tween-20, diluted 1:10 with MQ water) for 20 s, and then three times with 10 ml TTBS for 5 min each time. The TTBS solution was discarded and the "Transfer Membrane" was incubated with conjugated secondary antibody (bionated peroxidase labelled anti-rabbit antibody) (5 µl of secondary antibody in 5 ml of TTBS + 2 drops of solution A & 2 drops of solution B from Elite ABC Kit Vectastain) for 90 min. The "Transfer Membrane" was then washed 3 times with 10 ml TTBS for 5 min each time, and incubated with

peroxidase substrate kit (DAB Kit) for 10 min until colour was developed. The "Transfer Membrane" was then washed under running tap water five times and allowed to dry at room temperature.

In order to measure the molecular weights of the unknown protein samples, a calibration curve was made for protein standards by calculating the relative migration value (Rf) for each protein. This was done by measuring the migration distance of each protein standard on the stained Transfer Membrane from the loading point (used as a reference point). A calibration curve was constructed by plotting the Rf value for protein standards versus the logarithms of their corresponding molecular weights (Figure 2.1.7.3.1). The molecular weights of the unknown proteins were measured by calculating the Rf values and locating the point on the calibration curve which corresponds to that value.

2.1.7.4 Silver Staining

In order to show that the loaded sample (1 µl) contained protein, silver staining was carried out. The method used was essentially similar to that described by Heukeshoven and Dernick (1985) and Blum *et al.* (1987), with slight modification (PhastSystem Owner's Manual, 1986) which involved the use of sodium thiosulphate to decrease non-specific back ground staining. The sensitivity limit for SDS-PAGE separation of standard proteins is 0.3 to 0.5 ng protein per band. Alternatively, the use of glutaraldehyde with thiosulphate, alcohol and sodium acetate buffer results in a considerable increase in sensitivity, i.e. 0.05-0.1 ng protein per band (Heukeshoven and Dernick, 1988).

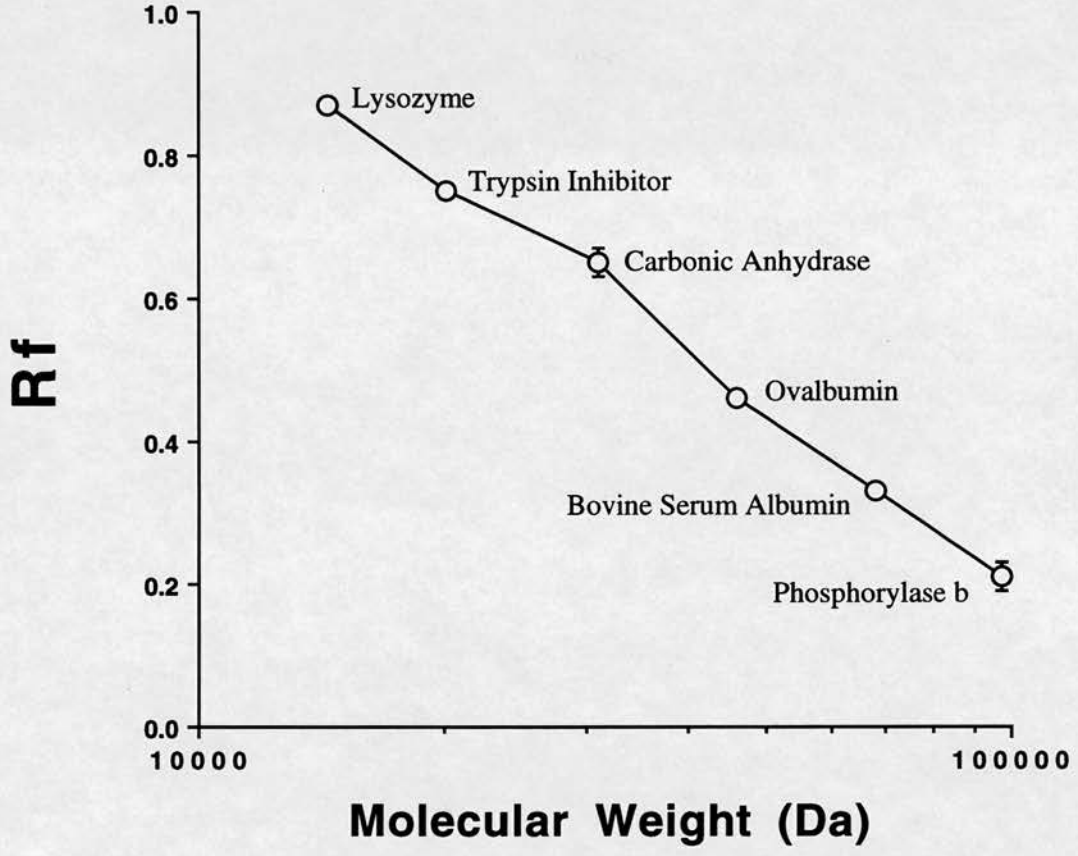


Figure 2.1.7.3.1. Standard curve for SDS-PAGE obtained with proteins from the Pharmacia Molecular Weight Calibration Kit. Each point indicates the mean (±SEM, n=4), although, in most instances the size of the points are greater than the standard error of the mean. Rf = the relative migration value.

The following solutions were prepared fresh on the day of use.

1. Wash solution. This consists of 10% ethanol, 5% acetic acid (HAc).

2. Developer, The contents of one formaldehyde ampoule (2% formaldehyde) were mixed with one bottle of 2.5% sodium carbonate. The mixture was shaken vigorously for 5 s.

2. Background reducer (pH 5 - 6). One packet of sodium thiosulphate (1.6 g) and 3.7 g Tris.HCl added to 100 ml reagent grade water.

3. Fixing Buffer. This consists of 10 ml 5% glutaraldehyde, 4 ml 1.6% sodium thiosulphate in water, 30 ml 95% ethanol and 5% isopropanol and 0.3 g of sodium acetate, made up to 100 ml with reagent grade water.

Silver staining was carried out in the "development unit" of a PhastSystem according to PhastSystem Owner's Manual (1986) as indicated in Table 2.1.7.4.1. The solutions were connected to the PhastSystem Developing Unit via tubes in numerical order and the programme was started. First, the gels were washed by Wash Solution for 2 and 4 min at 50°C. The gels were then exposed to Fixing Buffer for 6 min at 50°C. The gels were then washed twice with Wash Solution for 3 and 5 min at 50°C followed by two further washings with Reagent Grade Water for 2 min at 50°C. The gels were then exposed to Staining Solution for 6.5 min at 40°C followed by twice washing with Reagent Grade Water for 0.5 min at 30°C. Developer Solution was then run for 4.5 min at 30°C after which the gels were exposed to Background Reducing Solution for 2 min at 30°C. The Silver Staining process was terminated by exposing the gels to Stop Solution for 5 min at 50°C. When the silver staining process finished, the gel was taken out from the

Table 2.1.7.4.1. Process of Silver Staining, as described in the text.

Step Order	Solution	Tube order	Time (min)	Temp (°C)	Remarks
1	10% ethanol,5% HAc	3	2	50	Wash solution
2	10% ethanol,5% Hac	3	4	50	Wash solution
3	"Fixing Buffer"	4	6	50	High sensitivity fix solution
4	10% ethanol,5% HAc	3	3	50	Wash solution
5	10% ethanol,5% HAc	3	5	50	Wash solution
6	Reagent grade water	5	2	50	Wash solution
7	Reagent grade water	5	2	50	Wash solution
8	0.4% silver nitrate	6	6.5	40	Staining solution
9	Reagent grade water	5	0.5	30	Wash solution
10	Reagent grade water	5	0.5	30	Wash solution
11	Developer	7	0.5	30	Developing solution
12	Developer	7	4.0	30	Developing solution
13	Background reducer	8	2.0	30	Background reducing solution
14	5-10% glycerol	9	5	50	Stop solution

PhastSystem and left to dry at room temperature. The result indicated that the loaded amounts of samples contained proteins (Figure 2.1.7.3.2).

2.1.8 RADIOIMMUNOASSAY (RIA) OF PROSTAGLANDINS

2.1.8.1 Assay Procedure

Radioimmunoassay which was developed in the 1960s is a very powerful method for the measurement of PGs. The amount of [^{3}H]PG (tracer) used in each assay was sufficient to obtain counts of about 15000 - 20000 per tube when counted in a scintillation counter for 4 min ([^{3}H]$PGF_{2\alpha}$ and [^{3}H]6-keto-$PGF_{1\alpha}$) or for 10 min ([^{3}H]PGE_2). Tracer solutions were prepared by evaporating off the carrier solvent (i.e. methanol or acetonitrile : water) in a stream of air and then redissolving the residue in an appropriate quantity of diluent (see Section 2.2.7.6 for composition), so that a 50 µl aliquot of this solution provided the required number of counts.

The antiserum dilution chosen was that which gave between 60% - 80% binding of tracer PG in the absence of non-radioactive standard PG (i.e. zero standard). An aliquot was taken from a 1:100 stock solution of the antiserum in the appropriate diluent in order to prepare the final dilution required.

Standard PG solutions were made up in the appropriate diluent from a stock solution of 1 µg/ml in methanol; 200 µl of this solution were blown dry and redissolved in 20 ml of an appropriate diluent to give a final PG concentration of 10 ng/ml. Series of dilutions (nine for PGE_2 and 6-keto-$PGF_{1\alpha}$ and ten for $PGF_{2\alpha}$ assays) were then made from this PG concentration (i.e. 10 ng/ml) so that standard

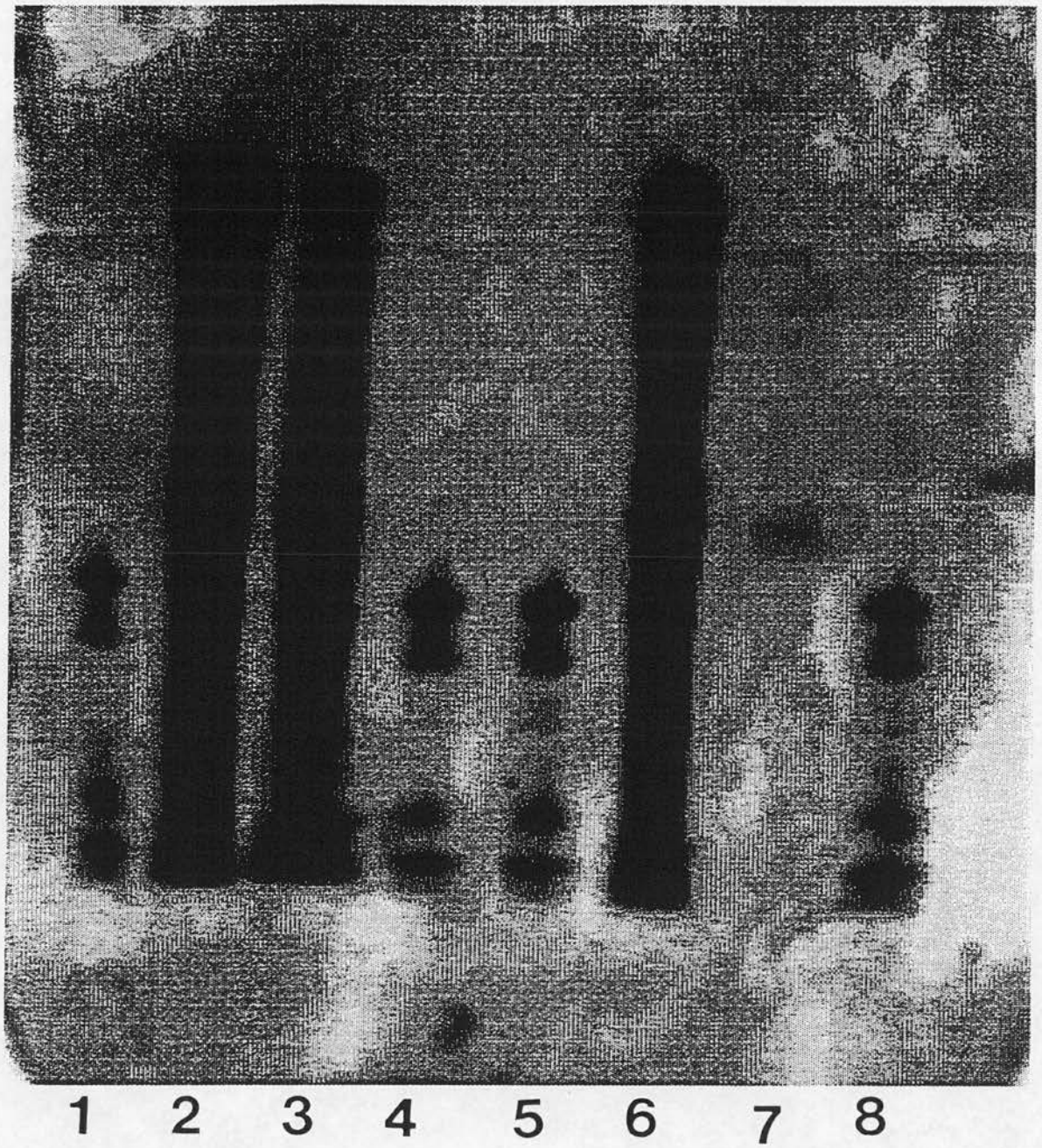


Figure 2.1.7.3.2. Silver staining of the soluble cell extracts obtained from the guinea-pig endometrium. Soluble cell extract proteins (7 μg) were resolved by one-dimensional SDS-PAGE and subjected to the Silver Staining process. Molecular Weight Marker (lanes 1, 4, 5 and 8), Day 6 endometrium (lanes 2 & 3) and Day 17 endometrium (lane 6).

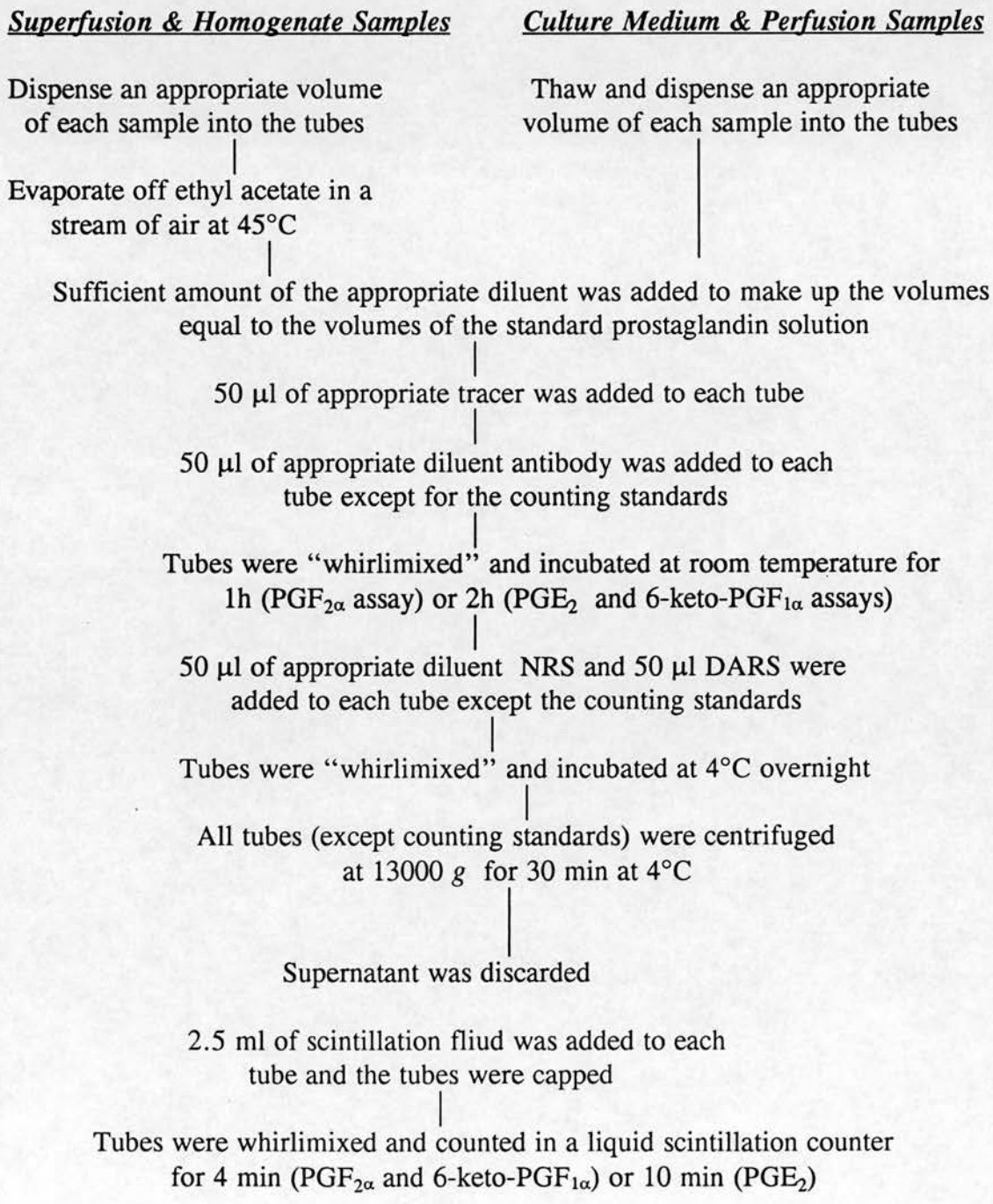
PG solutions ranging from 0.02 - 5.12 ng/ml for PGE_2 and 6-keto-$PGF_{1\alpha}$ standards, and ranging from 0.005 - 2.50 ng/ml for $PGF_{2\alpha}$ were obtained. The standard solutions were then kept at -20°C. From each of these standard solutions, 0.5 ml was dispensed in triplicate into 3 ml plastic tubes, as shown in Table 2.1.8.1.1. Counting standards (tubes 5 - 8) and non-specific binding standards (tubes 1 - 4) were also prepared and dispensed in quadruplicate as indicated in Table 2.1.8.1.1. The four counting standards when averaged gave an estimate of the number of radioactive counts within each tube. The non-specific binding standards estimate [^{3}H]PG binding to sites other than the specific PG binding sites, which include the plastic tubes, non-specific sites on the antiserum, and to other compounds used in the assay. The non-specific binding was always less than 10% of total binding in every assay. The last four tubes of each assay contained 500 µl of the appropriate diluent in duplicate (zero standards), and 500 µl of the appropriate PG (0.08 ng/ml for $PGF_{2\alpha}$ or 0.32 ng/ml for PGE_2 and 6-keto-$PGF_{1\alpha}$) standard solution in duplicate so that the inter-assay coefficient of variation could be calculated.

The assay procedure is outlined in the flow diagram (Figure 2.1.8.1.1). Appropriate volumes from the extracted samples in ethyl acetate from the superperfusion or homogenisation experiments were dispensed in duplicate into plastic tubes and then dried under a stream of air at 45°C; 0.5 ml diluent was then added to each tube so that the volumes in the standard and sample-containing tubes were the same. Samples obtained from the mesenteric vascular perfusion, and from tissue and cell culture were assayed directly without extraction; however an

Table 2.1.8.1.1 The concentration and volumes of standard solutions of $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ used to execute each radioimmunoassay standard curve.

Tube number	(ng PG/Tube) $PGF_{2\alpha}$	(ng PG/Tube) PGE_2 / 6-keto-$PGF_{1\alpha}$	Volume taken (µl)
1 -4	10	10	500
5 -8	-	-	-
9 -11	0.005	0.02	500
12 - 14	0.01	0.04	500
15 -17	0.02	0.08	500
18 - 20	0.04	0.16	500
21 - 23	0.08	0.32	500
24 - 26	0.16	0.64	500
27 - 29	0.32	0.128	500
30 - 31	0.64	2.56	500
32 - 35	1.28	5.12	500
36 - 38	2.56	-	500

Figure 2.1.8.1.1 The procedure used for the measurement of prostaglandins by radioimmunoassay.



appropriate amount of diluent was added to each tube so that the volumes in the standard and sample-containing tubes were the same. When tissue and cell culture samples were assayed, 100 µl of Medium 199 or Ham's F-10 medium were added into all tubes containing the standards as well as the zero standard tubes to minimise the effect of the medium on the PG assay.

Tubes containing the samples and standards were then treated in the same way throughout the assay. To each tube, 50 µl of the appropriate tracer and antiserum were added. Tubes were then "whirlimixed" and incubated at room temperature for 1 h (in case of the $PGF_{2\alpha}$ assay) or 2 h (in the case of the PGE_2 and 6-keto-$PGF_{1\alpha}$ assays). Normal rabbit serum (NRS; 50 µl; 1:140 dilution for 6-keto-$PGF_{1\alpha}$ and 1:100 dilutions for both $PGF_{2\alpha}$ and PGE_2) and donkey anti-rabbit serum (DARS; 50 µl; 1:10 dilution for all PGs) were then added to each tube and the tubes were once again "whirlimixed". NRS was added to ensure that, at dilutions of the antisera used, there was sufficient gamma-globulin present for adequate precipitation. DARS was added to separate free PG from antibody-bound PG. The tubes were then incubated at 4°C overnight. The following day, the tubes were centrifuged at 13000 *g* for 30 min at 4°C. The supernatant was poured off and discarded. 2.5 ml of scintillation fluid were added to each tube (see Section 2.2.7.6.iv). Tubes were then capped and "whirlimixed" to resuspend the pellet, after which they were counted for 4 min for the $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ assays or for 10 min for the PGE_2 assay using a Packard Liquid Scintillation Counter.

The % tracer bound was calculated from the formula:

$$\% \text{ bound} = (\text{counts in tube} \times 100)/(\text{average standard counts})$$

where the "average standard counts" is the average of the counts from tubes 5 - 8 (the counting standards). Data from the counter were passed directly into an IBM - XT personal computer programmed with the Packard Data Acquisition and Analysis system (PC-DAAS). The Spline Curve-Fit programme was used which processes the standards according to a Modified Smooth Spline Algorithm. This programme subtracted the amount of non-specific binding from all standards then calculated the co-ordinates for the curve of best fit. Using this standard curve, the computer calculated the amount of PG in each sample tube.

The intra-assay coefficient of variation was calculated using the formula:

Intra-assay coefficient of variation =

(standard deviation of sample duplicate x 100) / (mean value of sample duplicate)

This coefficient was calculated by the computer for each unknown sample assayed in duplicate. If the coefficient for the sample was greater than 12%, the sample was re-assayed.

The inter-assay coefficient of variation was calculated using the values obtained from tubes containing a known standard amount of the appropriate PG (0.32 ng/ml for PGE_2, and 6-keto-$PGF_{1\alpha}$; 0.08 ng/ml for $PGF_{2\alpha}$), which were included at the end of the assay, according to the formula below:

Inter-assay coefficient of variation =

(standard deviation of known PG standard x 100) / (mean of known PG standard)

The inter-assay coefficients of variation values obtained for the $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ were 6.1, 4.2 and 5.8, respectively.

2.1.8.2 Determination of Cross-Reactivities for PGs and Their Metabolites

Cross-reactivities for each of the prostaglandin antiserum have been determined previously in this laboratory (Dighe *et al.*, 1975; Poyser & Scott, 1980; Lytton & Poyser, 1982). The method used was such that standard curves for PGs and their different PG metabolites were set up. The concentration of the different PG or PG metabolite which produced a 50% fall in binding from the binding produced by a zero standard solution of the PG to which the antiserum was raised, was obtained and the percentage cross -reactivity was calculated using the formula:

% cross-reactivity =

$$\frac{\text{concentration of PG (to which the antisera was raised) giving a 50\% fall in zero standard binding}}{\text{concentration of a different PG or PG metabolite giving a 50\% fall in zero standard binding}} \times 100$$

Cross-reactivities of the antisera used with various prostanoids and their metabolites for $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ as determined previously (Dighe *et al.*, 1975; Poyser and Scott, 1980; Lytton and Poyser, 1982) are shown in Tables 2.1.8.2.1 to 2.1.8.2.3.

Table 2.1.8.2.1. Cross-reactivities with various prostanoids and their metabolites for the $PGF_{2\alpha}$ antiserum (rabbit 5, 6th bleed), measured at 30% binding of tracer.

Prostanoid	% Cross-Reactivity at 30% binding of tracer
$PGF_{2\alpha}$	100
$PGF_{1\alpha}$	28
PGE_2	0.2
PGE_1	0.5
PGA_2	<0.1
PGB_2	<0.1
PGD_2	0.4
15-keto-$PGF_{2\alpha}$	0.5
13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.2
6-keto-$PGF_{1\alpha}$	0.5
15-keto-PGE_2	<0.1
13,14-dihydro-15-keto-PGE_2	0.1
TXB_2	0.6

Table 2.1.8.2.2. Cross-reactivities with various prostanoids and their metabolites for the PGE_2 antiserum (rabbit R5, 6th bleed), measured at 30% binding of tracer.

Prostanoid	% Cross-Reactivity at 30% binding of tracer
PGE_2	100
PGE_1	94
PGA_2	13.6
PGB_2	72.7
PGD_2	0.4
$PGF_{1\alpha}$	4.3
$PGF_{2\alpha}$	3.8
15-keto-$PGF_{2\alpha}$	0.1
13,14-dihydro-15-keto-$PGF_{2\alpha}$	<0.1
6-keto-$PGF_{1\alpha}$	0.3
15-keto-PGE_2	0.2
13,14-dihydro-15-keto-PGE_2	0.4
TXB_2	0.2

Table 2.1.8.2.3. Cross-reactivities with various prostanoids and their metabolites for the 6-keto-$PGF_{1\alpha}$ antiserum (rabbit NP1, 6th bleed), measured at 30% binding of tracer.

Prostanoid	% Cross-Reactivity at 30% binding of tracer
6-keto-$PGF_{1\alpha}$	100
$PGF_{1\alpha}$	0.4
PGE_2	4.2
PGE_1	1.1
PGA_2	<0.1
PGB_2	<0.1
PGD_2	<0.1
$PGF_{2\alpha}$	<0.1
13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.1
15-keto-$PGF_{2\alpha}$	<0.1
15-keto-PGE_2	0.1
13,14-dihydro-15-keto-PGE_2	0.1
TXB_2	<0.1

2.1.8.3 Standard Curves for Prostaglandin Assays

The standard curves obtained for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ are the results of averaging six consecutive assays for each PG. The values indicate the means $\pm$ s.e.m for each assay. The standard curves are shown in Figures 2.1.8.3.1 to 2.1.8.3.3.

2.2 MATERIALS

The sources of the materials are as follows:

2.2.1 Solvents

Absolute ethanol - J. Boroughs., London, U.K.

Acetone - Rathburn Chemicals Ltd, Walkerburn, U.K.

Acetonitrile - Rathburn Chemicals Ltd, U.K.

2-Ethoxyethanol (reagent grade) Fisons Scientific Equipment, Loughborough, U.K.

Ethyl acetate* - Rathburn Chemicals Ltd, Walkerburn, U.K.

Methanol - Rathburn Chemicals Ltd, Walkerburn, U.K.

Toluene (reagent grade) - Rathburn Chemicals Ltd, Walkerburn, U.K.

Chloroform ($CHCl_3$)

* Redistilled prior to use

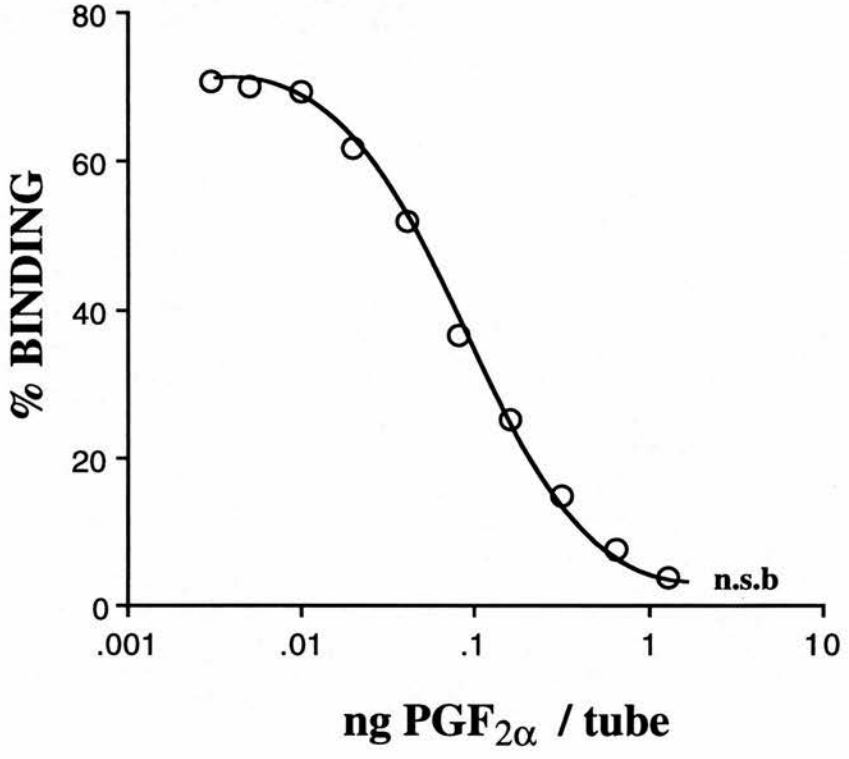


Figure 2.1.8.3.1. Standard curve for $PGF_{2\alpha}$ radioimmunoassay. The data indicate the mean (±SEM, n=6) although, in most instances, the size of the point is greater than the standard error of the mean. n.s.b. = Non-specific binding

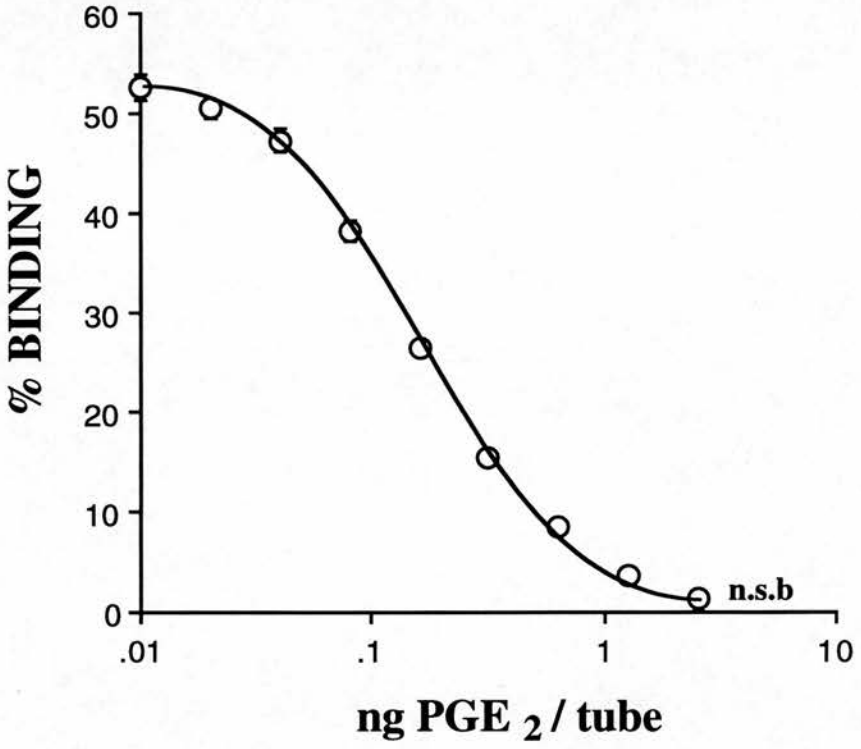


Figure 2.1.8.3.2. Standard curve for PGE_2 radioimmunoassay. The data indicate the mean (±SEM, n=6) although, in most instances, the size of the point is greater than the standard error of the mean. n.s.b. = Non-specific binding

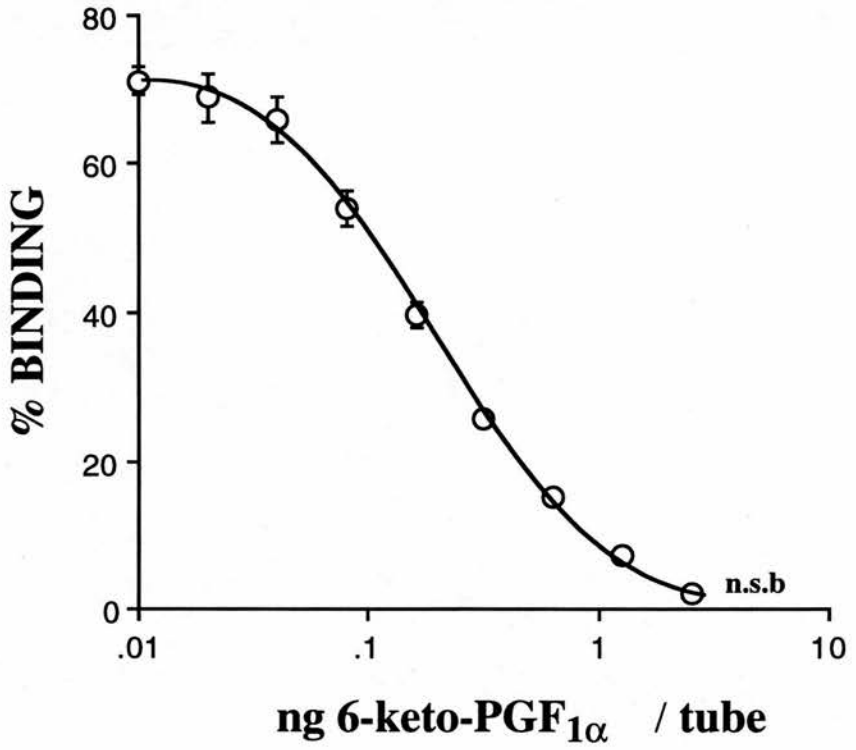


Figure 2.1.8.3.3. Standard curve for 6-keto-$PGF_{1\alpha}$ radioimmunoassay. The data indicate the mean (±SEM, n=6) although, in most instances, the size of the point is greater than the standard error of the mean. n.s.b. = Non-specific binding

2.2.2 Radioactive Compounds

[5,6,8,9,11,12,14,15(n)-^{3}H] Prostaglandin $F_{2\alpha}$ (Specific Activity 202 - 208 Ci/mmol).

[5,6,8,11,12,14,15(n)-^{3}H] Prostaglandin E_2 (Specific Activity 185 - 200 Ci/mmol).

6-keto-[5,8,9,11,12,14,15(n)-^{3}H] Prostaglandin $F_{1\alpha}$ (Specific Activity 170 - 180 Ci/mmol).

All [^{3}H] PG stock solutions were diluted to 5 μCi/ml and stored at -20°C before being used for radioimmunoassay. [^{3}H]$PGF_{2\alpha}$ and [^{3}H]PGE_2 were diluted in methanol and [^{3}H]6-keto-$PGF_{1\alpha}$ was diluted in acetonitrile:water (9:1) for storage.

All radioactive compounds were supplied by Amersham International Ltd., Cardiff, U.K.

2.2.3 Chemicals

A23187 (Calcium ionophore) - Calbiochem-Behring Corp., Nottingham, U.K.

Acetonitrile - Sigma Chemicals Co., Poole, Dorset, U.K.

Actinomycin D - Sigma Chemicals Co., Poole, Dorset, U.K.

Amphotericin B Solution (250 μg/ml) - Sigma Chemicals Co., Poole, Dorset, U.K.

Aristolochic acid - Sigma Chemicals Co., Poole, Dorset, U.K.

Berberine chloride - Sigma Chemicals Co., Poole, Dorset, U.K.

Bovine serum albumin (BSA) - Sigma Chemicals Co., Poole, Dorset, U.K.

Caffeine - Sigma Chemicals Co., Poole, Dorset, U.K.

Calcium chloride - B.D.H. Chemicals Ltd, U.K.

(CHAPS) [3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfonate] - Sigma Chemicals Co., Poole, Dorset, U.K.

Cycloheximide - Sigma Chemicals Co., Poole, Dorset, U.K.

Dantrolene sodium salt - Sigma Chemicals Co., Poole, Dorset, U.K.

D-Glucose - B.D.H. Chemicals Ltd, U.K.

Dithiothreitol (DTT) - Sigma Chemicals Co., Poole, Dorset, U.K.

DMSO (dimethyl sulfoxide) - Sigma Chemicals Co., Poole, Dorset, U.K.

EDTA (diaminoethanetetra-acetic acid)) - Fison's Scientific Equipment, Leics., U.K.

EGTA (Ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid) - Sigma Chemicals Co., Poole, Dorset, U.K.

Elite ABC Kit Vectastain^{R} - Vector Laboratories, Peterborough, U.K.

Forskolin [(7β-acetoxy-1α,6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one); from Coleus forskohlii] - Sigma Chemicals Co., Poole, Dorset, U.K.

Fura-2 acetoxymethyl ester (Fura-2/AM) - Sigma Chemicals Co., Poole, Dorset, U.K.

Gelatine - B.D.H. Chemicals Ltd, U.K.

Glycerol - Sigma Chemicals Co., Poole, Dorset, U.K.

Hank's Balanced Salt Solution (HBSS) - Sigma Chemicals Co., Poole, Dorset, U.K.

Hydrochloric acid (HCl) - B.D.H. Chemicals Ltd, U.K.

Ionomycin calcium salt (from Streptomyces conglobatus) - Sigma Chemicals Co., Poole, Dorset, U.K.

Indomethacin - Merck, Sharpe & Dohme Ltd, Hoddesdon, Herts., U.K.

Magnesium Sulphate - Sigma Chemicals Co., Poole, Dorset, U.K.

Medium 199 (plus Earle's salts & 2.20 g/l sodium bicarbonate) - Flow Laboratories, Irvine, U.K.

Melittin - Sigma Chemicals Co., Poole, Dorset, U.K.

2-Mercaptoethanol - Sigma Chemicals Co., Poole, Dorset, U.K.

Molecular weight marker - Sigma Chemicals Co., Poole, Dorset, U.K.

NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide - A Gift from Taisho Pharmaceutical Co., Ltd. Tokyo, JAPAN

Nutrient mixture Ham's F-10 - Sigma Chemicals Co., Poole, Dorset, U.K.

Oestradiol-17β - Sigma Chemicals Co., Poole, Dorset, U.K.

Penicillin G potassium salt - ICN Biochemicals Inc. Aurora, Ohio, USA.

Peroxidase labelled anti-rabbit antibody - Amersham International Ltd., Cardiff, U.K.

Peroxidase substrate kit (DAB SK-4100) - Vector Laboratories, Peterborough, U.K.

PGH Synthase 2 Polyclonal Antiserum - Cascade Biochem Ltd., Berkshire, U.K.

Phospholipase A_2 (from Naja naja venom) - Sigma Chemicals Co., Poole, Dorset, U.K.

Platelet activating factor (PAF) - Sigma Chemicals Co., Poole, Dorset, U.K.

Potassium chloride - B.D.H. Chemicals Ltd, U.K.

Potassium phosphate - Sigma Chemicals Co., Poole, Dorset, U.K.

PPO (2,5-diphenyloxazole) - B.D.H. Chemicals Ltd, U.K.

Progesterone - Sigma Chemicals Co., Poole, Dorset, U.K.

Prostaglandin $F_{2\alpha}$ - Sigma Chemicals Co., Poole, Dorset, U.K.

Prostaglandin E_2 - Sigma Chemicals Co., Poole, Dorset, U.K.

6-keto-Prostaglandin $F_{1\alpha}$ - Sigma Chemicals Co., Poole, Dorset, U.K.

Puromycin - Sigma Chemicals Co., Poole, Dorset, U.K.

Ruthenium red (Ruthenium oxychloride ammoniated) - Sigma Chemicals Co., Poole, Dorset, U.K.

Ryanodine - Calbiochem-Novabiochem, Nottingham, U.K.

Sodium azide - Hopkin and Williams Ltd, Essex, U.K.

Sodium chloride - Fison's Scientific Equipment, Leics., U.K.

Sodium dihydrogen orthophosphate - B.D.H. Chemicals Ltd, U.K.

Sodium dodecyl sulphate (SDS) - Sigma Chemical Ltd, U.K.

Sodium hydrogen carbonate - B.D.H. Chemicals Ltd, U.K.

Sodium hydroxide - B.D.H. Chemicals Ltd, U.K.

Streptomycin sulphate - Sigma Chemicals Co., Poole, Dorset, U.K.

Sucrose - B.D.H. Chemicals Ltd, U.K.

Thapsigargin - Sigma Chemicals Co., Poole, Dorset, U.K.

Theophylline - Sigma Chemicals Co., Poole, Dorset, U.K.

TMB-8 (3,4,5-trimethoxybenzoic acid,8-(dimethylamino)octyl ester hydrochloride) - Sigma Chemicals Co., Poole, Dorset, U.K.

Trifluoperazine dihydrochloride - Sigma Chemicals Co., Poole, Dorset, U.K.

Tris (hydroxymethyl)methylamine, (Tris) - B.D.H. Chemicals Ltd, U.K.

Tris HCl - Sigma Chemicals Co., Poole, Dorset, U.K.

Tryptan blue solution (0.4%) - Sigma Chemicals Co., Poole, Dorset, U.K.

Tween-20 - Sigma Chemicals Co., Poole, Dorset, U.K.

W-7 (N-(6-aminohexyl)-3-chloro-1-naphthalenesulfonamide hydrochloride) - Sigma Chemicals Co., Poole, Dorset, U.K.

2.2.4 Antibodies

Donkey anti-rabbit serum (DARS) & normal rabbit serum (NRS) - Scottish Antibody Production Unit, Carluke, Scotland, U.K.

$PGF_{2\alpha}$ antiserum, PGE_2 antiserum, and 6-keto-$PGF_{1\alpha}$ antiserum were all raised in this laboratory in rabbits (Dighe *et al.*, 1975; Dighe *et al.*, 1978a; Dighe *et al.*, 1978b).

Stock solutions of antisera and NRS were stored at -20°C. Stock solutions were diluted with the appropriate diluent and stored at 4°C before use.

2.2.5 Gases

95% air : 5% CO_2

95% O_2 : 5% CO_2

All gases were supplied by British Oxygen Co. Ltd., Guilford, U.K.

2.2.6 Other Materials

Petri dishes (vented, 5 cm diameter) - Sterilin Ltd., Teddington, U.K.

Cell culture plates (24-well) - Nunc A/S, Kamstrupvej 90, K-D-4000 Roskilde, Denmark.

Plastic tubes (3 ml) and caps - Sarsted Ltd., Leicester, U.K.

Micropipette tips - L.I.P. Ltd., Shipley, U.K.

Eppendorf microfuge tubes - Scot Lab. Ltd., Glasgow, Scotland, U.K.

Sample Applicator 8/1 PhastGel^{R} - Pharmacia, Uppsala, Sweden

10%-15% SDS Gels PhastGel^{R} - Pharmacia, Uppsala, Sweden

Filter Paper (50 x 50 mm) PhastGel^{R} - Pharmacia, Uppsala, Sweden

SDS Buffer Strips PhastGel^{R} - Pharmacia, Uppsala, Sweden

Immobilon-P^{TM} "Transfer Membrane" (Pore size 0.45 μM) - Pharmacia, Uppsala, Sweden

2.2.7 Composition of Solutions

2.2.7.1 Krebs solution*	**for 1 litre**
D-Glucose	2.1 g
$NaHCO_3$	2.1 g
NaCl	6.9 g
10% KH_2PO_4	1.6 ml
10% KCl	3.5 ml

10% $MgSO_4.7H_2O$	1.4 ml
1 M $CaCl_2$	2.5 ml

* $CaCl_2$ was omitted from nominally calcium-free Krebs solution

2.2.7.2 McEwans Solution	**for 1 litre**
D-Glucose	2.1 g
Sucrose	4.2 g
$NaHCO_3$	2.1 g
NaCl	7.6 g
10% KH_2PO_4	1.4 ml
10% KCl	4.2 ml
1 M $CaCl_2$	2.5 ml

2.2.7.3 Modified Medium 199 (With Earle's Salts & 2.20 g/l $NaHCO_3$), (M199)

The medium used for tissue culture in this thesis was modified Medium 199 including Earle's balanced salts solution plus 2.20 g/l sodium bicarbonate (EBSS) which was developed by Morgan *et al.* (1950) by the addition of a synthetic mixture of inorganic salts supplemented with glucose. The functions of EBSS in modified M199 are:

(i) Sodium bicarbonate (2.20 g/l) present to maintain pH buffer.

(ii) to maintain osmotic pressure.

(iii) to provide energy by the presence of glucose.

Medium 199 was further supplemented with antibiotics [penicillin (100 U/ml), streptomycin (100 μg/ml)], and amphotericin B (2.5 μg/ml) to prevent bacterial and fungal growth as well as L-glutamine (1.7 μM), an essential factor for stromal viability and good epithelial preservation (Kaufman *et al.*, 1980). The constituents of M199 are shown in Table 2.2.7.3.

2.2.7.4. Nutrient Mixture Ham's F-10

Cells were cultured in Ham's F-10 medium which was developed from nutrient mixture F-7 by Ham for the growth of diploid Chinese hamster ovary (strain CHD-3), strain CHL-1 Chinese hamster lung cells and strain S3-9-IV human Hela cell line (Ham, 1963). He reported that nutrient F-10 supplemented by fetal calf serum concentration to 10% or 15% could be used to obtain a good growth rate of the above mentioned cell lines as well as human diploid cell line and human white blood cells. Medium Ham's F-10 was not commercially available until late 1960's (Morton, 1970). The sodium bicarbonate was omitted from the original formulation and buffered with HEPES to maintain pH. The constituents of the medium Ham's F-10 are shown in Table 2.2.7.4.

2.2.7.5 Diluent Solution for PG Radioimmunoassays

(i) Diluent Solution for $PGF_{2\alpha}$ Radioimmunoassay

0.05 M Tris buffer pH 8.0

Table 2.2.7.3 Composition of Medium 199 modified with Earle's salts and 2.20 g/l sodium bicarbonate without glutamine.

Storage Temperature 2 - 8°C

Ingredient	mg/litre	Ingredient	mg/litre
L-Alanine	25.00	l-Inositol	0.05
L-Arginine.HCl	70.00	Menadione, $NaHSO_3.3H_20$	0.019
L-Aspartic acid	30.00	Nicotinamide	0.025
L-Cysteine.HCl.H_20	0.11	p-Aminobenzoic acid	0.05
L-Cystine.2HCl	26.00	Pyridoxine.HCl	0.05
L-Glutamic acid	75.00	Riboflavin	0.01
L-Glutamine	100.00	Thiamine.HCl	0.01
Glutathione	0.05	DL-αTocopherolphosphate disodium salt	0.01
Glycine	50.00	$CaCl_2$ (anhydrous)	200.00
L-Histidine.HCl.H_20	21.88	$Fe(NO_3)_3.9H_20$	0.72
L-Hydroxyproline	10.00	KCI	400.00
L-Isoleucine	20.00	$MgSO_4$ (anhydrous)	97.70
L-Leucine	60.00	Vitamin A acetate	0.14
L-Lysine.HCl	70.00	$NaHCO_3$	2200.00
L-Methionine	15.00	$NaH_2PO_4.H_2O$	140.00
L-Phenyialanine	25.00	Adenine sulphate	10.00
L-Proline	40.00	Adenylic acid	0.20
L-Serine	25.00	ATP, disodium salt	1.00
L-Threonine	30.00	Cholesterol	0.20
L-Tryptophan	10.00	2-Deoxyribose	0.50
L-Tryosine disodium salt.$2H_2O$	57.66	D-Glucose	1000.00
L-Valine	25.00	Guanine.HCl	0.30
Ascorbic acid	0.05	Hypoxanthine, Na	0.354
Biotin	0.01	D-Ribose	0.50
Calciferol	0.10	Sodium acetate (anhydrous)	50.00
D-Ca, pantothenate	0.01	Phenol red sodium salt	10.00
Choline chloride	0.50	Thymine	0.30
Folic acid	0.01	Tween 80	5.00
NaCl	6800.00	Uracil	0.30
Xanthine, Na	0.34		

Table 2.2.7.4. Composition of Nutrient mixture Ham's F-10 Medium. This medium contains ingredients at optimum concentrations for supporting the clonal growth of Chinese Hamster Ovary (CHO) cells. It is now a popular medium for the growth of fastidious cell lines.

Storage Temperature 20.8°C

Ingredient	**mg/litre**	**Ingredient**	**mg/litre**
L-Alanine	9.00	L-Threonine	3.57
L-Arginine.HCl	211.00	L-Tryptophan	0.60
L-Asparagine.H_2O	15.00	L-Tyrosine disodium salt.$2H_2O$	2.61
L-Aspartic acid	13.30	L-Valine	3.50
L-Cysteine.HCl	31.50	Biotin	0.024
L-Glutamic acid	14.70	D-Ca pantothenate	0.715
L-Glutamine	146.20	Choline chloride	0.698
Glycine	7.50	Folic acid	1.32
L-Histidine.HCl.H_2O	23.00	l-Inositol	0.541
L-Isoleucine	2.60	Nicotinamide	0.615
L-Leucine	13.00	Pyridoxine.HCl	0.206
L-Lysine.HCl	29.00	Riboflavin	0.376
L-Methionine	4.48	Thiamine.HCl	1.00
L-Phenylalanine	5.00	Vitamin B_{12}	1.36
L-Proline	11.50	$CaCl_2$ (anhydrous)	33.30
L-Serine	10.50	$CuSO_4$ (anhydrous)	0.0016
$FeSO_4.7H_2O$	0.834	$ZnSO_4.7H_2O$	0.0288
KCl	285.00	D-Glucose	1100.00
KH_2PO_4	83.00	Hypoxanthine, Na	4.74
$MgSO_4$ (anhydrous)	74.60	Lipoic acid	0.20
NaCl	7400.00	Phenol red sodium salt	1.20
Na_2HPO_4 (anhydrous)	153.70	Sodium pyruvate	110.00
$NaHCO_3$	1200.00	Thymidine	0.73

0.1 g/l Sodium azide

1.0 g/l Gelatine

"Tris diluent pH 8"

(ii) Diluent Solution for PGE_2 Radioimmunoassay

0.05 M Phosphate (6.9 g/l Na_2HPO_4 (anhydrous) + 1.7 g $NaH_2PO_4.2H_2O$/litre) buffer pH 7.5

0.1 g/l Sodium azide

1.0 g/l Gelatine

"Phosphate diluent pH 7.5"

(iii) Diluent Solution for 6-keto-$PGF_{1\alpha}$ Radioimmunoassay

0.05 M Tris buffer pH 6.8.

0.1 g/l Sodium azide

1.0 g/l gelatine

"Tris diluent pH 6.8"

All diluents were stored at 4°C.

(iv) Scintillation Fluid for PG Radioimmunoassay

1.5 litre Toluene

0.9 litre 2-Ethoxyethanol

10.5 g 2,5-Diphenoxyloxazole (PPO)

2.2.7.6 Solutions for SDS-PAGE and Western Blotting

2.2.7.6.1 Laemmli or Sample Buffer

62.5 mM Tris-HCl, pH 6.8	0.151 g
2% SDS	0.4 g
10 mM DTT (1 M stock)	0.2 ml
5% 2-mercaptoethanol	1.0 ml
10% glycerol	2.0 ml

Final Volume = 20 ml

2.2.7.6.2 Transfer Buffer

20 mM Tris	1.5 g Tris HCl
192 mM glycine	7.2 g glycine
20% methanol	100 ml methanol
deionised distilled water	400 ml MQ water

2.2.7.6.3 Tris Buffer Saline (TBS pH: 7.6)

20 mM Tris-HCl	4.84 g Tris
500 mM NaCl	58.48 g NaCl

Dissolved in 1.5 litre deionized H_2O, pH was adjusted to 7.5 with HCl, then volume was adjusted to 2 litre with deionized H_2O.

2.3 STATISTICAL PROCEDURES

It is obvious that the results of an experiment should be displayed in a form which makes them readily comprehensible to the reader. This can be done by presenting the results in terms of descriptive statistics which summarise certain aspects of the results. Two types of descriptive statistics are commonly used- one which gives a measure of central tendency (known as "the most typical value") and one which gives a measure of dispersion (the variability or spread) for a set of results.

The arithmetic mean most commonly used to measure "the most typical value". It is the average of a set of scores obtained by adding all scores together and dividing by the number of scores. Its advantage in theoretical terms is that it gives a better estimate of the population from which one sample of scores is drawn than the median, which is in turn better than the mode (Robson, 1977). The mean of a sample tends to differ less from the mean of the population than the median of the sample differs from the median of the population. This is because the mean extracts most information from the sample. One proof of this is that the mean would be altered by the alteration of any single one of the individual scores, i.e. the mean is dependent on the values of each of the scores in the sample. In contrast, both the median and the mode may be unaffected by changing the value of a number of scores or, on the other hand, they may be greatly affected by changing the value of a small number of the scores. Again in contrast to the mean, neither the median nor the mode is affected by extreme scores, either vary high or very low.

If the hypothesis that all samples in each experiments were drawn from the same population is true (for example treatment does not affect PG output), the within-

groups variance and between groups variance are both estimates of the same population variance and so should be about equal (Glantz, 1992). Therefore, F-test was carried out as follow:

$$F\ test = \frac{\text{population variance estimated from sample mean}}{\text{population variance estimated as average of the sample variance}}$$

Since both the numerator and the denominator are estimates of the same population variance, F should be about 1. For the random samples the value of F is equal to 1 (Glantz, 1992). If F is a big number, the variability between the samples is larger than the expected from the variability within the samples, so the hypothesis that all the samples were from the same population is rejected.

When there were more than two samples, the analysis of variance which is a multi-dimential "t" test was used. It allows to conclude only that the data are not consistent with the hypothesis that all the samples were drawn from a single population (Steel & Torrie, 1980). However, it does not help to decide which one or ones are most likely to differ from the others. To answer these questions a procedure that is specifically designed to test for differences in two groups: the "t" test or students "t" test was employed since standard error of the mean (s.e.m) quantifies the uncertainty of the estimate of the true population mean based on a sample. As the sample size increases, the uncertainty in the estimate of the difference between the test (treated) and the control (untreated) samples decreases relative to the difference of the means. To formalise this logic, "t" test is used which is calculated as:

$$\text{"t" test} = \frac{\text{difference in sample means}}{\text{standard error of difference of sample means}}$$

When this ratio is small, it was concluded that the data are compatible with the hypothesis that both samples were drawn from a single population and assesses that the treatment produced no effect.

"t" test is parametirc test since it is based on mean values and assumes that normal distribution is valid. Parametric methods generally provide more information about the treatment being studied and are more likely to detect a real treatment effect when the underlying population is normally distributed (Glantz, 1992).

This logic, while differing in emphasis from that used to develop the analysis of variance, is essentially the same (Glantz, 1992). In both cases, the relative magnitude of the differences in the sample means with the amount of variability that would be expected from looking within the samples were compared. To calculate "t" ratio it was necessary to determine (a) the difference of sample means and (b) the standard error of this difference.

The specific value of "t" one obtains from any two samples depends not only on whether or not there actually is a difference in the means of the populations from which the samples were drawn but also on which specific individuals happened to be selected for the samples. Thus, as for F, there will be a range of possible values that "t" can take, even when both samples are drawn from the same population. Since the means computed from the two samples will generally be close to the means of the population from which they were drawn, the value of "t" will tend to

be small when the two samples are drawn from the same population. Therefore, as for F, if the resulting value of "t" is "big" then the treatment had actually affected the samples. The critical value of "t" like F, have been tabulated and depends not only on the level of confidence with which one rejects the hypothesis of no difference- the P value- but also on the sample size. As with the F distribution, this depends on sample size which enters the table as the degree of freedom, which is equal to 2(n-1) for this "t" test, where n is the size of each sample. As the sample size increases, the value of "t" needed to reject the hypothesis of no difference decreases. The sampler the samples, the greater the difference demand by the "t" test; otherwise, since big differences occur more frequently as sample get smaller, the probability of getting a significant result would increase as the sample size decreases. With the "t" test, then, a big difference is needed to establish significant if the samples are small. As the sample size increases, smaller and smaller differences become significant. For instance, to be significant at the 5% level, the difference between samples of 4 would need to exceed 3.2 x SE-diff. But with samples of 12. a difference of 2.2 x SE-diff would be significant at this level.

All forms of analysis of variance, including the "t" test are based on the assumptions that the observations are drawn from normally distributed population in which the variance are the same even if the treatments changes the mean responses. These assumptions are often satisfied well enough to make analysis of variance an extremely useful statistical procedure.

The "t" distribution shares with normal distribution the properties of being symmetric and of extending from negative to positive infinity. However, it differs

from the normal in that it assumes different shapes depending on the number of degrees of freedom. By degrees of freedom is meant the quantity n-1, where n is the sample size upon which a variance has been based. It will be remembered that this quantity n-1 is the divisor in obtaining an unbiased estimate of the variance from a sum of squares (Skol & Rohlf, 1973).

As it was mentioned earlier, the analysis of although the analysis of variance allows to conclude that the data are not consistent with the hypothesis that all the samples were drawn from a single population, it does not help to decide which one or ones are most likely to differ from the others. An approach to the problem of comparing each treatment mean with every other treatment mean known as "Duncan's multiple range test" was used to test for a set of significant differences of increasing size, the size to depend upon the closeness of the mean after ranking, with the smallest value for adjacent mean and the largest value for extremes (Steel & Torrie, 1980). In this test the confidence levels are not appropriate; the notion of confidence is replaced by that of "protection levels" against finding false significant difference at various stages of testing. It uses a variable level depending on the number of means involved at any stage. The idea is that as the number of means under test increases, the smaller is the probability that they will all be alike.

In summary, since interest was on the mean (as it gives more information about a set of data) and the data was a continuos variable (scales) the Students "t" test was uesd, and where "t" test was not appropriate Duncan's multiple range test was used to determine any statistical significance between the values obtained from the experimental conditions.

SECTION THREE

3 RESULTS

This section deals, in full details, with all the results obtained from the experiments carried out for this thesis.

3.1 INVESTIGATION INTO THE ROLE OF INTRACELLULAR CALCIUM IN THE CONTROL OF PROSTAGLANDIN SYNTHESIS BY AND RELEASE FROM THE GUINEA-PIG UTERUS.

3.1.1 The Effects of Caffeine and Theophylline on Prostaglandin Output From The Day 7 and Day 15 Guinea-Pig Uterus Superfused *In Vitro*.

Introduction:

Prostaglandins (PG) produced by the uterus are involved in several reproductive processes. In many non-primate mammalian species, increased $PGF_{2\alpha}$ production by the endometrium is responsible for terminating the life-span of the corpus luteum and thereby regulating the length of an oestrous cycle or pseudopregnancy and, in some species, a pregnancy (see, Poyser, 1981). It has been shown that, in the guinea-pig, uterine prostaglandin synthesis is not mediated by cAMP (Poyser, 1987a), increased inositol turnover (Ning & Poyser, 1984) or a toxin-sensitive G-protein (Leckie & Poyser, 1990a, b). However, it has been postulated that the release of calcium from an internal store is involved in increased uterine

prostaglandin production (Riley & Poyser, 1987b). Hence, this set of experiments was designed to investigate whether caffeine (a releaser of calcium from an internal store) would affect PG output from the day 7 and day 15 guinea-pig uterus superfused *in vitro*.

Methods:

Tissue preparation and the experimental procedures were as described in Section 2.2.1. Briefly, guinea-pigs were killed (by stunning followed by exsanguination) on days 7 or 15 of the cycle. Each uterus was removed and separated into its two uterine horns. The uterine horns were blotted dry and weighed. Each uterine horn was opened by cutting longitudinally down the anti-mesometrial side and suspended in an organ bath with one end attached to an isotonic lever. Each uterine horn was superfused at 5 ml/min with Krebs solution (see Section 2.2.7.1 for composition) at 37°C, as described by Poyser and Brydon (1983), and was superfused initially for an equilibrium period of 60 min. Samples of superfusate were then collected for 10 min periods over the next 80 min (i.e 8 samples per uterine horn). After collection, the pH of each sample was lowered to 4.0 with 1 M HCl and the prostaglandins were extracted by shaking twice with 50 ml ethyl acetate, according to the method described by Poyser (1972). The two ethyl acetate fractions were combined and evaporated to dryness on a rotary evaporator. Each dried extract was redissolved in 10 ml ethyl acetate and stored at -20°C until assayed.

After the initial settling period of 60 min, uterine horns in each experiment were treated as described below. In experiment 1, caffeine (10 mM) was present in the

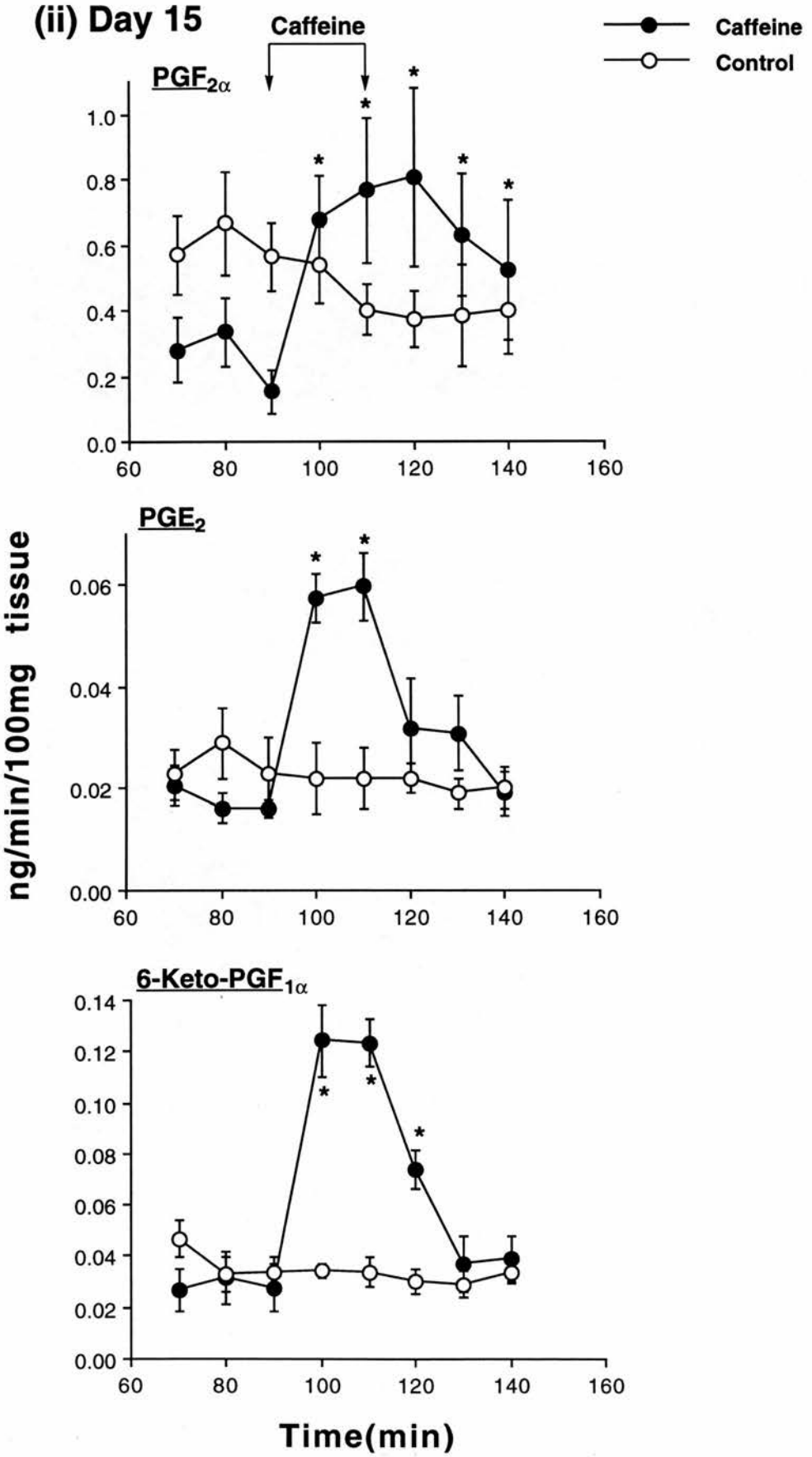
Krebs solution superfusing one uterine horn from each of the five animals on day 7 of the oestrous cycle and from five animals on day 15 of the oestrous cycle during the collection of samples 4 and 5. The other uterine horn from each animal was untreated and hence acted as control tissue. In experiment 2, theophylline (10 mM) was present in the Krebs solution superfusing one uterine horn from each of the four animals on day 7 of the oestrous cycle during the collection of samples 4 and 5. The other uterine horn from each animal was untreated and hence acted as the control tissue. In experiment 3, caffeine (1 mM) was present in the Krebs solution superfusing one uterine horn from each of the four animals on day 7 of the oestrous cycle during the collection of samples 4 and 5, and caffeine (0.1 mM) was present in the Krebs solution superfusing the other uterine horn from each of the four animals on day 7 of the oestrous cycle during the collection of samples 4 and 5. All the solutions of caffeine and theophylline were freshly made up in Krebs solution prior to use. The amount of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ in the uterine extracts were measured by radioimmunoassays as described in Section 2.1.8.

Statistical analyses:

Changes in PG output with time were analysed by Duncan's multiple range test.

Results:

Caffeine (10 mM) significantly ($p<0.05$, $n=5$) increased the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the day 7 and day 15 guinea-pig uterus, and of PGE_2 from the day 15 uterus (Figure 3.1.1.1). Theophylline (10 mM) significantly ($p<0.05$,


(ii) Day 15
Caffeine
Caffeine
Control
PGF2α
PGE2
6-Keto-PGF1α
ng/min/100mg tissue
Time(min)

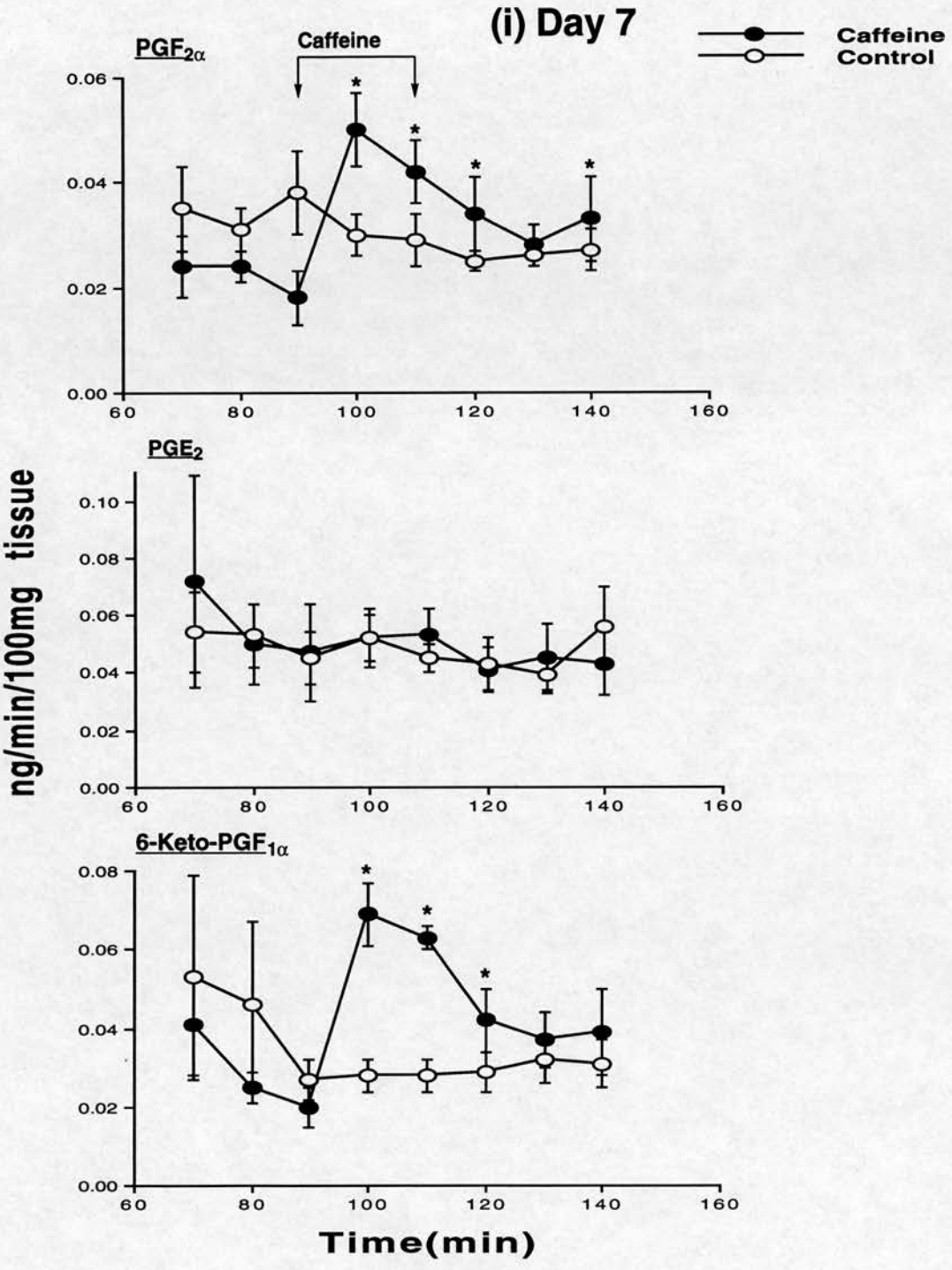


Figure 3.1.1.1 Effects of caffeine (10 mM) on mean (± SEM, n=5) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the (i) day 7 and (ii) day 15 guinea-pig uterus superfused in vitro. * Significantly ($p<0.05$) higher than before caffeine treatment.

n=4) increased the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but not that of PGE_2 from the day 7 guinea-pig uterus (Figure 3.1.1.2). Caffeine (1 mM but not 0.1 mM) significantly ($p<0.05$, n=4) increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus (Figure 3.1.1.3).

Discussion:

The basal outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus were typically low. However, by day 15 the output of $PGF_{2\alpha}$ from the uterus had increased approximately by 20-fold, whereas the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ showed very little changes. This is in agreement with previous reports by Poyser and Brydon (1983). Poyser (1983b) has reported that this selective increase in $PGF_{2\alpha}$ output is due to the effect of oestradiol on a progesterone-primed uterus.

Caffeine (10 mM) caused 3- to 4-fold increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the guinea-pig uterus on days 7 and 15, and of PGE_2 from the uterus on day 15 of the oestrous cycle. There was more variability in the outputs of $PGF_{2\alpha}$ and, to a lesser extent, of PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 15 uterus. This could be due to the fact that day 15 in one animal may not be exactly equivalent to day 15 in another animal, as an oestrous cycle may last from 15 to 18 days. Theophylline (10 mM) caused 3.6- to 4.3-fold increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but not of PGE_2 from the guinea-pig uterus on day 7. Caffeine (1 mM but not 0.1 mM) caused 2- to 3-fold increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the guinea-pig uterus on day 7 of the oestrous cycle.

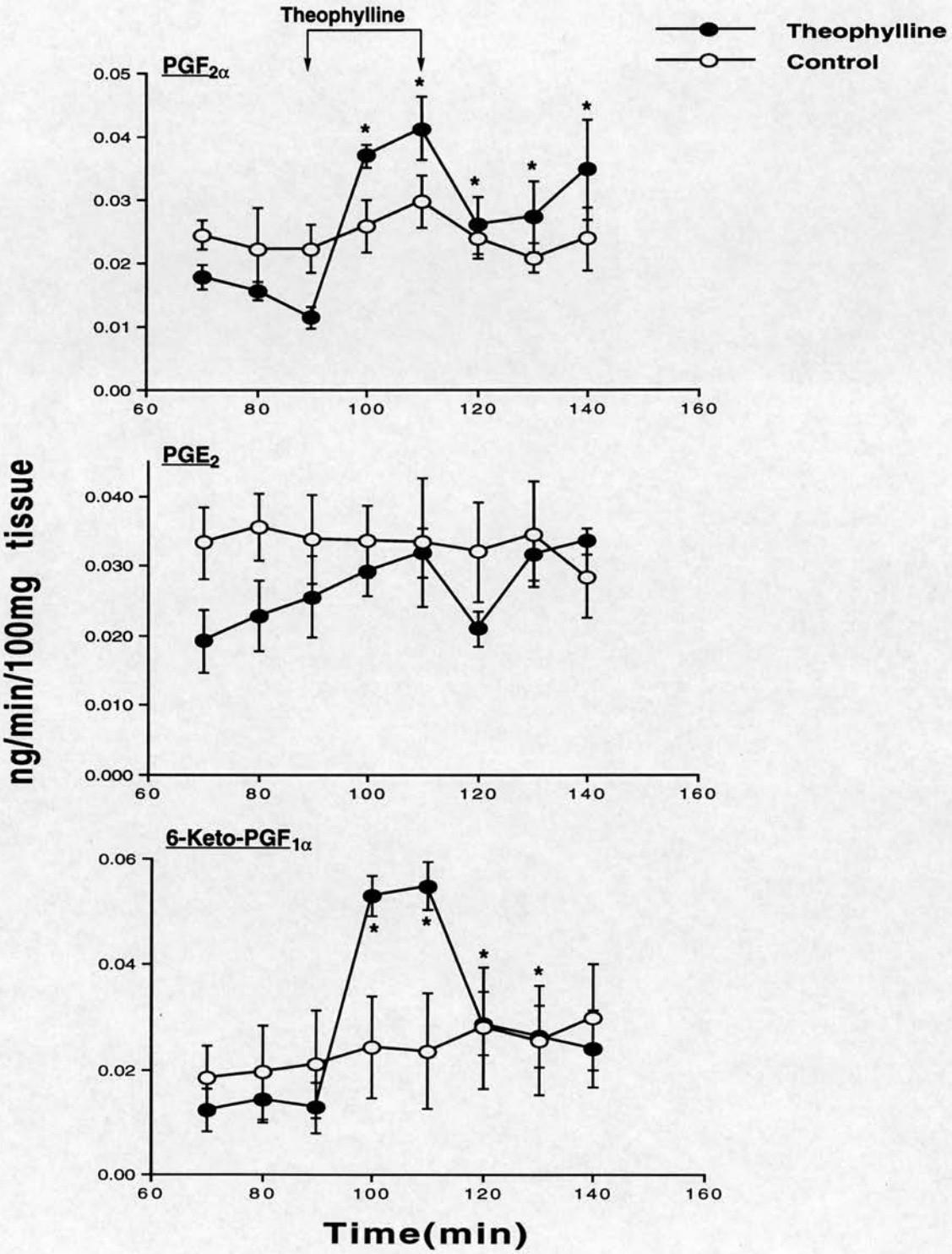


Figure 3.1.1.2 Effects of theophylline (10 mM) on mean (± SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro*. * Significantly ($p<0.05$) higher than before theophylline treatment.

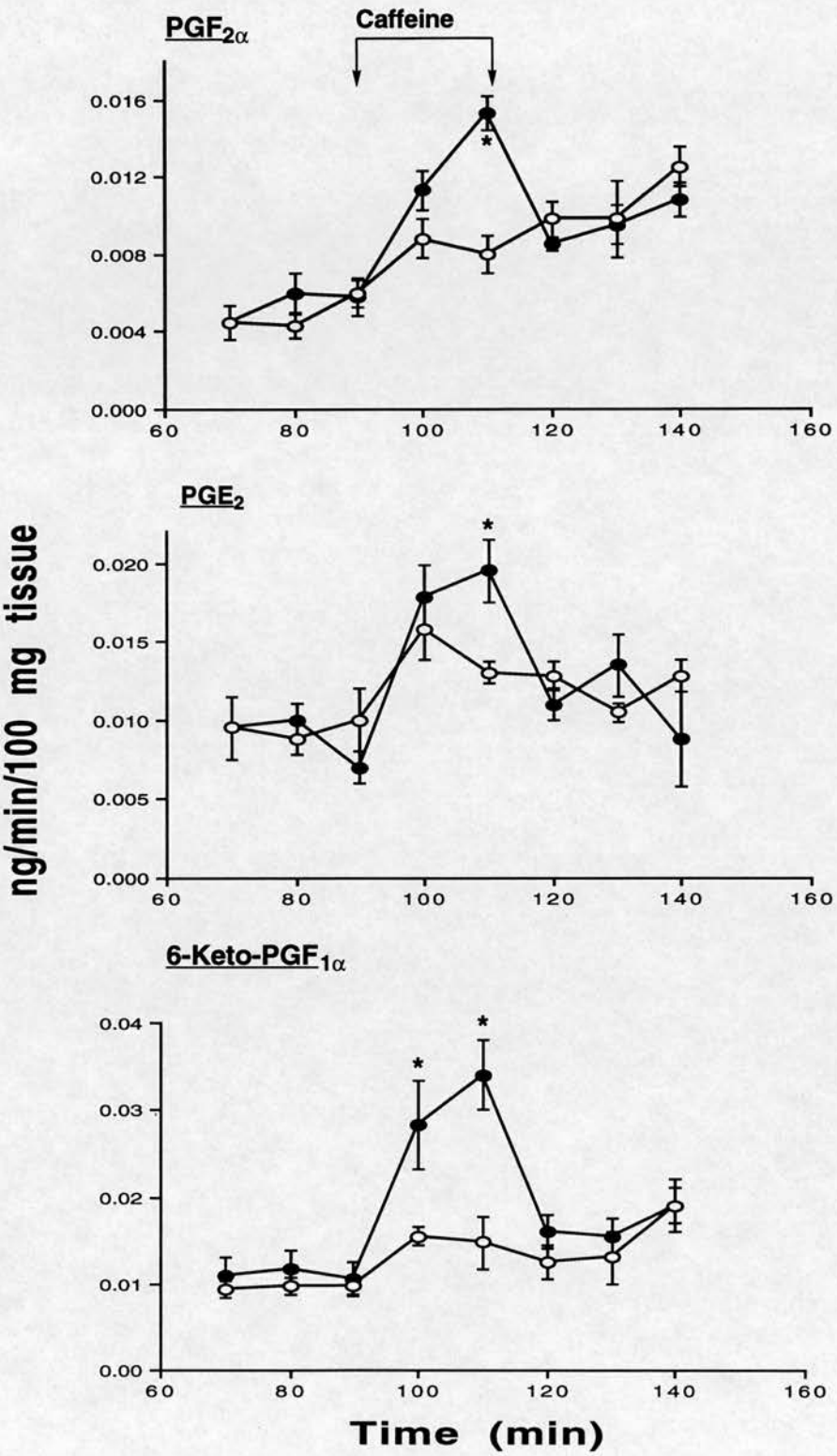


Figure 3.1.1.3 Mean (± SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the presence of caffeine (1 mM; ●) or caffeine (0.1 mM; o).

* Significantly ($p<0.05$) higher than before caffeine treatment.

Since caffeine and theophylline are phosphodiesterase inhibitors, they may be acting to increase adenosine 3´-5´-cyclic monophosphate [cyclic AMP; (cAMP)] concentrations in the uterus. However, it has been reported that increasing cAMP concentrations in the day 7 and day 15 guinea-pig uterus (by using forskolin) does not affect the outputs of $PGF_{2\alpha}$, PGE_2 or 6-keto-$PGF_{1\alpha}$ (Poyser, 1987a). Thus it is unlikely that effects of caffeine and theophylline in increasing uterine PG output are as a result of an increase in the cAMP concentration.

3.1.2 The Effects of Lack of Extracellular Calcium, an Intracellular Antagonist (TMB-8), Inhibitors of Intracellular Calcium Release (Ryanodine & Ruthenium Red), and Calmodulin Inhibitors (W-7 & Trifluoperazine) on Caffeine-Induced Increases in The Outputs of Prostaglandins From The Guinea-Pig Uterus Superfused *In Vitro*.

Introduction:

It has been reported in the rat, guinea-pig and man that the increase in endometrial PG synthesis is associated with increased activity of phospholipase A_2 (PLA_2), a calcium-dependent enzyme (Dey *et al.*, 1982; Downing & Poyser, 1983; Bonney, 1985). It has also been reported that in the guinea-pig, the increase in uterine PG production may involve the release of calcium from an internal pool (Leckie & Poyser, 1990b; Johnson & Poyser, 1991). However, Ning and Poyser (1984) have reported that there is no increase in inositol turnover in the guinea-pig endometrium at the end of the oestrous cycle when $PGF_{2\alpha}$ synthesis is high. This finding indicates that the stimulation of endometrial $PGF_{2\alpha}$ synthesis is not dependent on the generation of inositol-1,4,5-trisphosphate (IP_3) and, therefore, does not involve the release of calcium from an IP_3-sensitive store.

Studies on several cell types have indicated that only part of the calcium stored in the endoplasmic reticulum is IP_3-sensitive and there is another store of calcium which is sensitive to ryanodine and can be released by caffeine (see Berridge, 1993; Komori & Bolton, 1989; Zhang *et al.*, 1993; Sorimachi *et al.*, 1992; Cheek *et al.*, 1993). Considering all these reports and findings from the previous

set of experiments (see Section 3.1.1), the effect of caffeine on prostaglandin output from the guinea-pig uterus superfused *in vitro* in the absence of extracellular calcium was investigated. The effects of other factors which modulate intracellular calcium such as TMB-8 (an intracellular calcium antagonist, Malagodi & Chiou, 1974), calmodulin antagonists (W-7, Hidaka *et al.*, 1978; and trifluoperazine (TFP), Levin & Weiss, 1977), ryanodine (RY) and ruthenium red (inhibitors of caffeine-induced calcium release from the RYR-channel of the endoplasmic reticulum) (Vites & Pappano, 1994; see Casteels *et al.*, 1992; see Berridge, 1993) on caffeine-induced prostaglandin production by the guinea-pig uterus superfused *in vitro* have been investigated.

Methods:

Tissue preparation and the experimental procedure were as described in Section 2.2.1. After an initial settling period of 60 min, uterine horns in each experiment were treated as described below. In experiment 1, one uterine horn from each of the five animals on day 7 of the oestrous cycle was superfused with normal Krebs solution and the other uterine horn was superfused with Krebs solution from which the calcium chloride had been omitted (calcium-free Krebs solution). Caffeine (10 mM) was present in both types of Krebs solution during the collection of samples 4 and 5.

In experiments 2 to 6, both uterine horns from four or five guinea-pigs on day 7 of the cycle were superfused with normal Krebs solution, and caffeine (10 mM) was present in the solution superfusing both uterine horns during the collection of

samples 6 and 7. The following additions were made to the Krebs solution superfusing one uterine horn from each animal during the collection of samples 4 to 7: Exp 2: TMB-8 (150 μM; n=5); Exp 3: RY (2, 20 or 200 μM; n=4); Exp 4: ruthenium red (10 or 100 μM; n=4); Exp 5: W-7 (150 μM; n=4); Exp 6: TFP (100 μM; n=4). The other uterine horn from each animal was untreated and hence acted as the control tissue.

Poyser (1985a, b) has shown that concentrations of W-7 and TFP used in experiments 5 and 6, are sufficient to inhibit A23187-induced $PGF_{2\alpha}$ release from the guinea-pig uterus. Cheek *et al.* (1993) has shown that caffeine (1 to 80 mM) dose-dependently increased $[Ca^{2+}]_i$ in the presence or absence of extracellular calcium and this effect of caffeine is inhibited in a use-dependent fashion by ryanodine (10 μM). It has also been shown that caffeine- (1 & 10 mM) induced transient increase of $[Ca^{2+}]_i$ (Donoso *et al.*, 1994; Kuermmerle *et al.*, 1994) is blocked by 10 μM ryanodine (Usachev *et al.*, 1993), and ruthenium red (0.1 & 1 μM) (Kuemmerle *et al.*, 1994).

Solutions of each compound were freshly made up in Krebs solution prior to use, except for ryanodine. A concentrated solution of RY was prepared in ethanol and stored at -20°C. The appropriate concentration of RY in Krebs solution was prepared by adding up 0.25 ml of the ethanolic stock solution to 250 ml Krebs solution. The Krebs solutions superfusing the control uterine horn during the same period contained an equivalent amount of ethanol.

Prostaglandins were extracted from the samples of superfusate and stored at -20°C as described in Section 3.1.1. The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ in each extract were measured by radioimmunoassays as described in Section 2.1.8.

Statistical analyses:

Changes in the PG output with time were analysed by Duncan's multiple range test. Other comparisons were made using Student's *t* test, or if the variances of the two groups were significantly different by the Variance ratio *F* test, by a modified *t* test for unequal variances (see Steel & Torrie, 1980).

Results:

In the absence or presence of extracellular calcium, caffeine (10 mM) significantly ($p<0.05$) increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 7 guinea-pig uterine horns. However, the caffeine-induced increase in $PGF_{2\alpha}$ output tended to be less during the first 10 min period of treatment in calcium-free Krebs solution (Figure 3.1.2.1). TMB-8 caused small, but significant ($p<0.05$) increases in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 7 guinea-pig uterine horns, an effect reported previously (Poyser, 1985a). However, TMB-8 inhibited the caffeine-induced increase in $PGF_{2\alpha}$ output, but not the increases in the outputs of PGE_2 or 6-keto-$PGF_{1\alpha}$ (Figure 3.1.2.2).

Ryanodine alone had no effect on the outputs of $PGF_{2\alpha}$ and PGE_2 from the day 7 guinea-pig uterus, although RY (20 and 200 μM) significantly ($p<0.05$) increased the outputs of 6-keto-$PGF_{1\alpha}$. Ryanodine (2, 20, 200 μM) did not inhibit the

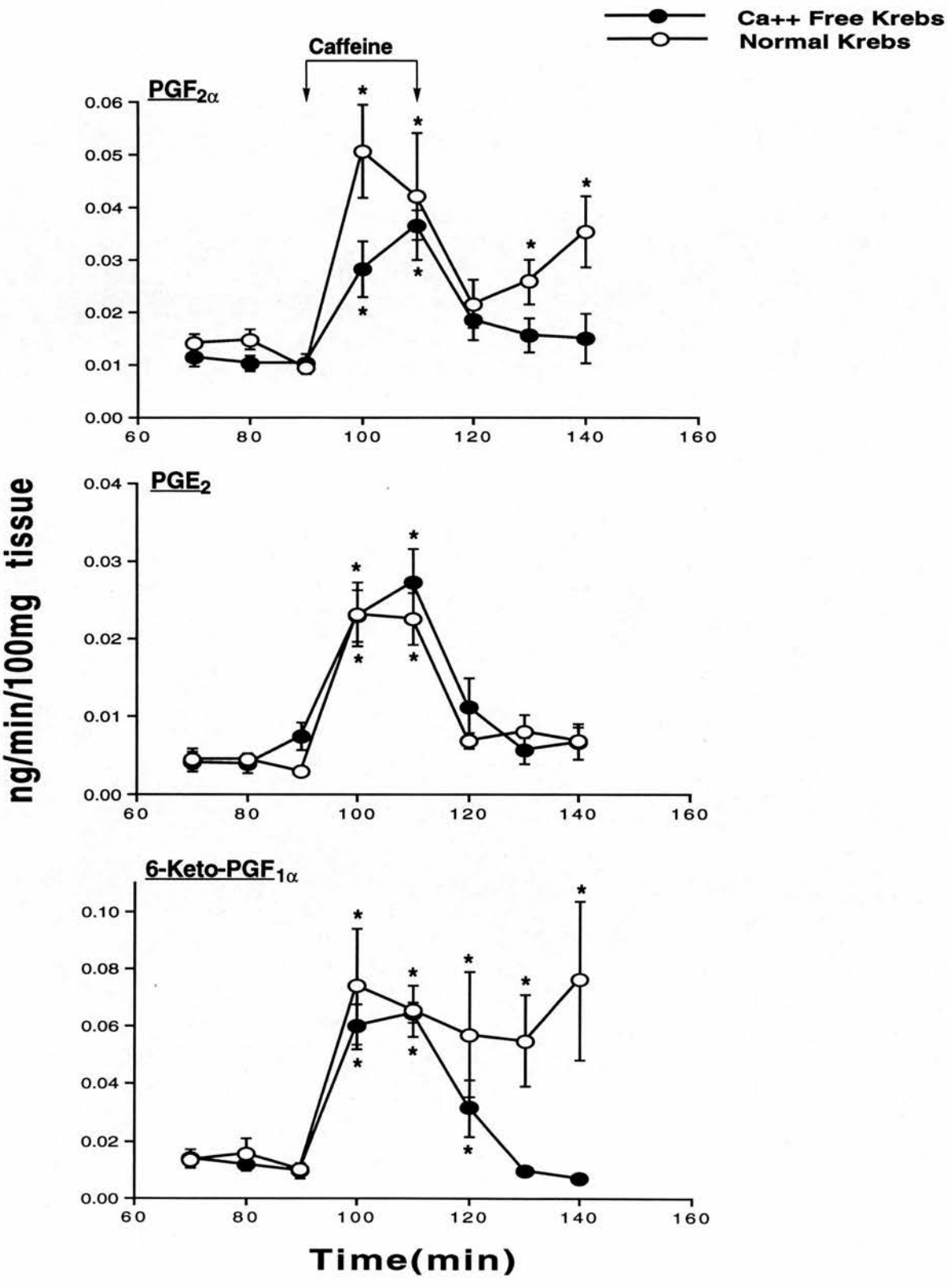


Figure 3.1.2.1. Effects of caffeine on mean (± SEM, n=5) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the presence or absence of extracellular calcium. * Significantly higher than before caffeine treatment.

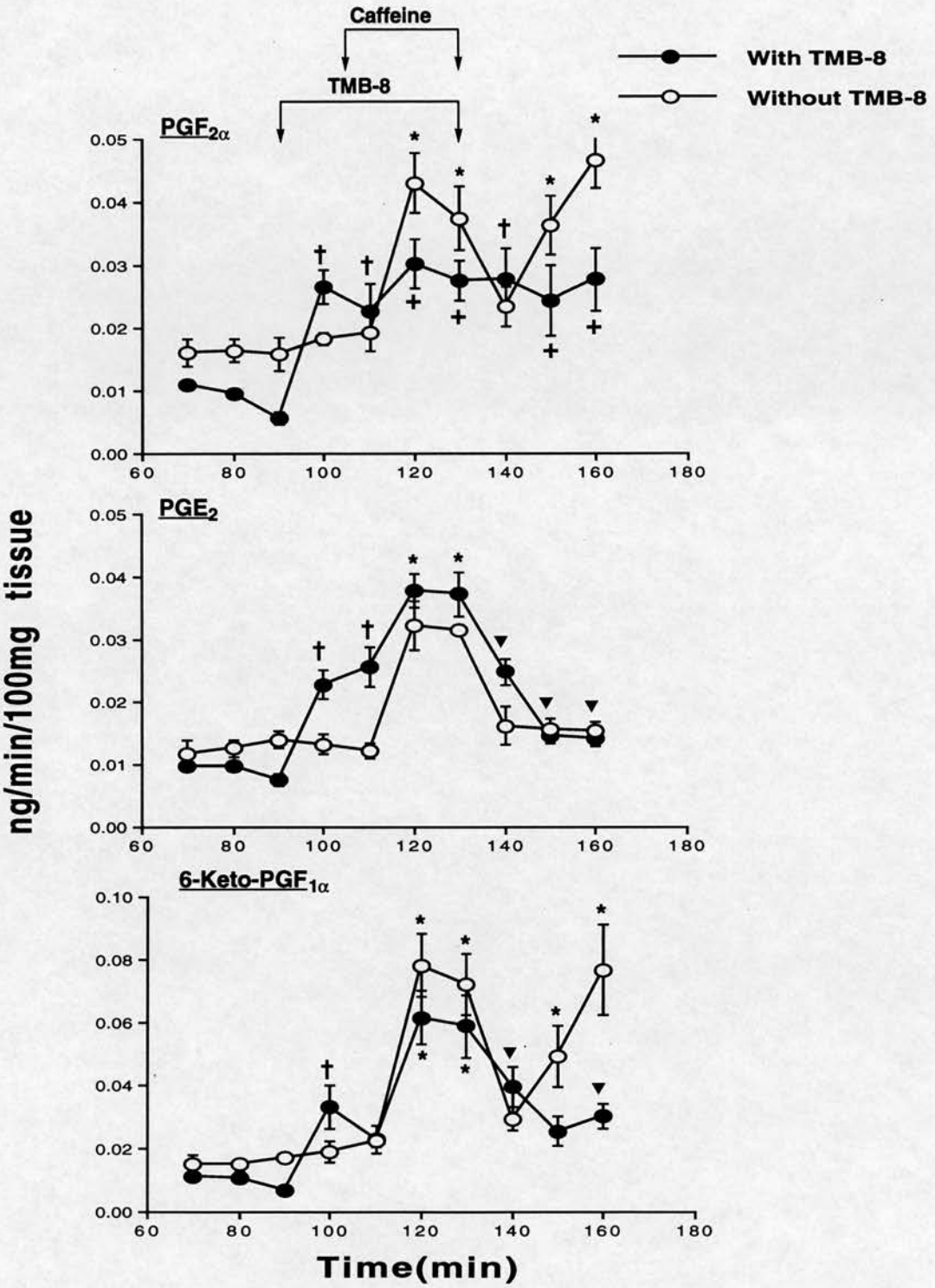
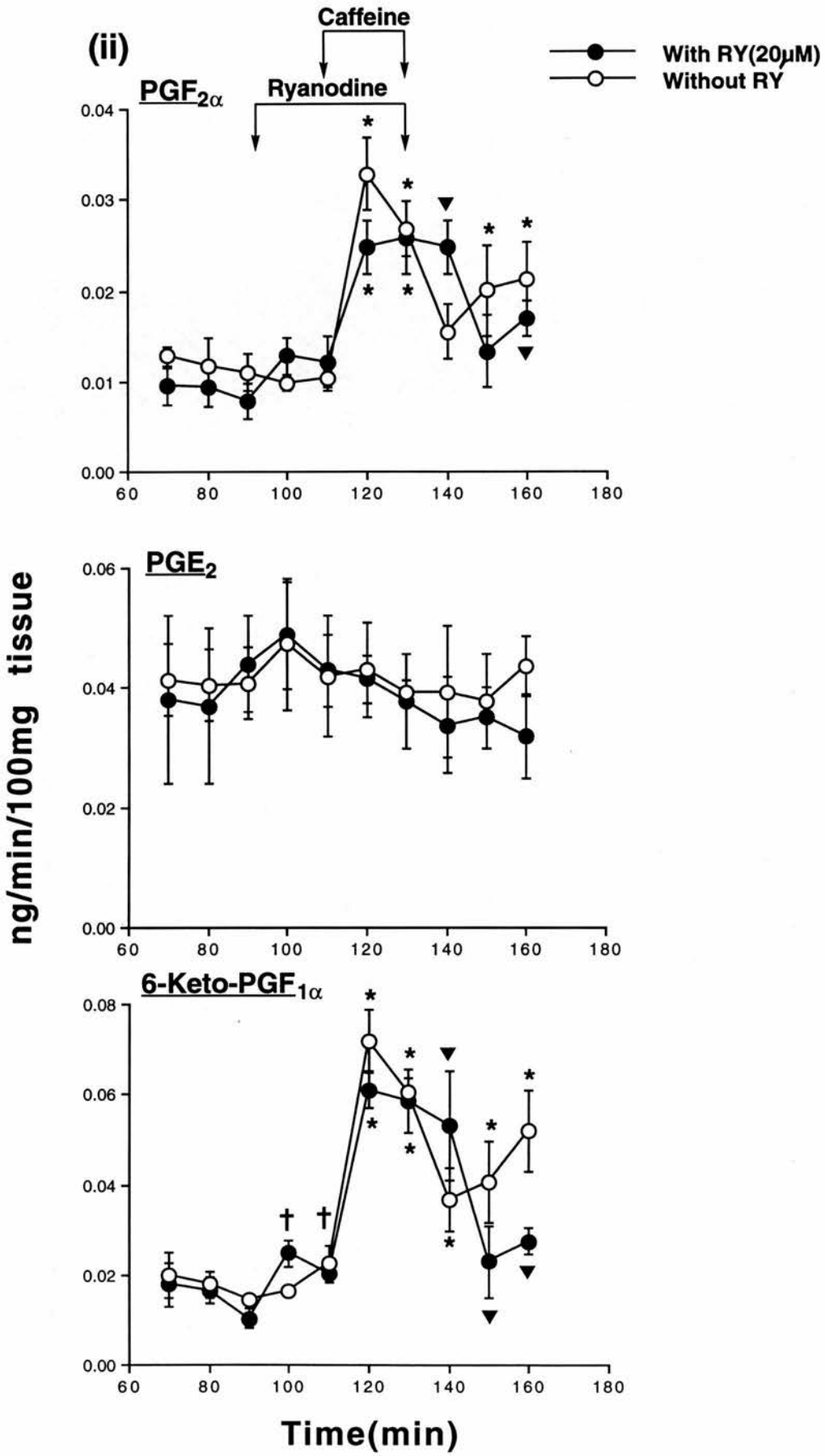


Figure 3.1.2.2 Effects of caffeine on the mean (± SEM, n=5) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the presence or absence of TMB-8. † Significantly ($p<0.05$) higher than before TMB-8 treatment alone. ✻ Significantly ($p<0.05$) increased by caffeine treatment. ▼ Significantly ($p<0.05$) lower following the end of caffeine treatment but significantly higher than before TMB-8 treatment.

caffeine-induced increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus (Figure 3.1.2.3). However, RY (20 & 200 μM) delayed both the increase and subsequent decrease in $PGF_{2\alpha}$ output produced by caffeine (10 mM) such that the 'peak of increased $PGF_{2\alpha}$ release' was flatter and broader. PGE_2 output was not affected by neither caffeine nor RY in this experiment (Figure 3.1.2.3).

Ruthenium red alone had no effect on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus. Ruthenium red (10 & 100 μM) did not inhibit the increase in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ induced by caffeine from the day 7 guinea-pig uterus (Figure 3.1.2.4).

Trifluoperazine (TFP) alone produced a small increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus, an effect reported previously (Poyser, 1985b). However, TFP had no effect on caffeine-induced increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus. In this experiment, PGE_2 output from the uterus increased after the caffeine treatment had finished in both the control (i.e. in the absence of TFP) and TFP-treated uterine horns (Figure 3.1.2.5). W-7 alone had no effect on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus. W-7 had no inhibitory effect on caffeine-induced increases in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus. In fact, W-7 caused a 3-fold potentiation of the caffeine-induced increase in $PGF_{2\alpha}$ output. W-7 also caused a much smaller potentiation of the increase in PGE_2 output but had no effect on the increase in 6-


(ii)
Caffeine
Ryanodine
With RY(20μM)
Without RY
PGF2α
PGE2
6-Keto-PGF1α
ng/min/100mg tissue
Time(min)

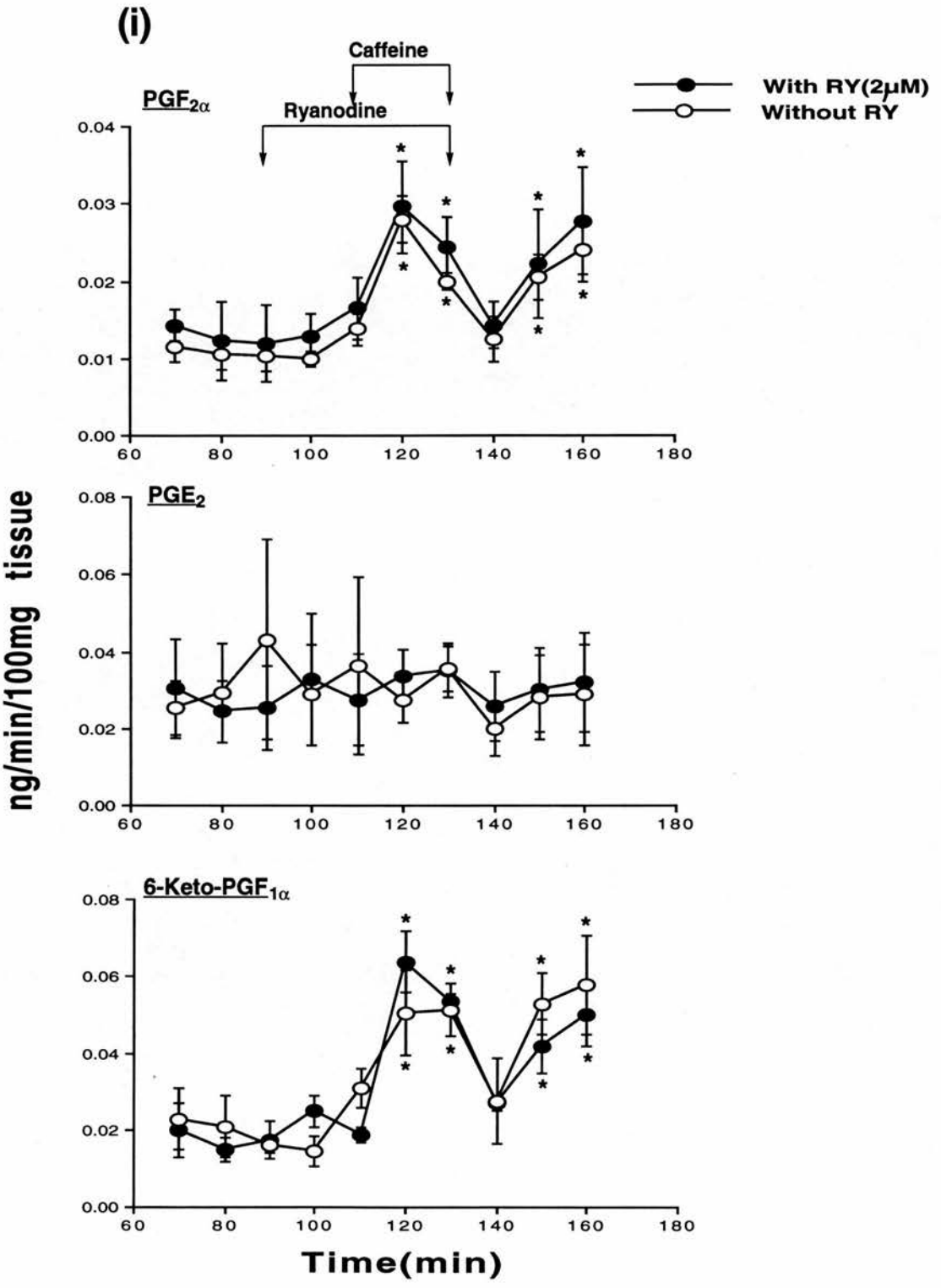


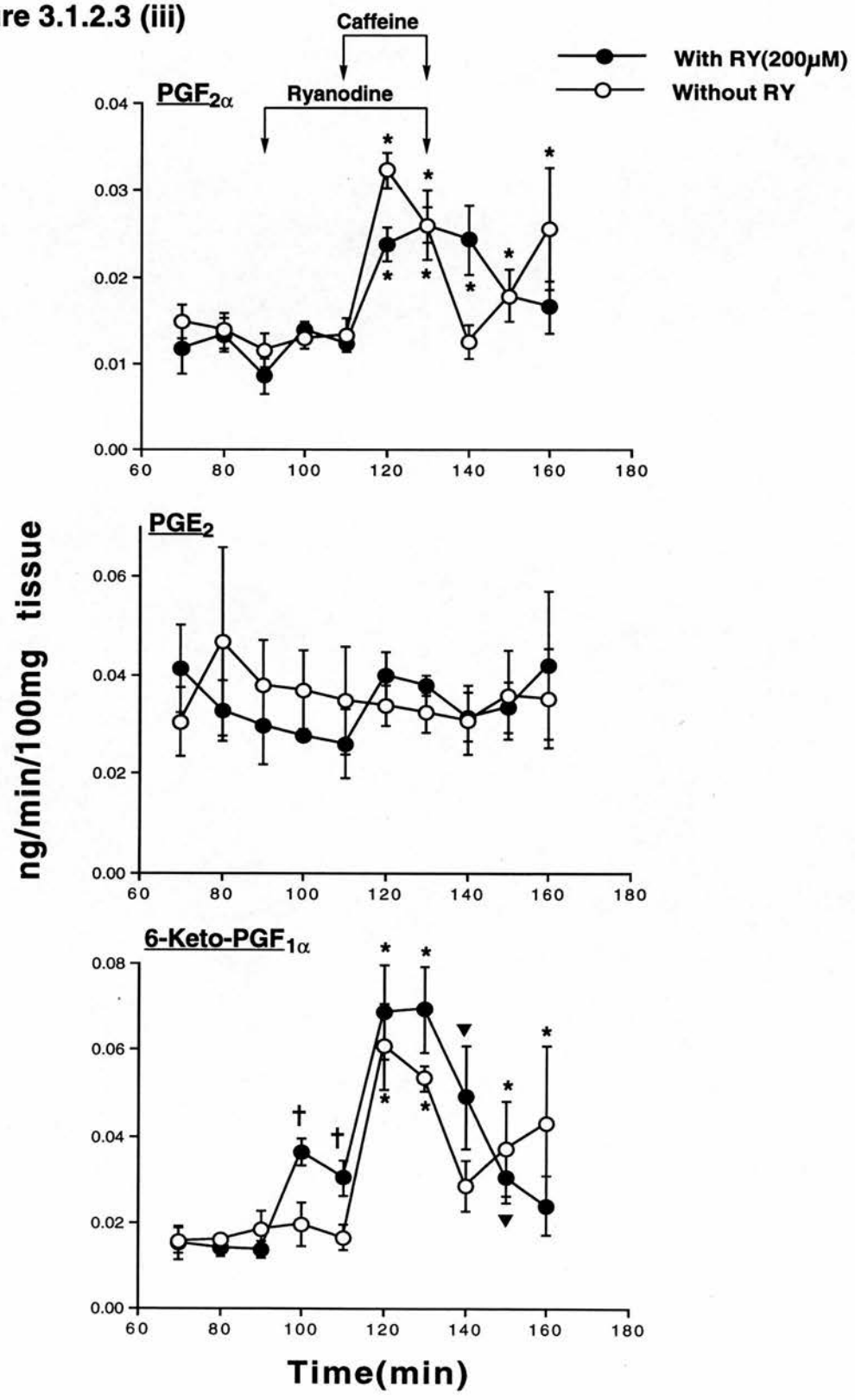
Figure 3.1.2.3. Effect of caffeine on the mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the absence and presence of ryanodine (RY) at concentrations of (i) 2 μM, (ii) 20 μM and (iii) 200 μM.

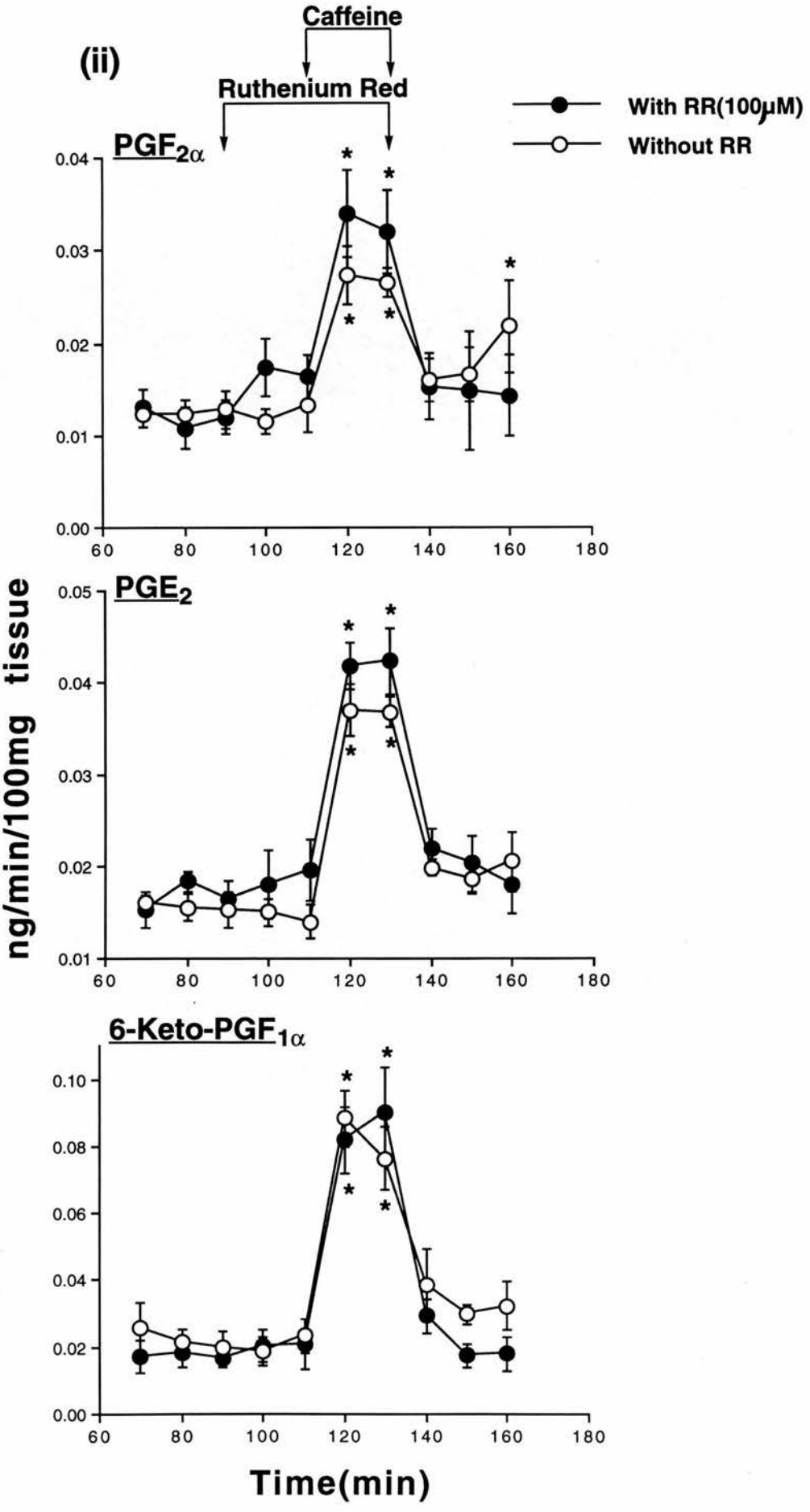
† Significantly ($p<0.05$) higher than before RY treatment.

✻ Significantly ($p<0.05$) higher than before caffeine treatment.

▼ Significantly ($p<0.05$) lower than following the end of caffeine treatment but significantly higher than before RY treatment.

Figure 3.1.2.3 (iii)




(ii)
Caffeine
Ruthenium Red
With RR(100μM)
Without RR
PGF2α
PGE2
6-Keto-PGF1α
ng/min/100mg tissue
Time(min)

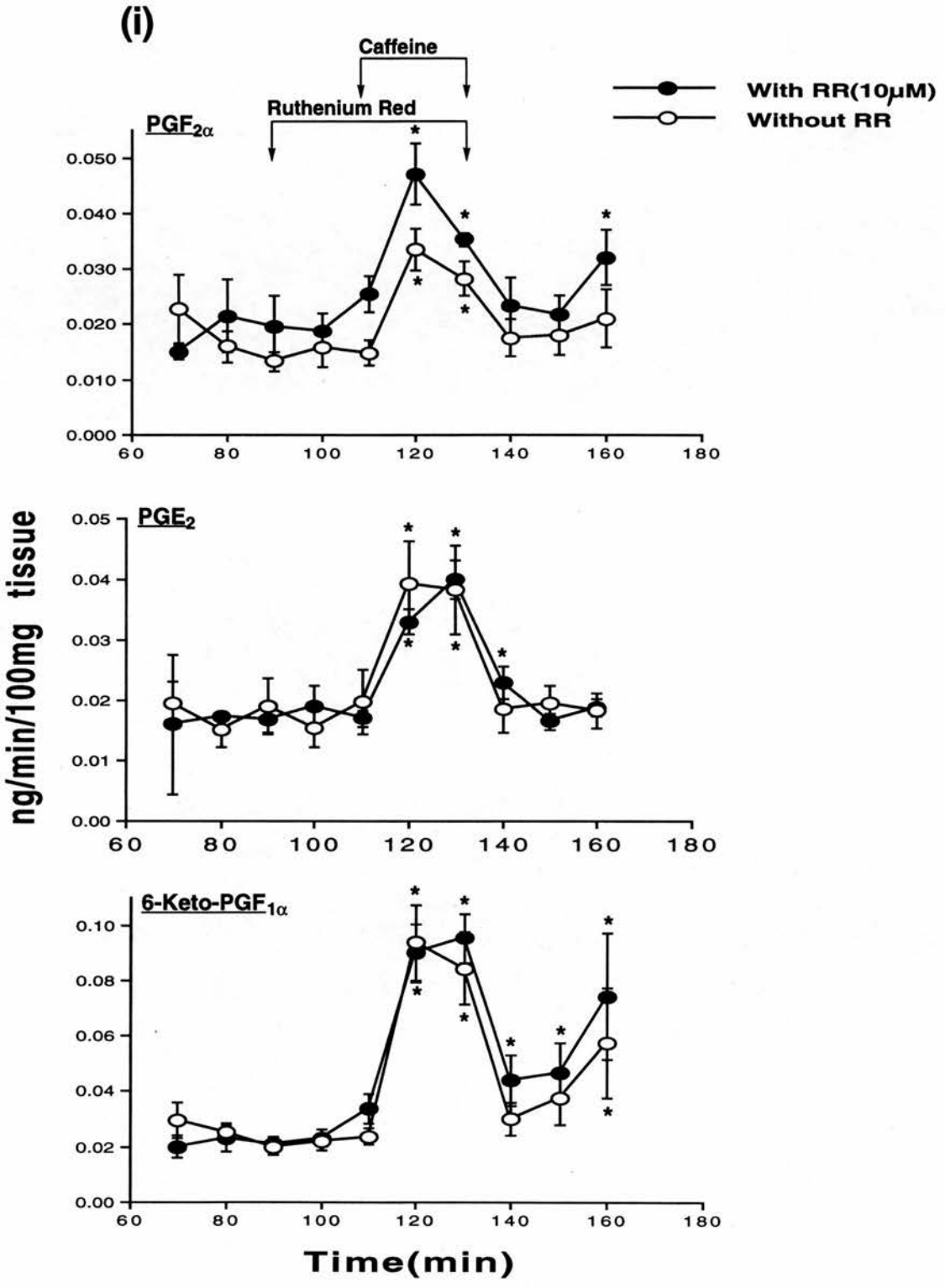
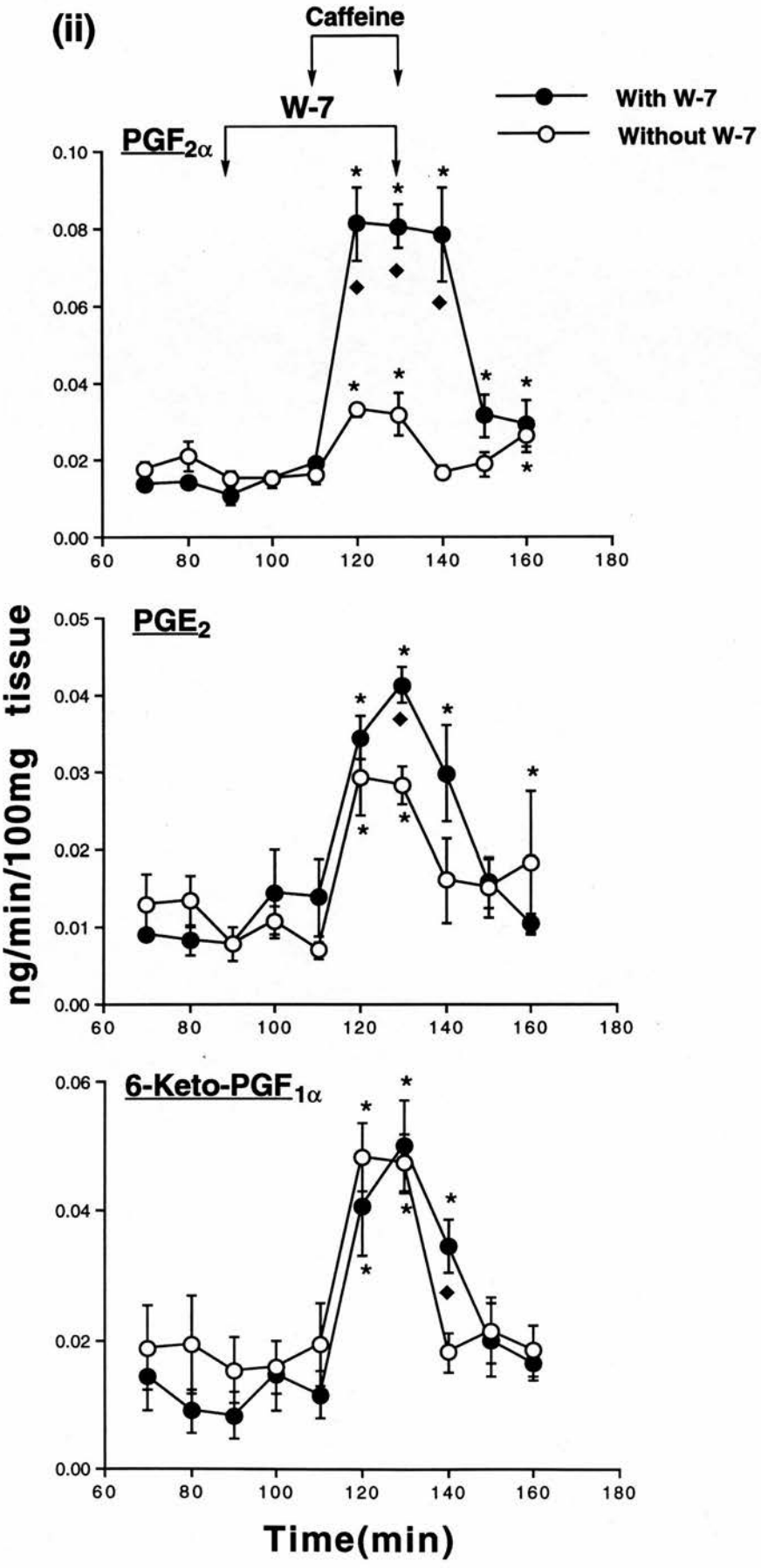


Figure 3.1.2.4. Effects of caffeine on the mean (± SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the absence and presence of ruthenium red (RR) at concentrations of (i) 10 μM and (ii) 100 μM. ✻ Significantly ($p<0.05$) higher than before caffeine treatment.


(ii)
Caffeine
W-7
With W-7
Without W-7
PGF2α
PGE2
6-Keto-PGF1α
ng/min/100mg tissue
Time(min)

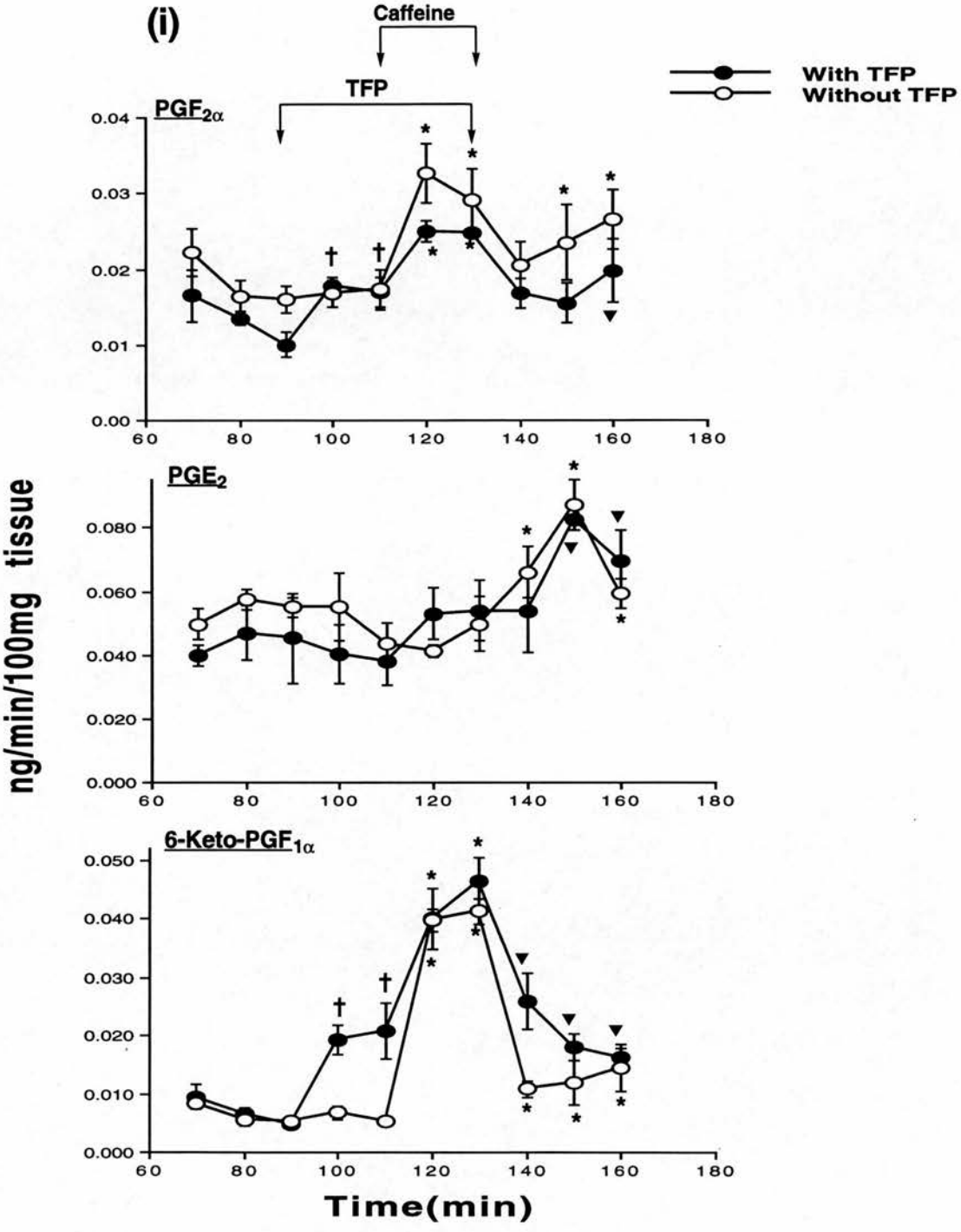


Figure 3.1.2.5 Effect of caffeine on the mean (± SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused in vitro in the absence and presence of (i) 150 μM trifluoperazine (TFP) and (ii) 100 μM W-7. † Significantly ($p<0.05$) higher than before TFP treatment alone. ✻ Significantly ($p<0.05$) higher than before caffeine treatment. ▼ Significantly ($p<0.05$) lower following the end of caffeine treament but significantly higher than before TFP treatment. ◆ Significantly ($p<0.05$) higher than corresponding control value (i.e. without W-7 treatment) for the same prostaglandin.

keto-$PGF_{1\alpha}$ output produced by caffeine from the day 7 guinea-pig uterus (Figure 3.1.2.5).

Discussion:

In some experiments caffeine stimulated PGE_2 output, whereas in other experiments it did not. It became apparent that this differential effect of caffeine on PGE_2 output from the guinea-pig uterus superfused *in vitro* was age related, i.e. caffeine was not effective in stimulating PGE_2 output in animals <4 month but stimulated PGE_2 output from older animals. The reason for this may be the fact that the basal PGE_2 output from younger animals was much higher than in the older animals and as a result caffeine was not able to further stimulate PGE_2 synthesis.

Caffeine in the presence or absence of extracellular calcium increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro*, suggesting that the effect of caffeine does not depend on the presence of extracellular calcium. However, when calcium-free Krebs solution was used, the increase in $PGF_{2\alpha}$ output but not the increases in PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs, tended to be slower then when calcium containing Krebs solution was used as the superfusing solution. This suggests that the action of caffeine on $PGF_{2\alpha}$ output, but not on PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs, may depend to some extend on the presence of extracellular calcium. Hence, the mechanism by which caffeine stimulates $PGF_{2\alpha}$ synthesis and release may be different from the mechanisms by which caffeine stimulates the synthesis and release of PGE_2 and PGI_2 (measured as

6-keto-$PGF_{1\alpha}$). TMB-8 (an intracellular calcium antagonist) completely abolished the caffeine-induced increase in the output of $PGF_{2\alpha}$, but had no effects on the caffeine-induced increases in the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$. These findings suggest that the stimulation of uterine $PGF_{2\alpha}$ synthesis and release in the guinea-pig, but not of PGE_2 and PGI_2 synthesis and release, by caffeine is dependent on intracellular calcium, or the stimulation of guinea-pig uterine PGE_2 and PGI_2 synthesis and release is dependent on an intracellular calcium pool which is not affected by TMB-8. Therefore, these results provide further evidence that the mechanism by which caffeine stimulates $PGF_{2\alpha}$ output is different from the mechanisms by which caffeine stimulates PGE_2 and PGI_2 outputs from the guinea-pig uterus.

The calmodulin antagonists (W-7 & TFP) did not inhibit the stimulatory effect of caffeine on uterine PG output, indicating that calmodulin does not mediate this stimulatory effect of caffeine. In fact, W-7 greatly potentiated the stimulatory effect of caffeine on $PGF_{2\alpha}$ output and, to a lesser extent, on PGE_2 outputs. W-7 had no effect on the caffeine-induced increase in 6-keto-$PGF_{1\alpha}$ output. TFP did not potentiate the stimulatory effect of caffeine on $PGF_{2\alpha}$ and PGE_2 outputs. This suggests that the potentiating effect of W-7 on caffeine-induced increases in $PGF_{2\alpha}$ and PGE_2 outputs from the guinea-pig uterus is not due to the inhibition of calmodulin. Also, caffeine-induced increases in $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from the guinea-pig uterus were affected to a different extent by W-7, providing further evidence that the mechanism by which caffeine stimulates $PGF_{2\alpha}$ synthesis and release is different from the mechanisms by which caffeine stimulates

PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis and release from the guinea-pig uterus. The reason for this potentiating effect of W-7 on caffeine-induced increase on uterine $PGF_{2\alpha}$ synthesis and release is not clear. A mechanism of calcium release has been identified in many tissues known as calcium-induced calcium release (CICR) (see McPherson & Campell, 1993a; Gyorke & Fill, 1993). This process is modulated by calcium, caffeine and RY. Cyclic ADP-ribose, an endogenous nucleotide, has recently been suggested as a second messenger which releases calcium from the ryanodine receptor (RYR) channel and through the CICR mechanism. Cardiac RYR is activated by cyclic ADP-ribose (Mesza'ros *et al.*, 1993) but inhibited by calmodulin (Smith *et al.*, 1989). The latter effect may explain the potentiating action of W-7 on caffeine-stimulated outputs of $PGF_{2\alpha}$ and PGE_2. However, Lee *et al.* (1994) reported that W-7 dose-dependently inhibits calcium release induced by cyclic ADP-ribose from *L.pictus* and *S.purpuratus* egg homogenates.

Caffeine induces intracellular calcium release from human vascular endothelial cells (Zhang *et al.*, 1993), skinned myocardial fibres of foetal and adult rats (Su & Shang, 1993), isolated snail neurones (Kostyuk & Kirischuk, 1993), rat pituitary cells (Tanaka & Tashjian, 1993), and bovine adrenal chromaffin cells (Cheek *et al.*, 1993) by acting on ryanodine receptors Types 1 and 2 (RYR-1 & RYR-2), an effect which is blocked by ruthenium red and high concentrations of RY (see Sorrentino & Volpe, 1993). Caffeine-induced increases in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus were not affected by ruthenium red or RY. This suggests that caffeine-induced increases in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus is not due to

that activation of RYR-1 or RYR-2 by caffeine. However, both RY and ruthenium red relaxed the uterine horns and abolished spontaneous contractions, suggesting that these two compounds were probably preventing the release of calcium from an intracellular calcium pool. Why RY (20 & 200 μM) alone should increase 6-keto-$PGF_{1\alpha}$ output and alter the time course of caffeine-induced rise in $PGF_{2\alpha}$ output is not clear and merits further investigation.

A third type of RYR (RYR-3) has been reported in mink lung epithelial cells (see Sorrentino & Volpe, 1993), and in rat brain and smooth muscle (McPherson & Campbell, 1993b) which, despite binding RY, is not activated by caffeine. Hence, it is unlikely that the activation of RYR-3 by caffeine is the mechanism by which caffeine exerts it effect on uterine PG output. Caffeine sensitive calcium stores are reported to be present in adrenal chromaffin cells (Sorimachi *et al.*, 1992), liver (Shoshan-Barmatz *et al.*, 1991), and pancreas (Schmid *et al.*, 1990). The receptor type involved in the pancreas is not sensitive to RY, but it is blocked by ruthenium red (Dehlinger-Kremer *et al.*, 1991). This indicates that the receptor type involved in the pancreas is not one of the three RYR-types described earlier. Also, in the present study RY or ruthenium red did not inhibit caffeine-induced increases of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$, indicating that caffeine effect on the guinea-pig uterus is not via activation of any caffeine and/or RY-sensitive calcium pools described so far. McNulty & Taylor (1993) reported an intracellular calcium pool in the hepatocytes which is insensitive to RY and theophylline, but is activated by caffeine and blocked by ruthenium red whereas Donoso *et al.* (1994) reported that both caffeine and theophylline mobilize intracellular calcium from an internal store

in cardiac myocytes. Thus, it is possible that the caffeine-sensitive calcium pool present in the hepatocytes is also present in the guinea-pig uterus which is involved in the caffeine-induced stimulation of uterine PG output. However, theophylline also induced a rise in PG outputs from the guinea-pig uterus, an effect which was not seen in hepatocytes (McNulty & Taylor, 1993), but it is seen in cardiac myocytes (Donoso *et al.*, 1994). Hence, it can be deduced that the mechanism by which theophylline induces PG output from the uterus may be different from that of caffeine or, if caffeine and theophylline are acting through the same mechanism, then the caffeine effects on hepatocytes and uterus are via different mechanisms. It can also be deduced that the mechanism by which caffeine and theophylline induce PG output from the uterus is the same as the mechanism by which caffeine and theophylline exert their effects on cardiac myocytes.

In summary, caffeine increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 and day 15 guinea-pig uterus superfused *in vitro*, an effect which is not dependent upon the presence of extracellular calcium. This uterine prostaglandin stimulatory effect of caffeine is not modulated by calmodulin antagonists or inhibitors of calcium release from the RYR channel. However, TMB-8 blocked the stimulatory effect of caffeine on the output of $PGF_{2\alpha}$ but not on the outputs of PGE_2 or 6-keto-$PGF_{1\alpha}$, suggesting that intracellular calcium may be involved in the effect of caffeine on $PGF_{2\alpha}$ synthesis.

3.1.3 The Effects of Caffeine, Theophylline and Ryanodine on Prostaglandin Output From Day 7 and Day 15 Guinea-Pig Endometrial Tissue in Culture.

Introduction:

The previous sets of experiments (see Sections 3.1.1 & 3.1.2) have indicated that caffeine and theophylline, but not ryanodine, have the ability to stimulate prostaglandin synthesis by and release from the day 7 and day 15 guinea-pig uterus superfused *in vitro*. It has been previously reported that a number of compounds, including PLA_2, phospholipase C (PLC), A23187 and sodium fluoride (NaF) (Poyser, 1987a, b; Leckie & Poyser, 1990a) increase PG outputs from the guinea-pig uterus superfused *in vitro*. However, Leckie and Poyser (1990b) have also reported a short-term stimulatory effect and a long-term inhibitory effect of NaF on PG outputs from the day 15, and to a lesser extent, from the day 7 guinea-pig endometrium in culture. Considering the above reports, this set of experiments were designed to investigate whether or not caffeine and theophylline would have different effects on PG outputs from the guinea-pig uterus in tissue culture in comparison to the stimulatory effect seen with *in vitro* superfusion.

Methods:

Endometrial tissue from the day 7 and day 15 guinea-pig uterus was removed and cultured as described in Methods Section 2.1.3. Briefly, uterine horns were removed from the guinea-pigs as described in Section 2.1.1 except that the whole procedure was carried out under aseptic conditions. After "opening up" each

uterine horn, endometrium was cut away from the myometrium, cut into approximately 1 mm^3 pieces, and then placed onto sterile lens paper lying across a stainless steel grid in a vented sterile dish which contained 4 ml of tissue culture medium (TCM) plus any other compound used according to the nature of the experiment. The amount of tissue placed in each petri dish varied between 19 - 35 mg. Petri dishes were then placed into racks which were in turn placed into modified Kilner jars. The jars were then gassed and incubated as described in Section 2.1.3. From each guinea-pig endometrium, 12-14 tissue cultures dishes (according to the nature of experiment) were prepared.

In experiment 1, uteri were removed from four day 7 guinea-pigs. Four of the petri dishes from each animal were left untreated (controls), and the remaining dishes were treated in duplicate with caffeine (Cf; 1 & 10 mM) and theophylline (Theo; 1 & 10 mM). In experiment 2, uteri were removed from four day 15 guinea-pigs. Four of the petri dishes from each animal were left untreated (controls), and the remaining dishes were treated in duplicate with Cf (1 & 10 mM), Theo. (1 & 10 mM). In experiment 3, uteri were removed from four day 7 guinea-pigs. Four of the petri dishes from each animal were treated with ethanol (20 μl) (controls), and the remaining dishes were treated with ryanodine (RY; 20 μM). These dishes were then divided equally; two dishes from the controls and one dish from each of the duplicate treatments were placed in each of the two modified Kilner jars and placed in the incubator. Culture medium was changed at 2, 8, and 24 h. Samples of culture medium were stored at -20°C before radioimmunoassay, without extraction,

for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$. At the end of the experiment, tissues were blotted dry and weighed.

Solutions of caffeine and theophylline were freshly made up in TCM solution prior to use. A concentrated solution of RY was prepared in ethanol and stored at -20°C. The appropriate concentration of RY was prepared by diluting 200-fold in TCM solution.

Statistical tests:

The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

After 2 h of culture, the major prostaglandins produced were 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ by the day 7 and day 15 guinea-pig endometrium, respectively. The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ produced by day 7 endometrium were 84, 46 and 339 pg/mg tissue/h, and by day 15 endometrium were 1541, 111 and 500 pg/mg tissue/h, respectively.

The prostaglandin outputs from the cultured tissues declined with time over 24 h. However, there was an increase in the output of $PGF_{2\alpha}$ between 8 and 24 h of culture from day 7 endometrium but not from day 15 endometrium in culture. The amounts of $PGF_{2\alpha}$ and PGE_2 produced by the endometrium obtained from day 15 of the oestrous cycle were 18.4- and 2.0-fold higher than the amounts produced by the endometrium obtained from day 7 of the cycle, respectively. There was no

difference in the outputs of 6-keto-$PGF_{1\alpha}$ from guinea-pig endometrium in culture between day 7 and day 15 of the cycle (Figures 3.1.3.1 & 3.1.3.2.).

For day 7 endometrium in culture, Cf (10 mM but not 1 mM) significantly ($p<0.05$, $n=4$) increased the outputs of $PGF_{2\alpha}$ by 1.5-fold after 8 h and 6-keto-$PGF_{1\alpha}$ up to 2.2-fold after 8 and 24 h of culture (Figure 3.1.3.1). Theo (10 mM but not 1 mM) significantly ($p<0.05$, $n=4$) increased the outputs of $PGF_{2\alpha}$ by up to 9-fold and PGE_2 by up to 6.2-fold after 8 and 24 h of culture (Figure 3.1.3.1). Theo (1 and 10 mM) significantly increased 6-keto-$PGF_{1\alpha}$ output by 1.3- to 14.8-fold after 8 and 24 h (Figure 3.1.3.1). Theo (10 mM) also significantly ($p<0.05$, $n=4$) increased 6-keto-$PGF_{1\alpha}$ output after 2 h of culture. The stimulatory effect of theophylline was between 1.7- to 6.6-fold greater than the stimulatory effect of caffeine on the same PG output at the same time.

For day 15 endometrium in culture, Cf (10 mM but not 1 mM) showed both an inhibitory and a stimulatory effect on $PGF_{2\alpha}$ output, but not on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$. Cf (10 mM) significantly ($p<0.05$, $n=4$) inhibited $PGF_{2\alpha}$ output after 2 h of culture (Figure 3.1.3.2). By 8 h, the inhibitory effect was reversed, and a significant ($p<0.05$, $n=4$) 1.6-fold rise in the output of $PGF_{2\alpha}$ was seen after 24 h of culture (Figure 3.1.3.2). Cf (10 mM) also stimulated the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ by 1.5-fold from the endometrium after 24 h of culture. Theophylline (1 mM) significantly ($p<0.05$, $n=4$) increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ by up to 2.6-fold after 8 and 24 h. Theophylline (10 mM) significantly ($p<0.05$, $n=4$) stimulated $PGF_{2\alpha}$ output by 2.1-fold, PGE_2

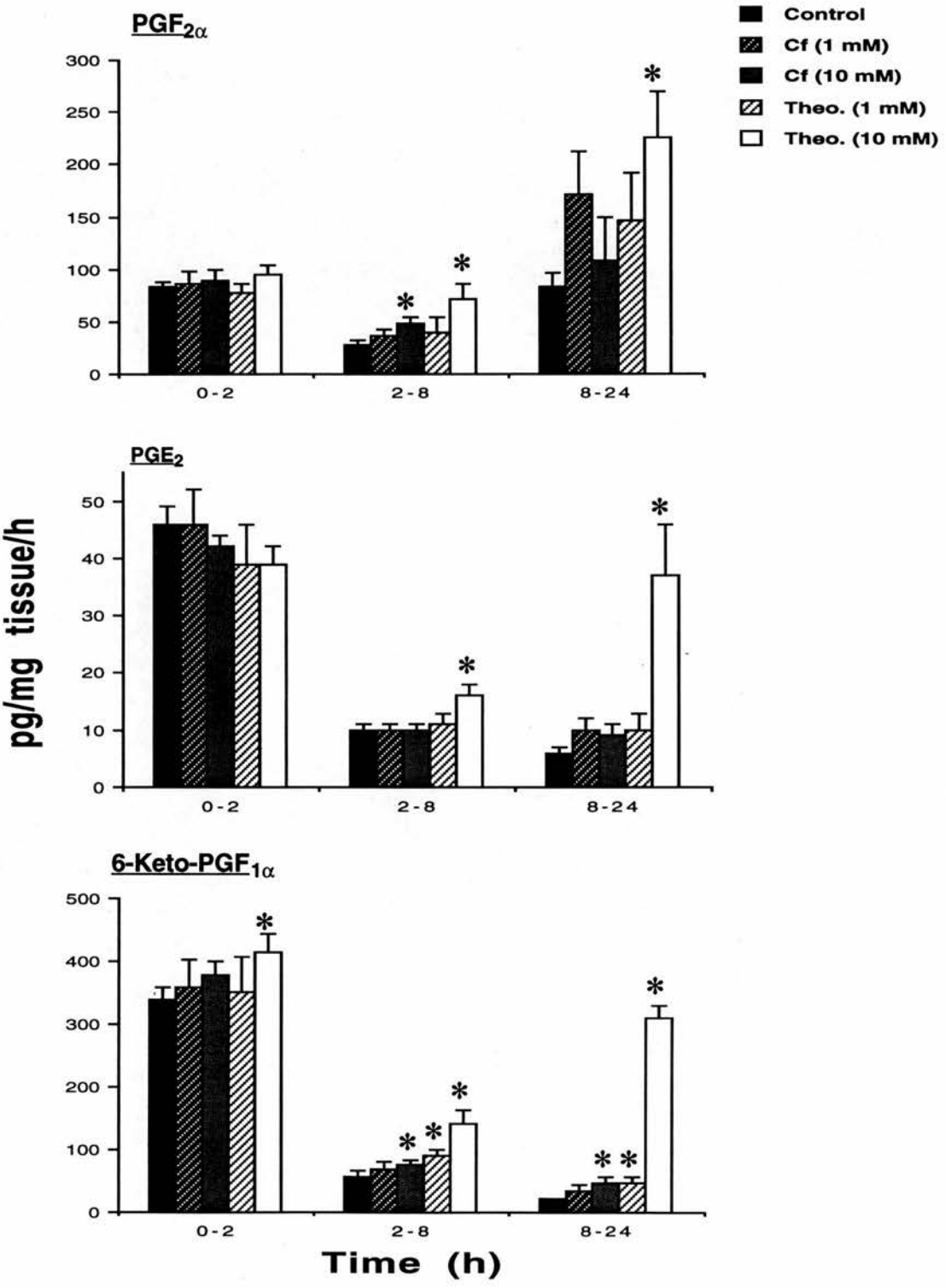


Figure 3.1.3.1 Effects of caffeine (Cf) and theophylline (Theo) on the mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig endometrium in culture.

* Significantly ($p<0.05$) increased by caffeine or theophylline treatment.

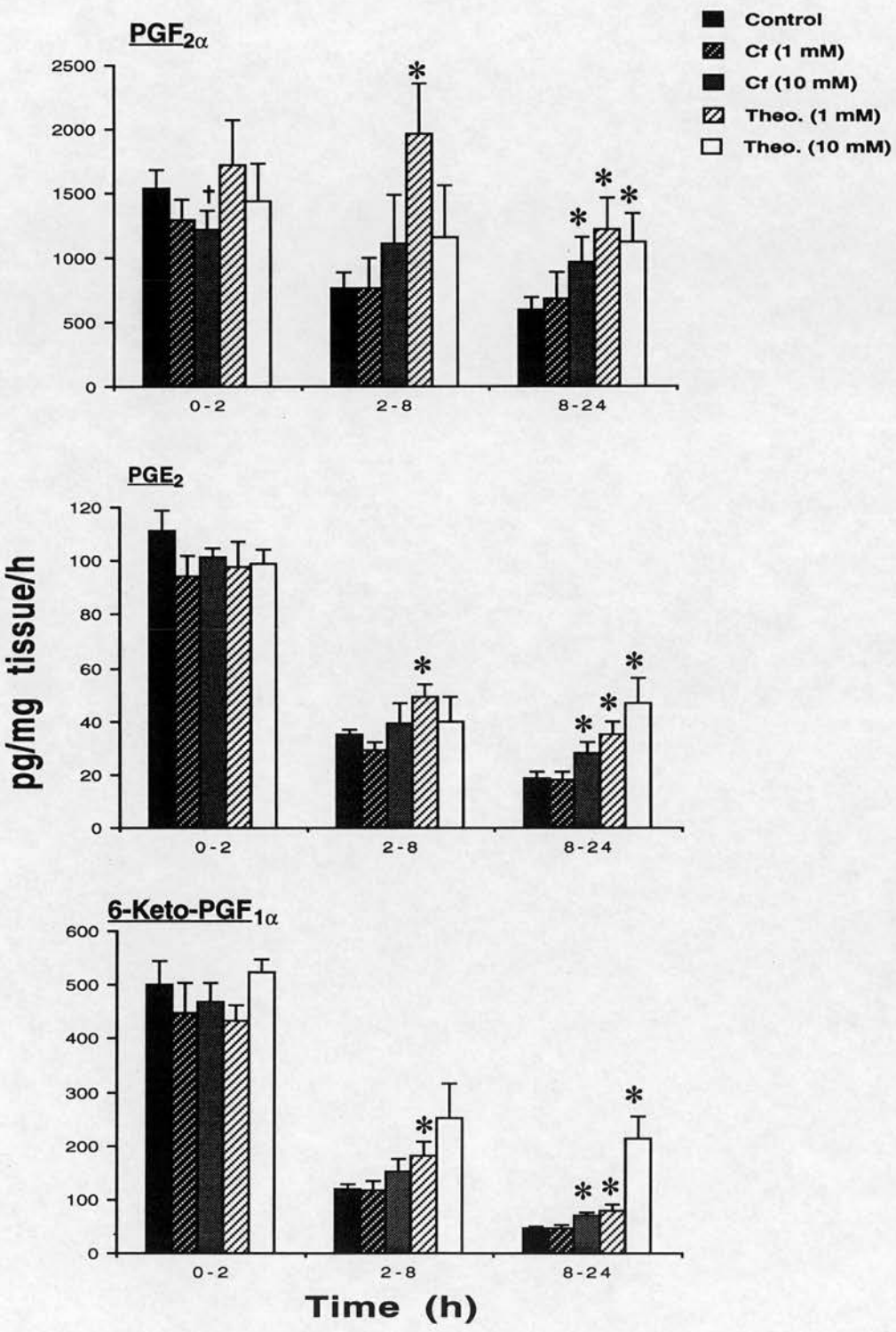


Figure 3.1.3.2. Effects of caffeine (Cf) and theophylline (Theo.) on the mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 15 guinea-pig endometrium tissue in culture.

† Significantly decreased by caffeine treatment.

* Significantly increased by caffeine or theophylline treatment.

output by 2.5-fold, and 6-keto-$PGF_{1\alpha}$ output by 4.7-fold after 24 h of culture (Figure 3.1.3.2). Ryanodine (20 μM) had no effect on the outputs of all three PG measured at the end of any culture period (Figure 3.1.3.3).

Discussion:

In this study, the amount of $PGF_{2\alpha}$ produced by the endometrium obtained from day 15 of the oestrous cycle was 18.4-fold higher than the amount produced by the endometrium obtained from day 7 of the cycle. The higher output of $PGF_{2\alpha}$ from the day 15 endometrium compared to day 7 endometrium is in agreement with the increase in $PGF_{2\alpha}$ concentrations in the utero-ovarian venous plasma of the guinea-pig at the end of the oestrous cycle (Blatchley *et al.*, 1972; Earthy *et al.*, 1975; Antonini *et al.*, 1976).

The decline in PG output from the endometrium in culture over a period of 24 h seen in this study has been previously reported (Riley & Poyser, 1989; Leckie & Poyser, 1991b). The increase in the $PGF_{2\alpha}$ output between 8 and 24 h of culture from day 7 endometrium seen in this study has also been reported elsewhere (Riley & Poyser, 1989). The reason for this increase may be the absence of an inhibitory influence of progesterone on day 7 endometrium *in vitro* (Riley & Poyser, 1989).

Caffeine at higher concentration caused both an inhibitory and a stimulatory effect on the output of $PGF_{2\alpha}$, but not on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$, from the guinea-pig uterus, a property which was not shared by theophylline. This suggests that caffeine affects PG output from the guinea-pig uterus in a time-dependent manner. In general, theophylline was more potent in stimulating PG

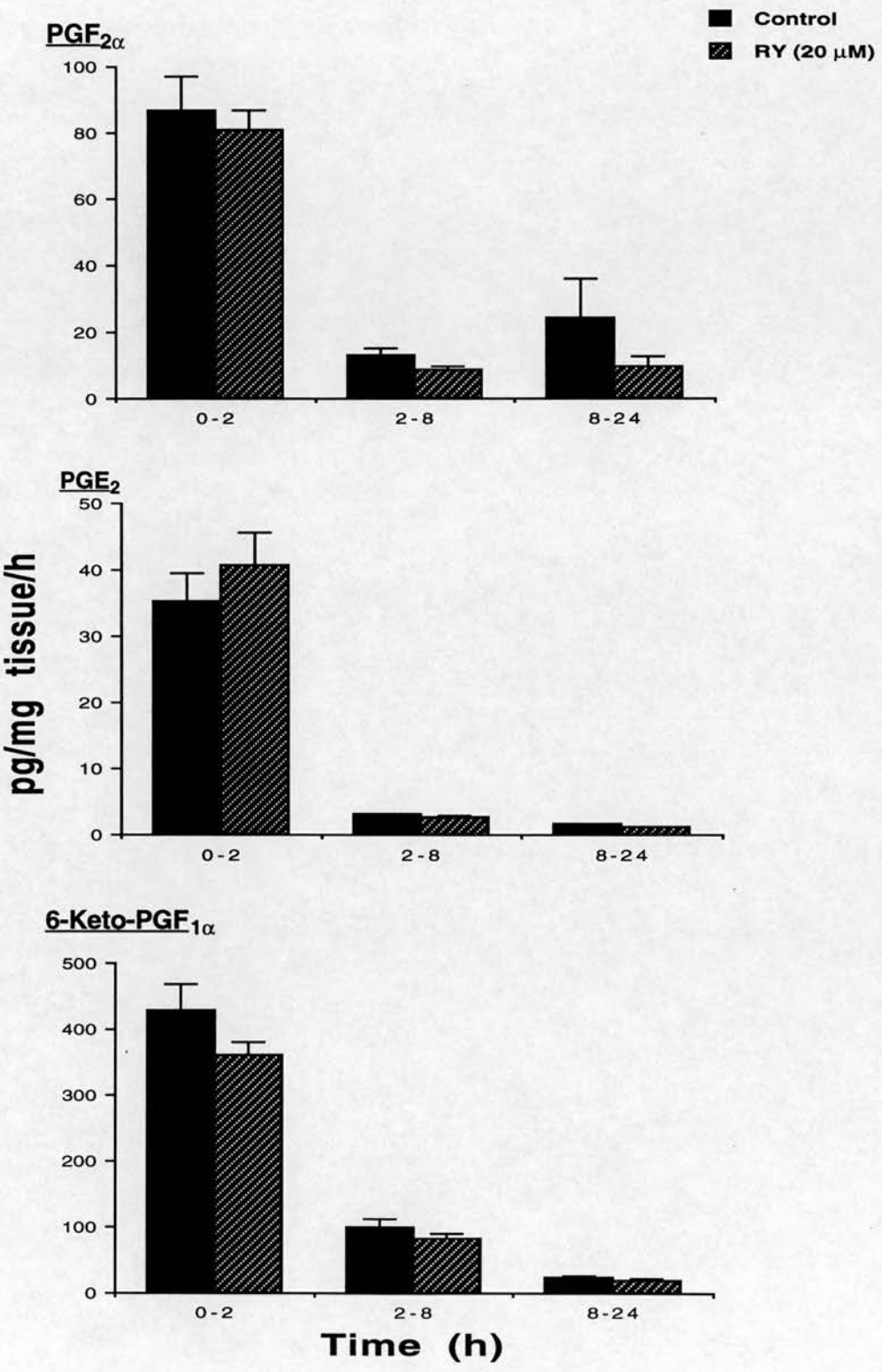


Figure 3.1.3.3. Effects of ryanodine (RY) on means (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 7 guinea-pig emdometrium in culture.

outputs from both days 7 and 15 endometrium in culture than caffeine. The findings that caffeine stimulated PG output from both the day 7 and day 15 guinea-pig uterus superfused *in vitro* (see Sections 3.1.1 & 3.1.2) and from day 7 and day 15 guinea-pig endometrial tissue in culture further support the hypothesis that mobilization of intracellular calcium may be involved in the stimulatory effects of caffeine and theophylline on PG output from the guinea-pig uterus. However, ryanodine had no effect on the outputs of prostaglandin from the day 7 and day 15 guinea-pig endometrium in culture further indicating that ryanodine-sensitive ryanodine receptor activation is not involved in prostaglandin synthesis by and release from the guinea-pig uterus.

3.1.4 The Effects of Caffeine, Theophylline and Ryanodine on Prostaglandin Production by Day 7 and Day 15 Guinea-Pig Epithelial and Stromal Cells in Culture.

Introduction:

Previous studies on prostaglandin production by the guinea-pig uterus were mainly conducted on either *in vitro* tissue superfusion (Poyser and Brydon, 1983; Poyser, 1984; Poyser, 1987; Leckie & Poyser, 1990a), tissue homogenates (Poyser, 1979, Poyser, 1983b, Poyser & Riley, 1987) or tissue culture (Riley & Poyser, 1987a, b; Riley & Poyser, 1989; Leckie & Poyser, 1990b). It has been shown that the amount of $PGF_{2\alpha}$ produced by the guinea-pig uterus superfused *in vitro* (Poyser & Brydon, 1983), by endometrial homogenates (Poyser, 1983b), and by endometrium in culture (Riley & Poyser, 1989; see Section 3.1.3) depends on the stage of the oestrous cycle.

The determination of PG output from endometrial cells has previously been reported in human (Schatz *et al.*, 1985; Smith & Kelly, 1988), cows (Fortier *et al.*, 1988), ewes (Cherny & Findlay, 1990), and mare (Watson *et al.*, 1992), but not in guinea-pigs. Studies on endometrial cell cultures of human (Bonney *et al.*, 1991) and of mares (Watson *et al.*, 1992) have suggested that the PG output from endometrial cells differs between species, and that there is a difference in response to activators between epithelial and stromal cells.

Cells were isolated and cultured from the guinea-pig endometrium in order to identify the major endometrial cell type producing $PGF_{2\alpha}$, PGE_2, and 6-keto-

$PGF_{1\alpha}$, as well as the effects of stage of the oestrous cycle. Since methylxanthines showed different actions on the outputs of prostaglandins from guinea-pig uterine tissue under culture conditions compared with *in vitro* superfusion, their effect on the PG output from the endometrial stromal and epithelial cells was also investigated.

Methods:

Cells were isolated from the endometrium of four day 7 and four day 15 guinea-pigs, and were cultured as described in Section 2.1.5. Briefly, the uterine horns were prepared as described in Section 2.1.3. All procedures were carried out under aseptic conditions. Endometrium was separated from the myometrium and chopped into fragments of about 0.5 mm^3 in 10 ml cell culture medium (CCM) (see Section 2.1.3 for composition) containing 2 mg/ml collagenase type I in a sterile conical flask. The endometrial pieces were incubated at 37°C in a shaking (230 oscillations /min) Grant Water bath for 2 h. After dissociation, the undigested fragments were collected by sedimentation and discarded. Epithelial and stromal cells were separated by centrifugation and re-suspended in 30 to 40 ml of CCM. Aliquots (2.5 ml) of epithelial cells were dispensed into each of 12 wells of a 24-well cell culture plate (2.3×10^3 - 4.5×10^3 cells per ml), and 2.5 ml aliquots of stromal cells were dispensed into the remaining 12 wells of the 24-well cell culture plate (2.2×10^5 - 5.6×10^5 cells per ml). Cell culture plates were then incubated at 37°C in a humidified atmosphere of 5% CO_2 : 95% air.

Culture medium was collected after 3 days of culture, denoted as the "first 3-day period" (3D1), and after 6 days of culture, denoted as the "second 3-day period" (3D2). The cultured cells were then treated in duplicate. Two wells containing each cell type were untreated (controls), and the remaining wells were treated with caffeine (1 & 10 mM), theophylline (1 & 10 mM) or ryanodine (20 μM). The cells were cultured for a further 24 h, and the culture medium was changed after 2, 8, and 24 h. Samples of culture medium were stored at -20°C before radioimmunoassay, without extraction, for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$.

Solutions of caffeine and theophylline were freshly made up in CCM solution prior to use. A concentrated solution of RY was prepared in ethanol and stored at -20°C. The appropriate concentration of RY was prepared by diluting 200-fold in CCM solution.

Statistical tests:

The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

For endometrial cells obtained from the day 7 guinea-pig uterus (hereafter will be called the day 7 cells), the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ but the not of PGE_2 from 3D2 epithelial cells were significantly ($p<0.05$, $n=4$) higher than those from 3D1 epithelial cells. There were no significant changes in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from stromal cells between 3D1 and 3D2. For both time

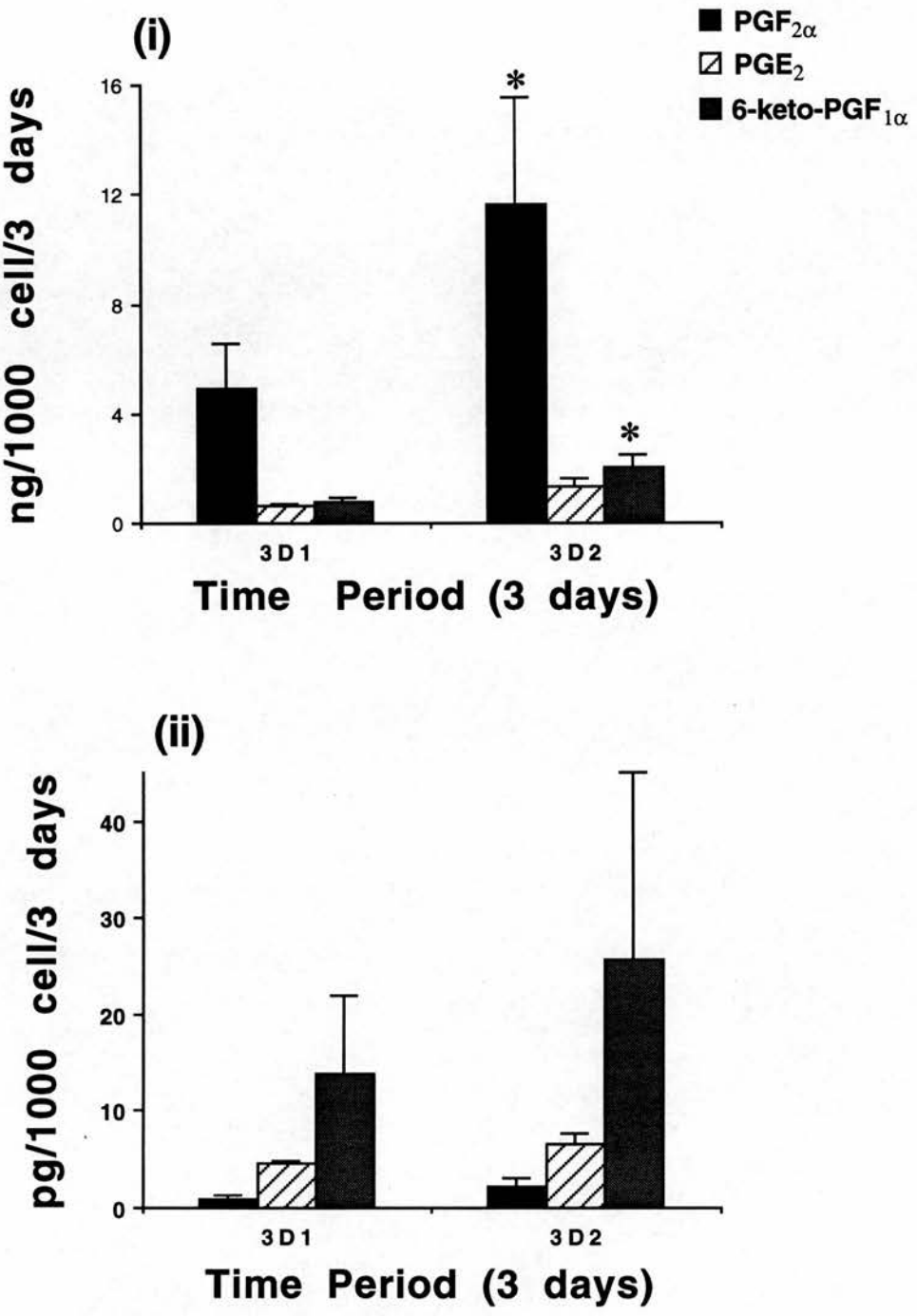


Figure 3.1.4.1. The basal outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured (i) epithelial and (ii) stromal cells of day 7 guinea-pig endometrium. The culture medium was collected on the third (3D1) and sixth (3D2) days of culture. The data indicate the mean (±SEM, n=4).
* Significantly ($p<0.05$) higher than the corresponding value obtained from the first 3 days of cell culture for the same PG. (i.e. 3D1).

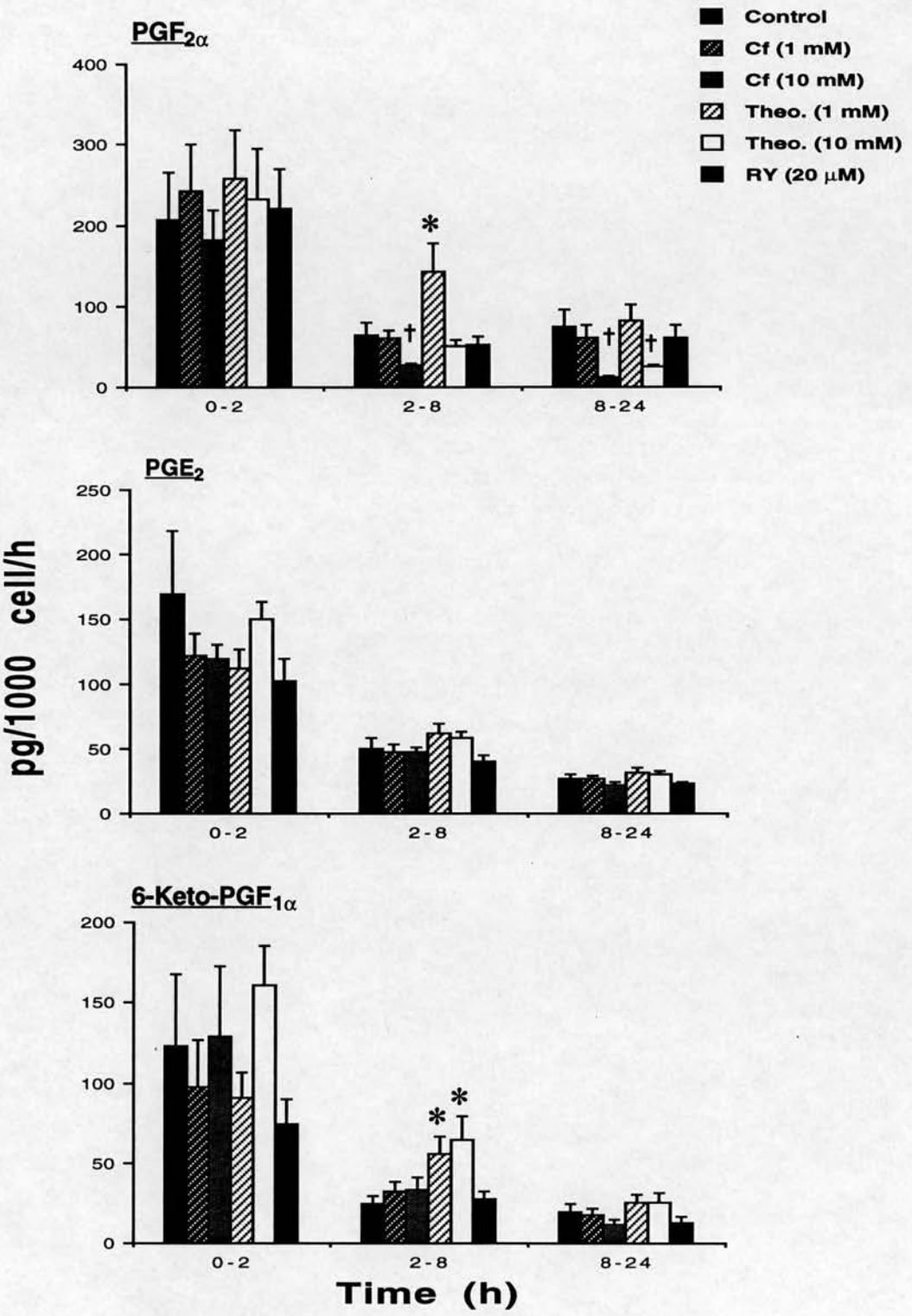


Figure 3.1.4.2. Effects of caffeine (Cf), theophylline (Theo) and ryanodine (RY) on means (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured epithelial cells obtained from day 7 endometrium. * Significantly ($p<0.05$) increased by treatment. † Significantly ($p<.0.05$) inhibited by treatment.

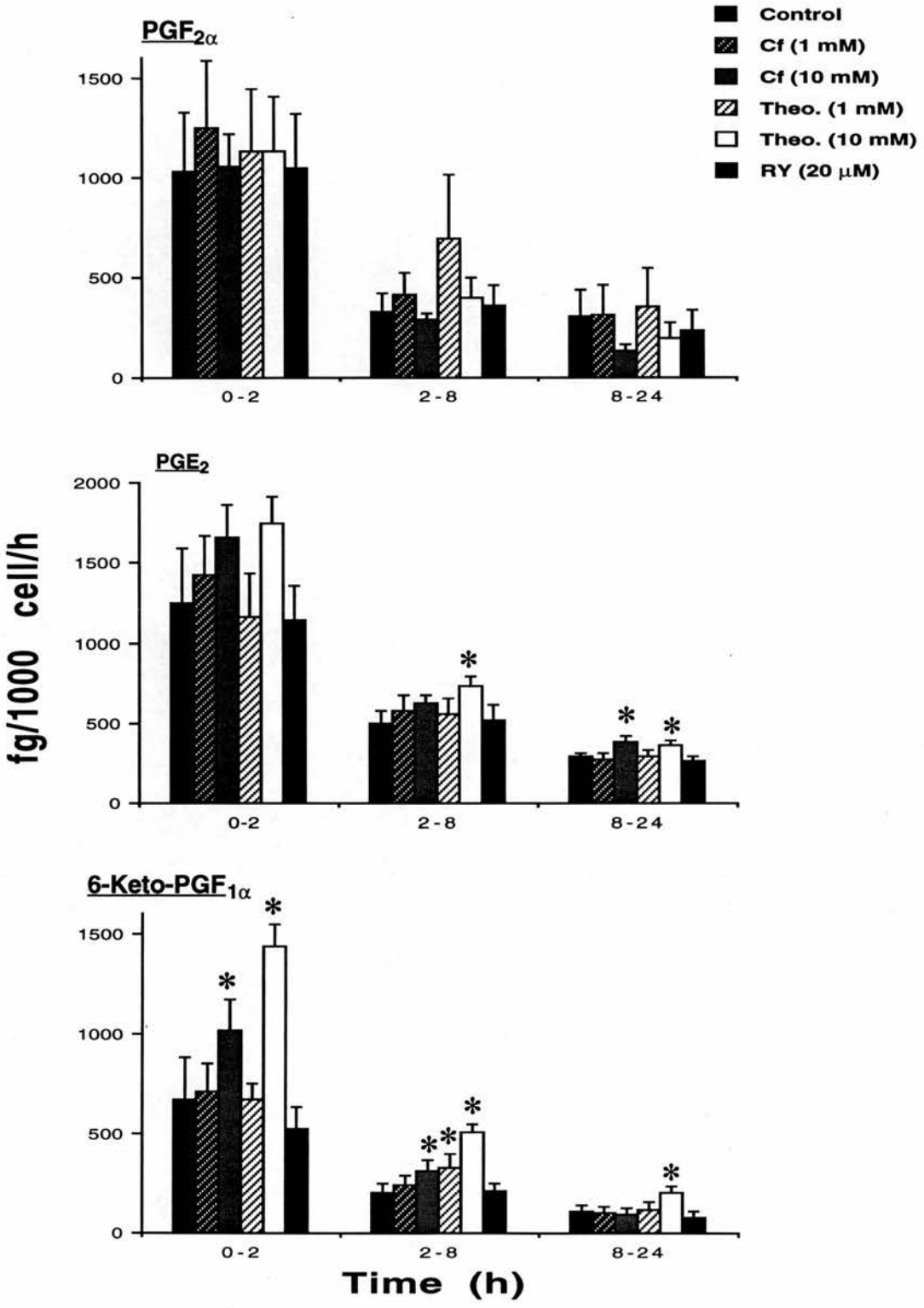


Figure 3.1.4.3. Effects of caffeine (Cf), theophylline (Theo) and ryanodine (RY) on means (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured stromal cells obtained from day 7 endometrium.

* Significantly ($p<0.05$) increased by treatment.

periods, the outputs of all 3 PGs were very much higher ($p<0.05$) from epithelial cells than from stromal cells (Figure 3.1.4.1). Similarly, on the final day of cell culture after 24 h, the basal PG outputs from the day 7 epithelial cells were 200-, 170-, and 150-fold greater than from the stromal cells for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$, respectively (Figures 3.1.4.2 & 3.1.4.3).

For epithelial cells, caffeine (10 mM but not 1 mM) significantly ($p<0.05$, $n=4$) decreased the output of $PGF_{2\alpha}$ after 8 and 24 h of culture (Figure 3.1.4.2). Theophylline (10 mM) significantly ($p<0.05$, $n=4$) decreased the output of $PGF_{2\alpha}$ after 24 h of culture. Theophylline (1 mM) significantly ($p<0.05$, $n=4$) increased $PGF_{2\alpha}$ output after 8 h of culture. Theophylline (1 & 10 mM) significantly ($p<0.05$, $n=4$) increased the output of 6-keto-$PGF_{1\alpha}$ by up to 2.7-fold after 8 h of culture. There were no significant changes in the outputs of any of the three prostaglandins measured due to RY treatment (Figure 3.1.4.2).

For stromal cells, caffeine and theophylline had no significant effects on $PGF_{2\alpha}$ output (Figure 3.1.4.3). Caffeine (10 mM) significantly ($p<0.05$, $n=4$) increased the outputs of PGE_2 by 1.3-fold after 24 h, and 6-keto-$PGF_{1\alpha}$ by up to 1.5-fold after 2 and 8 h of culture. Theophylline (10 mM) significantly ($p<0.05$, $n=4$) increased the output of PGE_2 by up to 1.5-fold after 8 and 24 h of culture. Theophylline (10 mM) caused a significant ($p<0.05$) increase in the output of 6-keto-$PGF_{1\alpha}$ by 1.9- to 2.5-fold after 2, 8, and 24 h of culture (Figure 3.1.4.3). Theophylline (1 mM) and ryanodine (20 μM) had no effects on the outputs of any of the 3 PGs measured from stromal cells in culture (Figure 3.1.4.3).

For endometrial cells obtained from the day 15 guinea-pig uterus (hereafter will be called the day 15 cells), 6-keto-$PGF_{1\alpha}$ output from 3D2 epithelial cells was significantly ($p<0.05$, $n=4$) higher than that from 3D1 epithelial cells. There were no changes in the outputs of PGF2a and PGE2 from the epithelial cells between 3D1 and 3D2. Also there were no significant changes in the outputs of $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ from stromal cells between 3D1 and 3D2 (Figure 3.1.4.4). On the final day of cell culture, the basal outputs of $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ after 24 h were 800-, 330-, and 330-fold greater from day 15 epithelial cells than from day 15 stromal cells, respectively (Figures 3.1.4.5 & 3.1.4.6).

For day 15 epithelial cells, caffeine (10 mM) significantly ($p<0.05$, $n=4$) decreased the output of $PGF_{2\alpha}$ after 2, 8 and 24 h of culture. Theophylline (1 mM but not 10 mM) significantly ($p<0.05$, $n=4$) increased the outputs of PGE_2 by 1.5-fold and 6-keto-$PGF_{1\alpha}$ by 2.3-fold after 8 h of culture. Caffeine (1 mM) and ryanodine (20 μM) had no significant effects on the outputs of the PGs measured (Figure 3.1.4.5).

For stromal cells, there were no significant changes in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ after 2, 8 and 24 h treatment with caffeine (1 & 10 mM), theophylline (1 & 10 mM) or RY (20 μM) (Figure 3.1.4.6). However, the outputs of PGE_2 were significantly ($p<0.05$, $n=4$) increased by caffeine (1 & 10 mM) by up to 1.3-fold after 2 h, by theophylline (10 mM) by up to 1.4-fold after 2 and 24 h, and by theophylline (1 mM) by up to 1.6-fold after 8 and 24 h of culture (Figure 3.1.4.6).

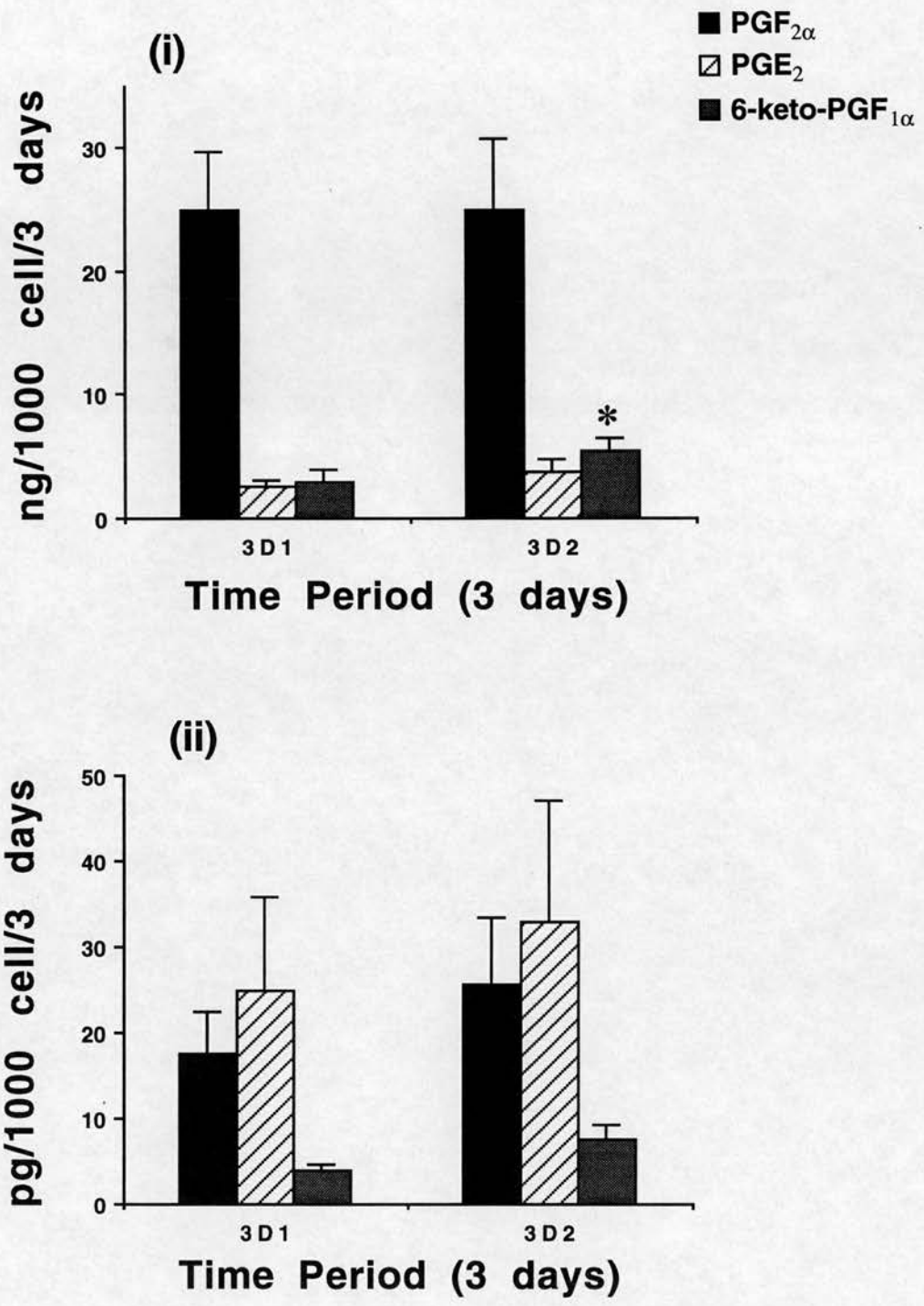


Figure 3.1.4.4. The basal outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured (i) epithelial and (ii) stromal cells of day 15 guinea-pig endometrium. The culture medium was collected on the third (3D1) and sixth (3D2) days of culture. The data indicate the mean (±SEM, n=4). * Significantly ($p<0.05$) higher than the corresponding value obtained from the first 3 days of culture for the same PG (i.e. 3D1).

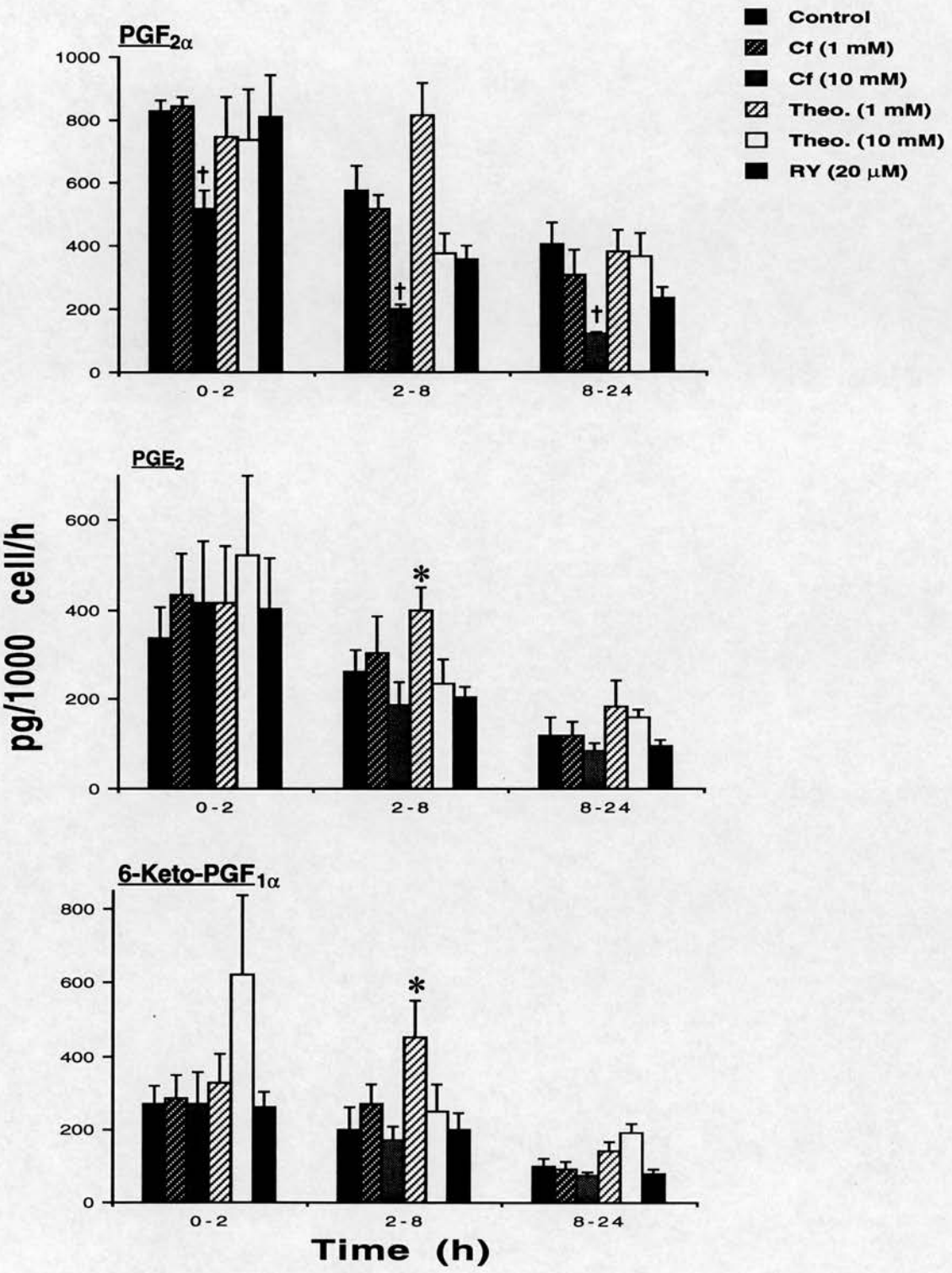


Figure 3.1.4.5. Effects of caffeine (Cf), theophylline (Theo) and ryanodine (RY) on means (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured epithelial cells obtained from the day 15 guinea-pig endometrium.

* Significantly ($p<0.05$) increased by theophylline treatment.

† Significantly ($p<0.05$) decreased by caffeine treatment.

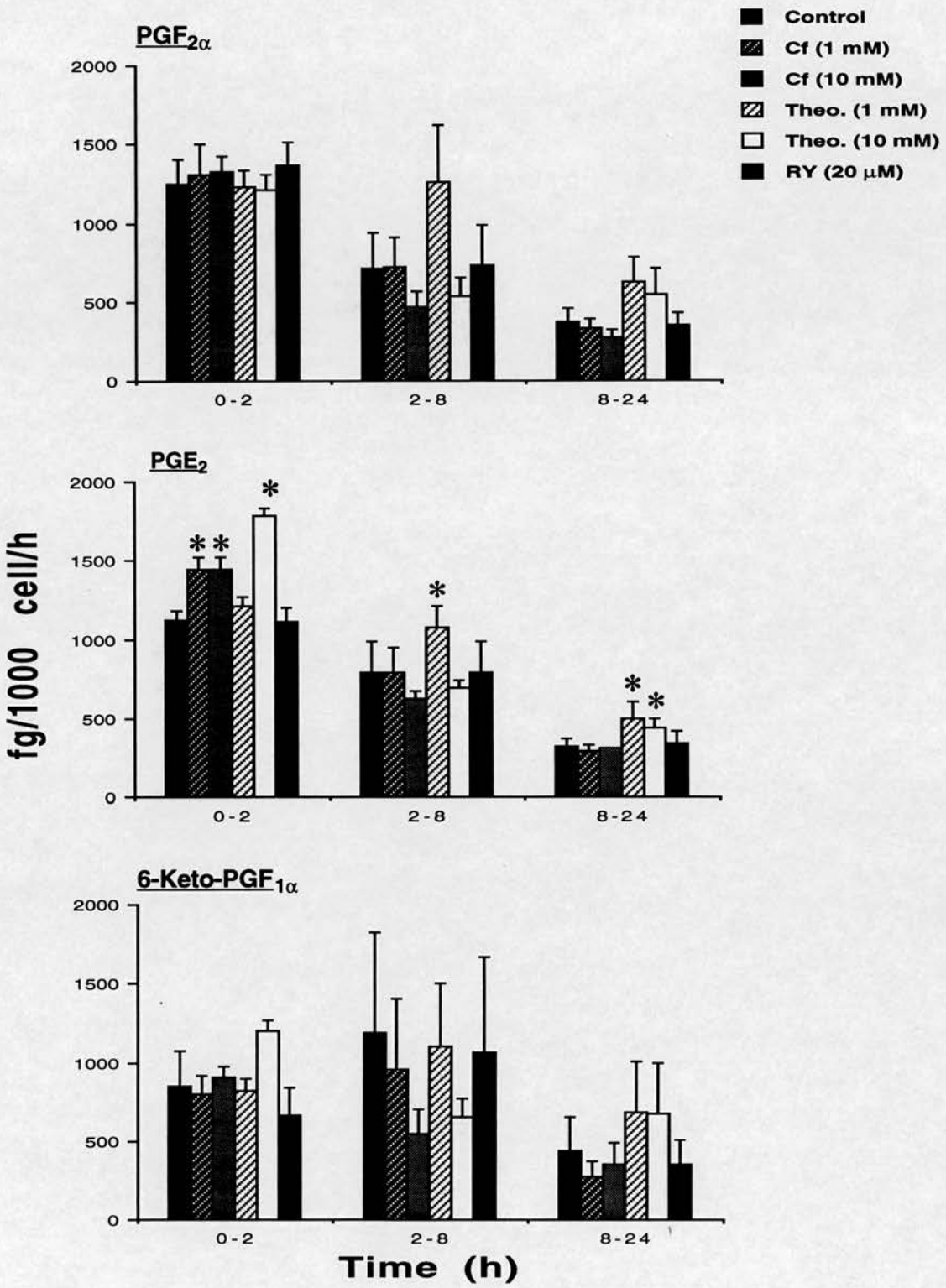


Figure 3.1.4.6. Effects of caffeine (Cf), theophylline (Theo) and ryanodine (RY) on mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured stromal cells obtained from day 15 guinea-pig endometrium.
* Significantly ($p<0.05$) increased by caffeine or theophylline treatment.

Discussion:

In the previous studies on the guinea-pig, the amount of prostaglandins produced by uterine homogenates (Poyser & Riley, 1987a), by the perfused uterine horn (Poyser & Brydon, 1983; Poyser, 1987c; Leckie & Poyser, 1990a) and by cultured endometrial tissue (Leckie & Poyser, 1990b; Riley & Poyser, 1987a, b) have been measured. By isolating endometrial cell types, it was apparent that the outputs of prostaglandins from both the epithelial and stromal cells were 2.5- to 8-fold higher on day 15 than on day 7 of the cycle, a difference which has been reported for both the superfused uterus (Poyser & Brydon, 1983) and cultured endometrial tissue (Leaver & Seawright, 1982; Riley & Poyser, 1987a). This difference between days of the cycle was more pronounced after 8 and 24 h of cell culture.

After 2 h of culture, the basal outputs of $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ were 200-, 170-, and 150-fold greater from epithelial cells than from stromal cells obtained from day 7 guinea-pig endometrium. Similarly, the basal outputs of $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ were 800-, 305-, and 300-fold greater from epithelial cells than from stromal cells obtained from day 15 guinea-pig endometrium. These findings indicate that, in the guinea-pig, the epithelial cells are the major cell type involved in the synthesis and release of prostaglandins in particular $PGF_{2\alpha}$ from the endometrium. This difference in PG output between cell types has also been reported in the mare after 24 h but not after 2 and 8 h of cell culture (Watson *et al.*, 1992).

In many species such as human (Smith & Kelly, 1988, 1989), cow (Fortier *et al.*, 1988), mare (Watson *et al.*, 1992) and sheep (Kim & Fortier, 1995), endometrial

epithelial and stromal cells have been shown to possess different characteristics with regard to prostaglandin production. Watson *et al.* (1992) have reported a gradual increase in the amounts of PGs produced by the epithelial and stromal cells of mare over a 24 h period. In the present study the outputs of all three PGs from the day 7 cells decreased rapidly after 2 h of cell culture, with no changes between 8 and 24 h of culture, but PG outputs from the day 15 cells declined between 2 and 8, and 8 and 24 h of culture. The reason for the gradual increase in the amounts of PGs produced by mare endometrial cell in culture is that the time period of culture has not been taken into account by Watson *et al.* (1992) such, that by increasing the time of culture, more PG was produced by the cells.

Kim and Fortier (1995) have reported that, by 36 h of culture, sheep epithelial cells produce more $PGF_{2\alpha}$ (by about 40-fold) than the stromal cells, whereas stromal cells produce more PGE_2 (by about 35-fold). However, in the present study, the amounts of $PGF_{2\alpha}$ and PGE_2 produced by epithelial cells obtained from the day 7 and day 15 guinea-pig endometrium were much greater than the amounts of $PGF_{2\alpha}$ and PGE_2 produced by stromal cells in culture, suggesting that the relative amounts of $PGF_{2\alpha}$ and PGE_2 produced by endometrial cells is species specific.

In the previous sets of experiments (see Sections 3.1.1 & 3.1.2), caffeine and theophylline increased the outputs of $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ from the guinea-pig uterus superfused *in vitro*. The stimulatory effects of caffeine and theophylline were also apparent on day 7 endometrium in tissue culture (see Section 3.1.3). Caffeine had both a stimulatory and an inhibitory effect on the

output of $PGF_{2\alpha}$ from the day 15 endometrium in culture (see Section 3.1.3). However, in this set of experiments caffeine inhibited, in particular, the output of $PGF_{2\alpha}$ from epithelial cells obtained from day 7 and day 15 endometrium.

It has been previously reported that sodium fluoride (NaF) produces both a stimulatory and an inhibitory effect on the outputs of prostaglandins from the guinea-pig uterus (Leckie & Poyser, 1990a, b). It has been shown that NaF inhibits protein synthesis in the guinea-pig endometrium in culture (Leckie & Poyser, 1990b), so the inhibitory effect of NaF on PG output from the guinea-pig endometrium in culture is thought to be due to its ability to inhibit protein synthesis (Leckie & Poyser, 1990b). The differences in the effects of methylxanthines on the outputs of prostaglandins from the superfused guinea-pig uterus, endometrium tissue culture and endometrial cells in culture may be explained by assuming that these compounds possess a 'short term' effect which was seen on superfusion and a 'long term' effect which was apparent in culture conditions (particularly in cell culture condition). It has been shown that caffeine affects protein synthesis. In the rat striatum caffeine induces neurotensin and CCK mRNA synthesis (Schiffmann & Vanderhaeghen, 1993). However, in rat brain caffeine decreases glial cell numbers (Marret *et al.*, 1993). Hence, it is possible that, in the long term, caffeine inhibits $PGF_{2\alpha}$ output by modulating protein synthesis from the guinea-pig endometrial cells in culture, as it has been shown that protein synthesis inhibitors decrease prostaglandin output from the cultured guinea-pig endometrial tissue (Poyser & Riley, 1987; Riley & Poyser, 1989). However, this suggestion requires further study. One other explanation is that, to see a true effect of methylxanthines on

guinea-pig uterine PG output, it is necessary that the epithelial and stromal cells should be in direct contact with each other. Interactions between stromal and epithelial cells (Roberts *et al.*, 1988) and between individual stromal cells (Parmley *et al.*, 1990) have been shown in the human endometrial cycle. Roberts *et al.* (1988) have reported four significant changes in the transition from early proliferative (days 5 to 9) to early secretory (days 15 to 19) phases. These changes include: (1) an increase in the number and size of lamina densa distribution, (2) an increase in the number and size of gap junctions, (3) an increase in the number of epithelial cells, and (4) an increase in close contacts between stromal and epithelial cells (Roberts *et al.*, 1988). Finally, the ineffectiveness of ryanodine in producing a change in PG output from the epithelial and stromal cells derived from guinea-pig endometrium once again indicates that ryanodine-sensitive RYR channels are not involved in the synthesis and release of prostaglandins from the guinea-pig endometrium.

3.1.5 The Effects of TMB-8 and Calmodulin Inhibitors (W-7 & Trifluoperazine) on The Action of Caffeine on PG Output, and The Effects of PLA_2 and A23187 on PG Output From Cultured Endometrial Cells of The Day 7 Guinea-Pig Uterus.

Introduction:

The effects of calmodulin inhibitors (W-7 & TFP) on basal PG output, and their effects on A23187-induced PG output from the guinea-pig uterus superfused *in vitro* (Poyser, 1985a, b) have previously been reported. In the earlier experiment (see Section 3.1.2), it was observed that W-7 or TFP had no inhibitory effects on the caffeine-induced rise in the outputs of prostaglandins from guinea-pig uterine horns superfused *in vitro*, and TMB-8 inhibited only the caffeine stimulated rise in $PGF_{2\alpha}$ output without affecting the increases in outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$. In fact, W-7, but not TFP, potentiated the caffeine effect on $PGF_{2\alpha}$ synthesis by and release from the guinea-pig uterus. However, in the previous experiment (see Section 3.1.4), it was seen that caffeine had a stimulatory and an inhibitory effect on the outputs of prostaglandins from endometrial cells in culture. Hence, this experiment was designed to investigate whether calmodulin inhibitors and TMB-8 would have different effects on the outputs of PG from cultured endometrial cells in comparison to *in vitro* superfusion of the uterine horns. The possible effects of these agents on caffeine-induced changes in the outputs of prostaglandin from endometrial cells in culture were also investigated.

Phospholipase A_2 and the calcium ionophore, A23187, increase the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 and day 15 guinea-pig uterus superfused *in vitro* (Poyser, 1987a; 1991). A23187 has been reported to have differential effects on human endometrial epithelial and stromal cells in culture. A23187 stimulates arachidonic acid release from human epithelial but not from stromal cells in culture (Bonney *et al.*, 1991). Here, it was decided to investigate the actions of PLA_2 and A23187 on cultured endometrial cells so that a comparison could be made regarding their effects on the superfused uterine horn and cultured endometrial cells.

Methods:

Epithelial and stromal cells from the endometrium of four day 7 guinea-pigs were isolated and cultured as described in Section 2.1.5. Culture medium was changed every 3 days and on the sixth day cells were treated in duplicate. In experiment 1, two wells containing each cell type were untreated (controls), and the remaining cells were treated with caffeine (10 mM), W-7 (100 μM), TFP (150 μM), caffeine (10 mM) plus W-7 (100 μM) or caffeine (10 mM) plus TFP (150 μM). In experiment 2, two wells from each cell type were untreated (controls), and the remaining cells were treated with caffeine (10 mM), TMB-8 (150 μM), caffeine (10 mM) plus TMB-8 (150 μM), PLA_2 (2 U/ml), or A23187 (1 μg/ml). Culture medium was changed at 2, 8, and 24 h. The concentrations of the PLA_2 and A23187 used in this experiment have been shown to stimulate PG output from the guinea-pig uterus superfused *in vitro* (Poyser, 1985a, b; Poyser & Ferguson,

1993). Samples of culture medium were stored at -20°C before radioimmunoassay, without extraction, for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$. Solutions of caffeine, TMB-8, W-7, TFP and PLA_2 were freshly made up in CCM solution prior to use. A concentrated solution of A23187 was prepared in ethanol and stored at -20°C. The appropriate concentration of A23187 was prepared by diluting 100-fold in CCM solution.

Statistical tests:

The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

Experiment 1:

For epithelial cells, caffeine (10 mM) significantly ($p<0.05$, $n=4$) inhibited the output of $PGF_{2\alpha}$ from epithelial cells after 2, 8 and 24 h of culture (Figure 3.1.5.1). The output of $PGF_{2\alpha}$ was significantly ($p<0.05$, $n=4$) inhibited by W-7 (100 μM) after 8 and 24 h, and by TFP (150 μM) after 2, 8 and 24 h of culture. Caffeine plus W-7 and caffeine plus TFP also significantly ($p<0.05$, $n=4$) inhibited the output of $PGF_{2\alpha}$ after 2, 8 and 24 h of culture. There were no significant changes in the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial cells in culture due to any of these treatments, except W-7 alone which significantly ($p<0.05$, $n=4$) inhibited 6-keto-$PGF_{1\alpha}$ output after 8 h of culture (Figure 3.1.5.1).

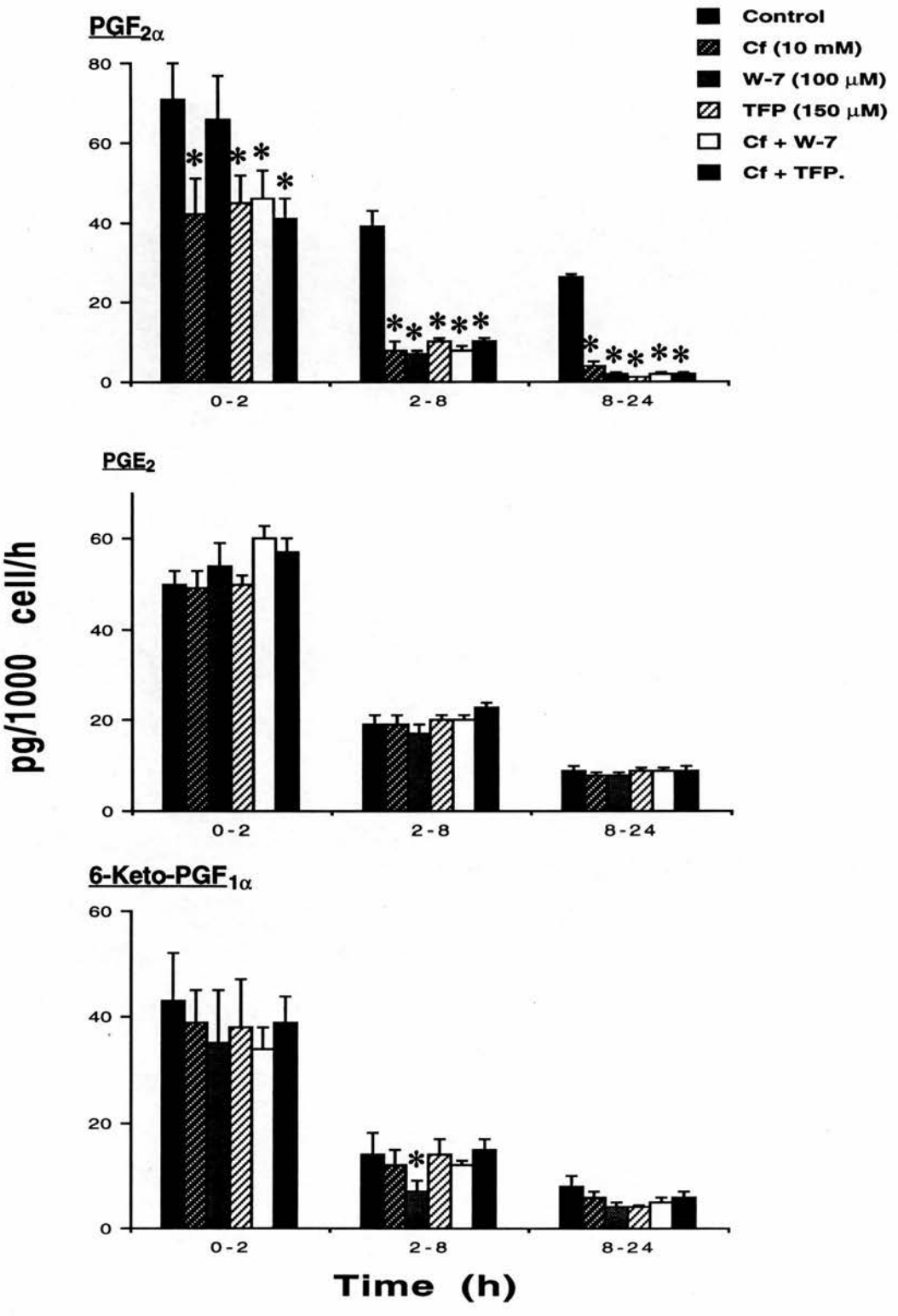


Figure 3.1.5.1. Effects of caffeine (Cf), W-7, trifluoperazine (TFP) and caffeine in the presence of W-7 or TFP on mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured epithelial cells obtained from the day 7 guinea-pig endometrium.

* Significantly ($p<0.05$) lower than the corresponding control value.

For stromal cells, the output of $PGF_{2\alpha}$ was significantly ($p<0.05$, $n=4$) inhibited by caffeine after 2, 8 and 24 h, by W-7 after 8 and 24 h and by TFP after 2, 8 and 24 h of culture (Figure 3.1.5.2). Caffeine plus W-7 and caffeine plus TFP also significantly ($P<0.05$, $n=4$) inhibited the output of $PGF_{2\alpha}$ after 8 and 24 h of culture. There were no significant changes in the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from stromal cells in culture due to these treatments, except W-7 and TFP alone which significantly ($p<0.05$, $n=4$) inhibited the output of 6-keto-$PGF_{1\alpha}$ after 24 h of culture (Figure 3.1.5.2).

Experiment 2:

For epithelial cells, caffeine (10 mM) significantly ($p<0.05$) inhibited the output of $PGF_{2\alpha}$ after 2, 8 and 24 h of culture (Figure 3.1.5.3). TMB-8 (150 μM) significantly ($p<0.05$, $n=4$) inhibited the output of $PGF_{2\alpha}$ after 8 and 24 h of culture. Caffeine and TMB-8 alone had no significant effect on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial cells in culture. Caffeine plus TMB-8 significantly ($p<0.05$, $n=4$) decreased the output of $PGF_{2\alpha}$ after 2, 8 and 24 h of culture. Caffeine plus TMB-8 had no effect on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$. PLA_2 (2 U/ml) significantly ($p<0.05$, $n=4$) increased the outputs of $PGF_{2\alpha}$ by 1.7-fold after 24 h, PGE_2 by 1.6- to 2.6-fold after 2, 8 and 24 h, and 6-keto-$PGF_{1\alpha}$ by 1.2-fold after 24 h of culture. Calcium ionophore (A23187; 1 μg/ml) significantly ($p<0.05$, $n=4$) increased the output of $PGF_{2\alpha}$ by 1.7-fold after 2 h but significantly ($p<0.05$, $n=4$) decreased the output of $PGF_{2\alpha}$ after 24 h

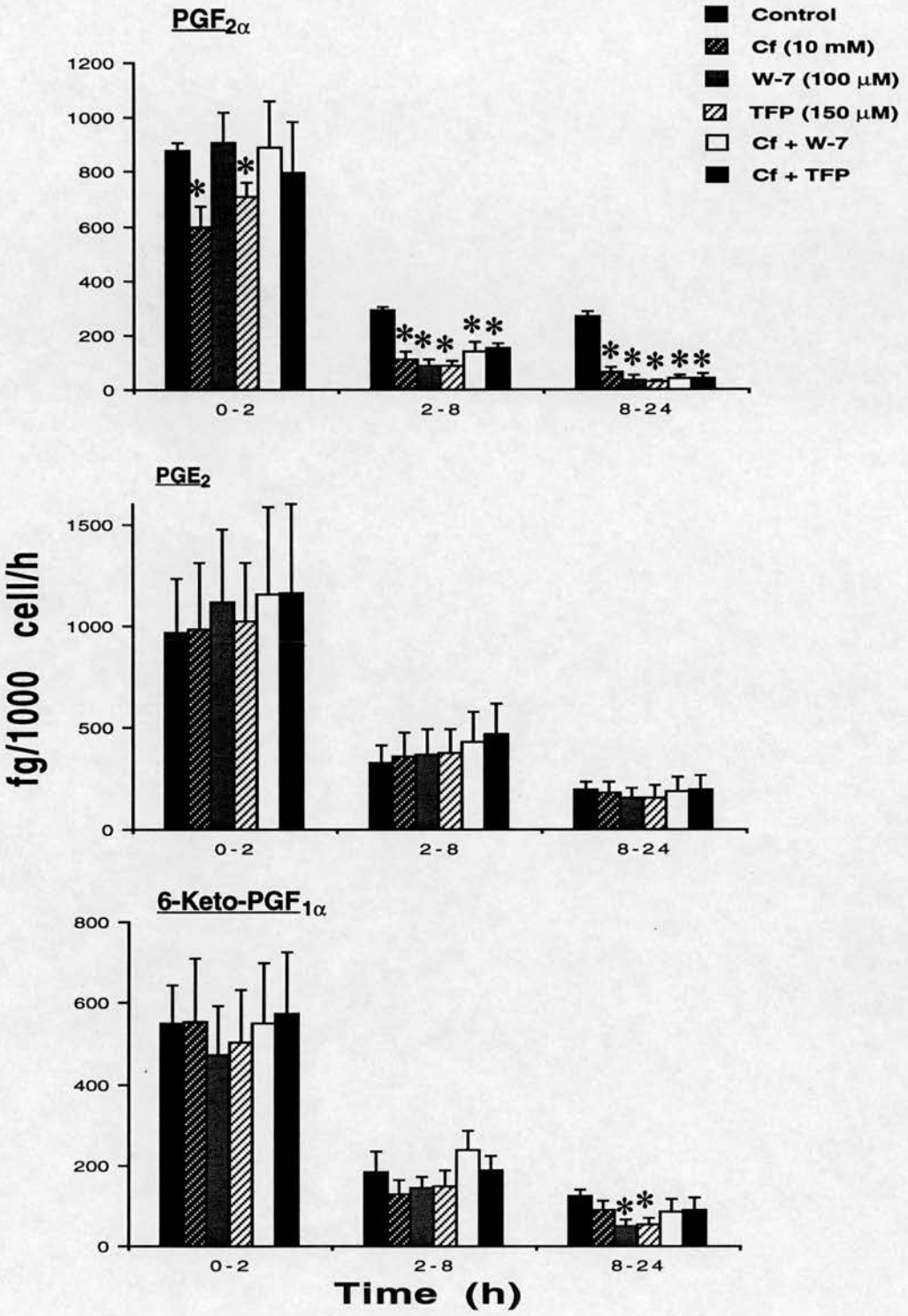


Figure 3.1.5.2. Effects of caffeine (Cf), W-7, trifluoperazine (TFP) and caffeine in the presence of W-7 or TFP on mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured stromal cells obtained from day 7 guinea-pig endometrium.

* Significantly ($p<0.05$) lower than the corresponding control value.

of culture. A23187 had no effect on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ (Figure 3.1.5.3).

For stromal cells, caffeine had no effect on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$, except it significantly ($p<0.05$, $n=4$) increased the output of PGE_2 by 1.2-fold after 24 h of cell culture (Figure 3.1.5.4). TMB-8 alone had no effect on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured stromal cells. Caffeine plus TMB-8 also had no effect on the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but significantly ($p<0.05$, $n=4$) increased the output of PGE_2 by 1.2- to 1.3-fold after 2 and 24 h but not after 8 h of culture. PLA_2 caused significant ($p<0.05$, $n=4$) increases in the outputs of $PGF_{2\alpha}$ by 1.7-fold and 6-keto-$PGF_{1\alpha}$ by 1.4-fold after 24 h of culture, and in the output of PGE_2 by 1.5- to 1.6-fold after 2, 8 and 24 h of culture (Figure 3.1.5.4). A23187 Significantly ($p<0.05$, $n=4$) increased the output of $PGF_{2\alpha}$ by 1.3-fold after 2 h but significantly ($p<0.05$, $n=4$) decreased $PGF_{2\alpha}$ output after 24 h of culture. A23187 also significantly ($p<0.05$, $n=4$) decreased the output of 6-keto-$PGF_{1\alpha}$ after 8 and 24 h of culture. A23187 had no effect on PGE_2 output from the stromal cells in culture (Figure 3.1.5.4).

Discussion:

It has been previously reported that the increase in the endometrial prostaglandin synthesis and release in the rat (Day *et al.*, 1982), guinea-pig (Downing & Poyser, 1983), and human (Bonney, 1985) is associated with an increase in the activity of phospholipase A_2, a calcium dependent enzyme. In the guinea-pig, it is thought that the activation of PLA_2 by calcium (Downing & Poyser, 1983) leads to the

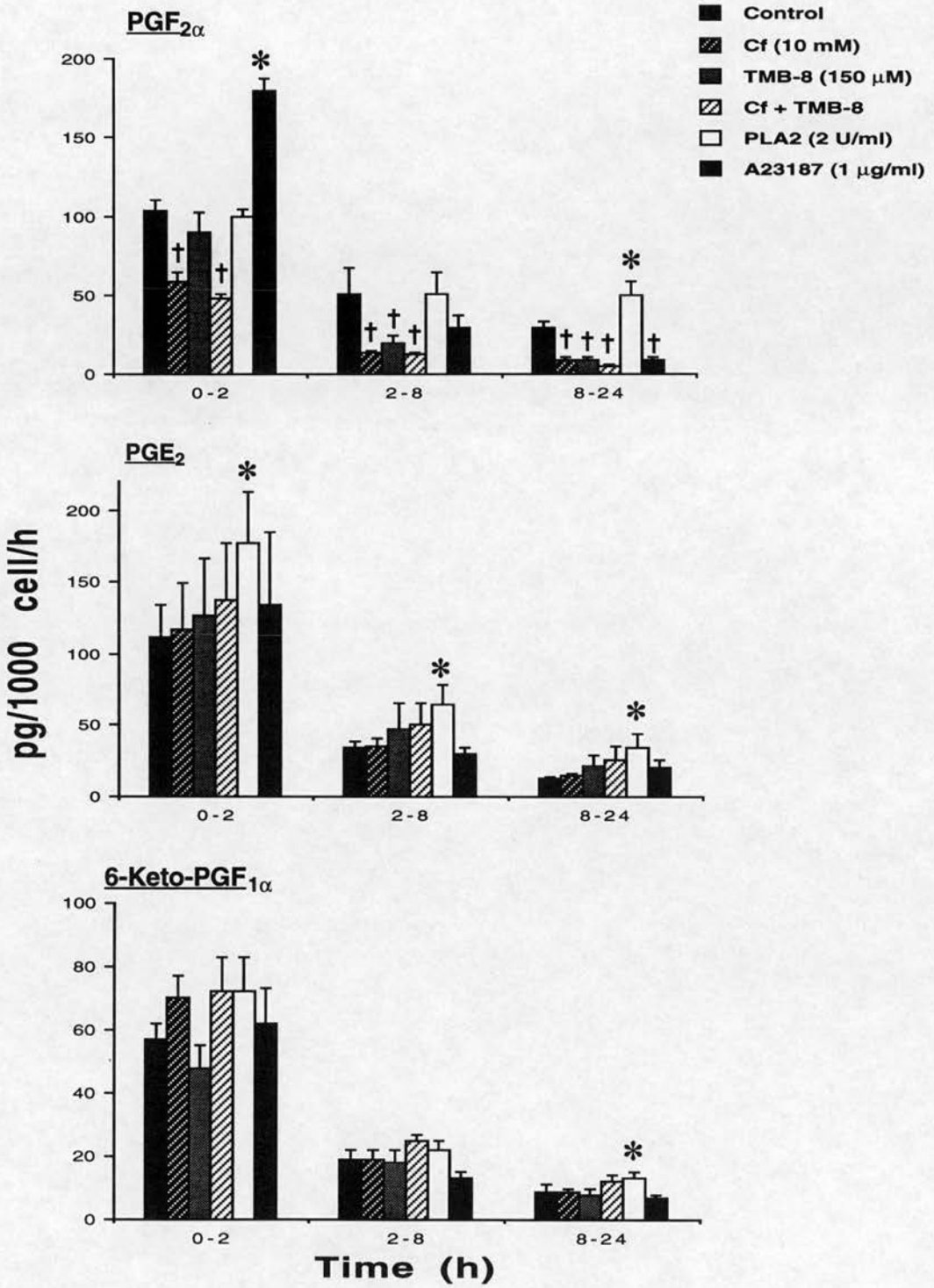


Figure 3.1.5.3 Effects of caffeine (Cf), TMB-8, PLA_2, A23187 and caffeine in the presence of TMB-8 on mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured epithelial cells obtained from day 7 guinea-pig endometrium. * Significantly ($p<0.05$) higher than the corresponding control value. † Significantly ($p<0.05$) lower than the corresponding control value.

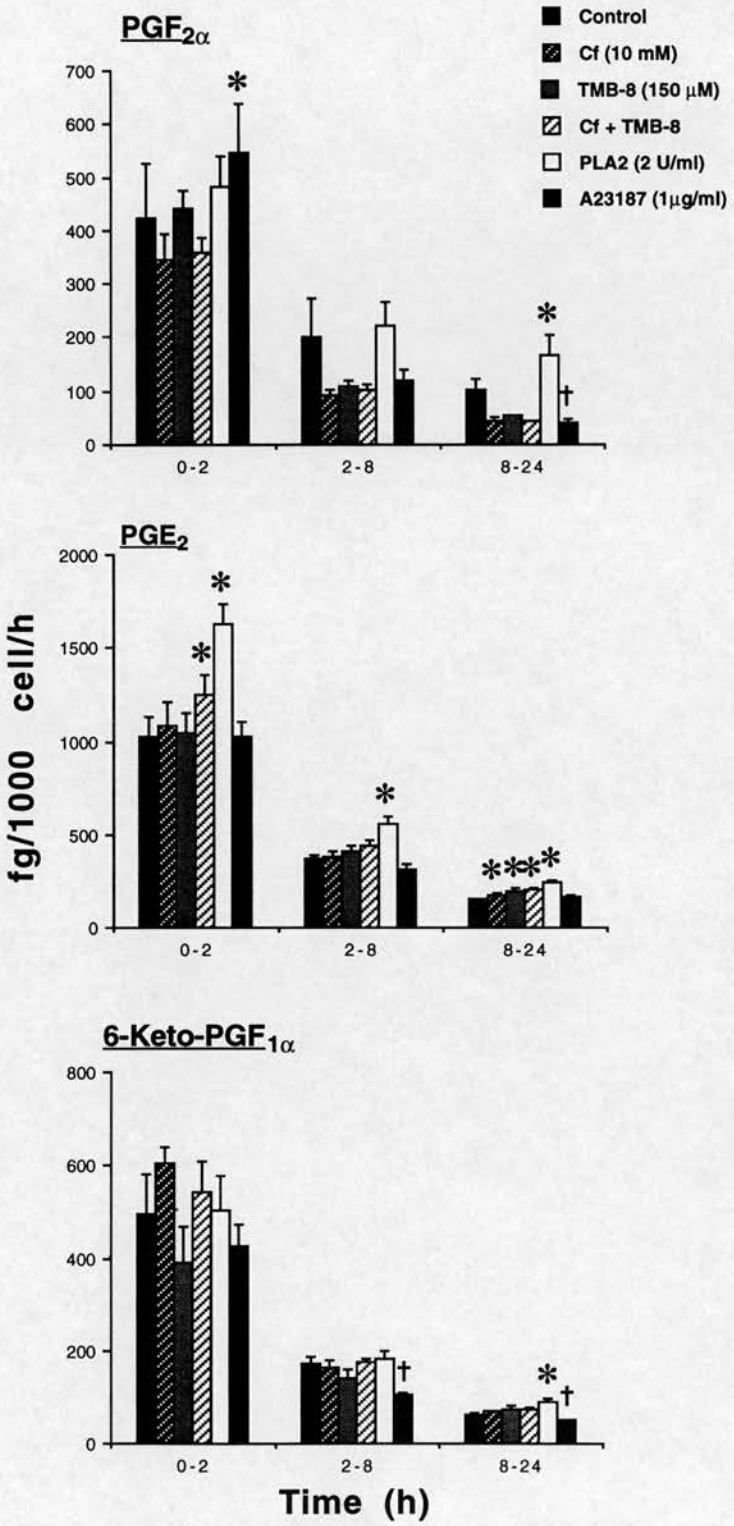


Figure 3.1.5.4. Effects of caffeine (Cf), TMB-8, PLA2, A23187 and caffeine in the presence of TMB-8 on mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured stromal cells obtained from day 7 guinea-pig endometrium.

* Significantly ($p<0.05$) higher than the corresponding control value.

† Significantly ($p<0.05$) lower than the corresponding control value.

release of arachidonic acid from phosphatidylcholine and phosphatidylethanolamine (Ning & Poyser, 1984) which, in turn, results in increased $PGF_{2\alpha}$ synthesis by and release from the endometrium. Poyser (1987a, b) also suggested that A23187-induced increase in PG output from the guinea-pig uterus is via activation of PLA_2. PLA_2 and A23187 have been reported to increase $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs from superfused guinea-pig uterine horns (Poyser, 1991). In this study, the PLA_2 stimulated the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from epithelial and stromal cells only after 24 h of cell culture, whereas PLA_2 stimulated the output of PGE_2 from both cell types after 2, 8 and 24 h of culture. These findings suggest that the exogenous PLA_2 tends to preferentially stimulate PGE_2 synthesis by and release from the guinea-pig endometrial cells. The reason for this limited action of PLA_2 on $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ output from cultured endometrial cells remains to be identified.

The effect of A23187 on PG outputs was somewhat unexpected. The outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial and stromal cells were not affected by A23187. Nevertheless, during the first 2 h of cell culture, A23187 stimulated $PGF_{2\alpha}$ output from cultured epithelial and stromal cells, an effect reported for perfused uterine horns (Poyser, 1987a, b; Poyser, 1991; Poyser & Ferguson, 1993). By 24 h of cell culture, this stimulatory effect was reversed and inhibition of $PGF_{2\alpha}$ output from both epithelial and stromal cells was seen. Following repeated stimulation, a partial refractoriness to A23187 has been shown on PG output from the guinea-pig uterus superfused *in vitro* (Poyser, 1991; Poyser & Ferguson, 1993). This refractoriness is thought to occur at the level of arachidonic

acid release, since there is no refractoriness to repeated stimulations with arachidonic acid as regards uterine prostaglandin production (Poyser, 1991). There appears to be one or several bound pools of arachidonic acid which are releasable but which take about 3-5 h to refill (Poyser, 1991). Thus, it seems that the lack of a stimulatory effect of A23187 on $PGF_{2\alpha}$ output from cultured cells after 8 h of culture was probably due to this refractoriness. However, this phenomenon of refractoriness does not explain the inhibitory effect of A23187 on the output of prostaglandins after 24 h of cell culture.

It has been reported that A23187 affects epithelial and stromal cells differently. A23187 stimulated a dose- and time-dependent release of arachidonic acid from human endometrial glands without affecting stromal cells (Bonney *et al.*, 1991). In the present study, A23187 stimulated PG output from both epithelial and stromal cells after 2 h of culture indicating a species difference with regard to A23187 activity on the uterine tissue.

TMB-8 causes a transient increase in the output of $PGF_{2\alpha}$ from the guinea-pig uterus superfused *in vitro* (Poyser, 1985b; Leckie & Poyser, 1991a; see Section 3.1.2). However, in this present set of experiments, TMB-8 inhibited $PGF_{2\alpha}$ synthesis by and release from cultured epithelial cells and, to a lesser extent, from stromal cells suggesting a role for intracellular calcium in uterine PG output. Caffeine alone and TMB-8 alone increased PGE_2 output from epithelial cells after 24 h but not after 2 and 8 h of culture. However, caffeine plus TMB-8 significantly increased PGE_2 output from epithelial cells after 2 h of culture, suggesting that in

the short term, these compounds have synergistic stimulatory effect on the output of PGE_2 from cultured epithelial cells.

Calmodulin inhibitors (W-7 and TFP) have been shown to inhibit A23187-induced prostaglandin output from the guinea-pig uterus superfused *in vitro* (Poyser, 1985a, b). In this study, the calmodulin antagonists (W-7 and TFP) inhibited the output of $PGF_{2\alpha}$ and to a much lesser extent the output of 6-keto-$PGF_{1\alpha}$ from both epithelial and stromal cells in culture suggesting that calmodulin is involved in the basal PG output from the cultured day 7 guinea-pig endometrial cells.

3.1.6 The Effects of Ryanodine, Dantrolene, Thapsigargin and Berberine on The Actions of Caffeine on Cultured Epithelial and Stromal Cells Obtained From Day 7 Guinea-Pig Endometrium.

Introduction:

Dantrolene is reported to be a potent muscle relaxant and an inhibitor of ryanodine-binding to the skeletal type ryanodine-receptor (RYR) (Pessah *et al.*, 1986; Shoshan-Barmatz *et al.*, 1991), but does not inhibit RY binding to rat brain RY receptors (Smith & Nahroski, 1993). Thapsigargin (TG) decreases intracellular calcium uptake in rabbit pancreatic acinar cells (Van de Put *et al.*, 1993), depletes calcium from the sarcoplasmic reticulum (Chen & Van Breemen, 1993), and abolishes agonist-induced calcium uptake (Kuemmerle *et al.*, 1994). Berberine is reported to block calcium release from the sarcoplasmic reticulum (Chiou *et al.*, 1991). In the previous experiments the effects of caffeine on PG output from endometrial cells were somewhat (see Sections 3.1.4 & 3.1.5) different from its action on PG output from perfused uterine horns (see Sections 3.1.1 & 3.1.2). However, RY alone consistently had no effect on PG output from superfused uterine horns, and from endometrial epithelial and stromal cells in culture (see Sections 3.1.1, 3.1.2 & 3.1.4). Thus, this set of experiments was designed to investigate whether these substances which modulate concentration of free intracellular calcium would affect the outputs of PG from the guinea-pig uterus.

Methods:

Epithelial and stromal cells from the day 7 guinea-pig endometrium were isolated and cultured as described in Section 2.1.5. Culture medium was changed every 3 days and on the sixth day cells in culture were treated in duplicate. In experiment 1, two wells containing each cell type were untreated (controls), and the remaining cells were treated with caffeine (10 mM), dantrolene (300 μM), RY (20 μM), caffeine (10 mM) plus dantrolene (300 μM) or caffeine (10 mM) plus RY (20 μM). In experiment 2, two wells containing each cell type were untreated (controls), and the remaining wells were treated with caffeine (10 mM), thapsigargin (1 μM), berberine (20 μM), caffeine (10 mM) plus thapsigargin (1 μM) and caffeine (10 mM) plus berberine (20 μM). Culture medium was changed after 2, 8, and 24 h of culture. Samples of culture medium were stored at -20°C before radioimmunoassay, without extraction, for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$.

Solutions of caffeine, dantrolene and berberine were freshly made up in CCM solution prior to use. Concentrated solutions of RY and thapsigargin were prepared in ethanol and DMSO, respectively and stored at -20°C. The appropriate concentration of RY and thapsigargin were prepared in CCM solution. These compounds were diluted 200- and 150-fold with CCM before use, respectively. Usachev *et al.* (1993) has shown that, in rat sensory neurones, caffeine-evoked $[Ca^{2+}]_i$ transients were effectively blocked by RY (10 μM) and dantrolene (10 μM). However, Smith and Nahorski (1993) have reported that [^{3}H]RY-binding to cortical membrane in the rat brain was enhanced by caffeine (10 mM) and inhibited

by ruthenium red (10^{-7} - 10^{-4} M), whereas dantrolene (300 μM) was ineffective. Xu and Forsberg (1993) have shown that thapsigargin (1 μM) induced increase in $[Ca^{2+}]_i$ an effect which was blocked by pre-treatment with 40 mM caffeine. Berberine (10^{7} - $3x10^{-5}$ M) causes dose-dependent relaxation of phenylephrine-induced contraction (Chiou *et al.*, 1991). Therefore, the concentrations of ryanodine, dantrolene, thapsigargin and berberine chosen in the present study were in the expected range to produce effects.

Statistical tests:

The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

In experiment 1, caffeine (10 mM) and caffeine (10 mM) plus RY (20 μM) caused a significant ($p<0.05$, $n=4$) decrease in the output of $PGF_{2\alpha}$ from epithelial cells after 8 and 24 h in culture (Figure 3.1.6.1). Caffeine (10 mM) also significantly ($p<0.05$, $n=4$) inhibited the output of 6-keto-$PGF_{1\alpha}$ from epithelial cells after 8 h of culture. Caffeine had no effect on PGE_2 output from epithelial cells. Caffeine (10 mM) significantly ($p<0.05$, $n=4$) stimulated the outputs of PGE_2 after 2 and 8 h and 6-keto-$PGF_{1\alpha}$ after 2, 8 and 24 h from stromal cell in culture (Figure 3.1.6.1). Caffeine had no effect on $PGF_{2\alpha}$ output from stromal cells.

Dantrolene (300 μM) alone had no effect on $PGF_{2\alpha}$ output from epithelial cells, but significantly ($p<0.05$, $n=4$) increase the output of $PGF_{2\alpha}$ from stromal cells by

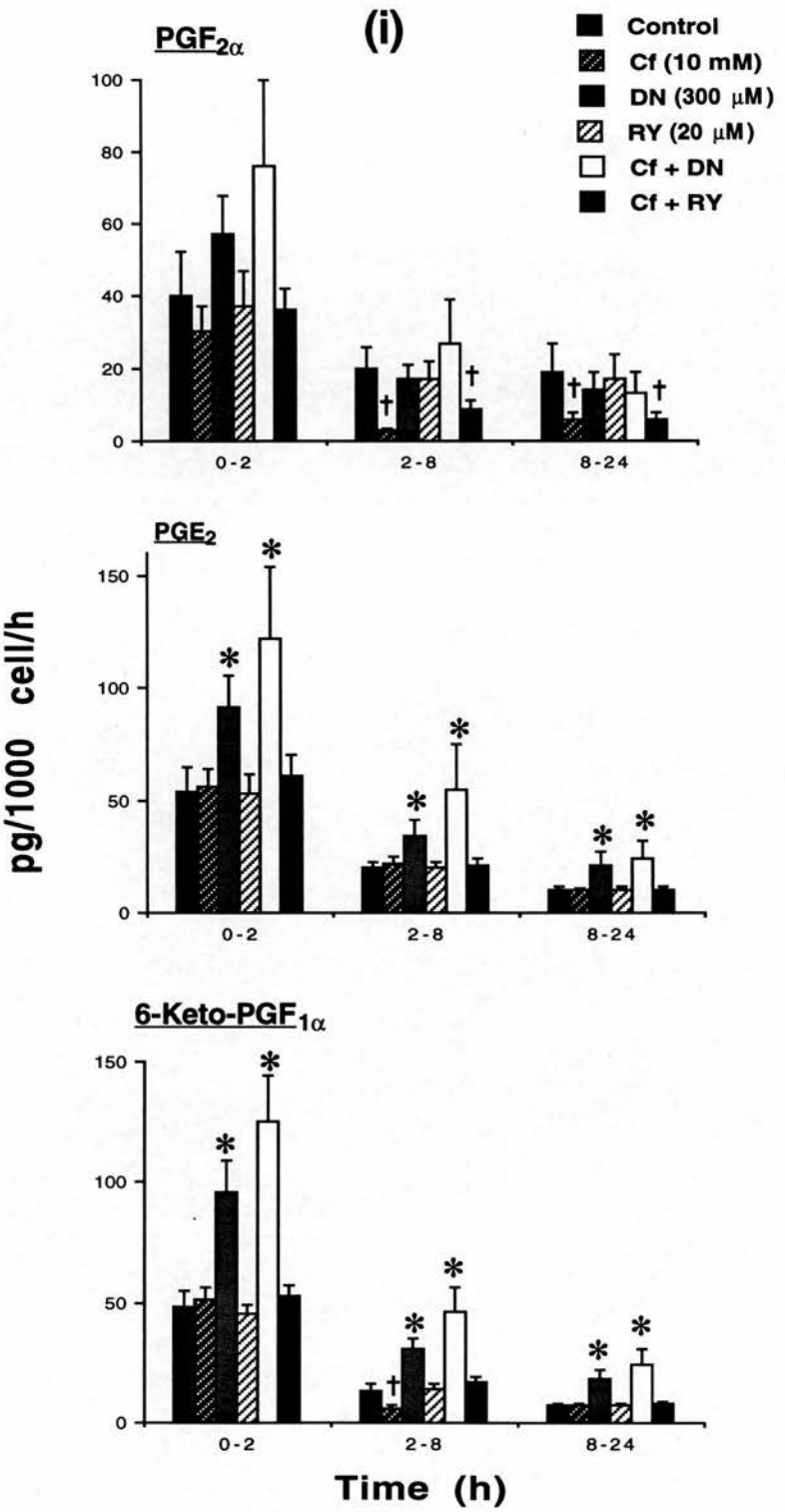


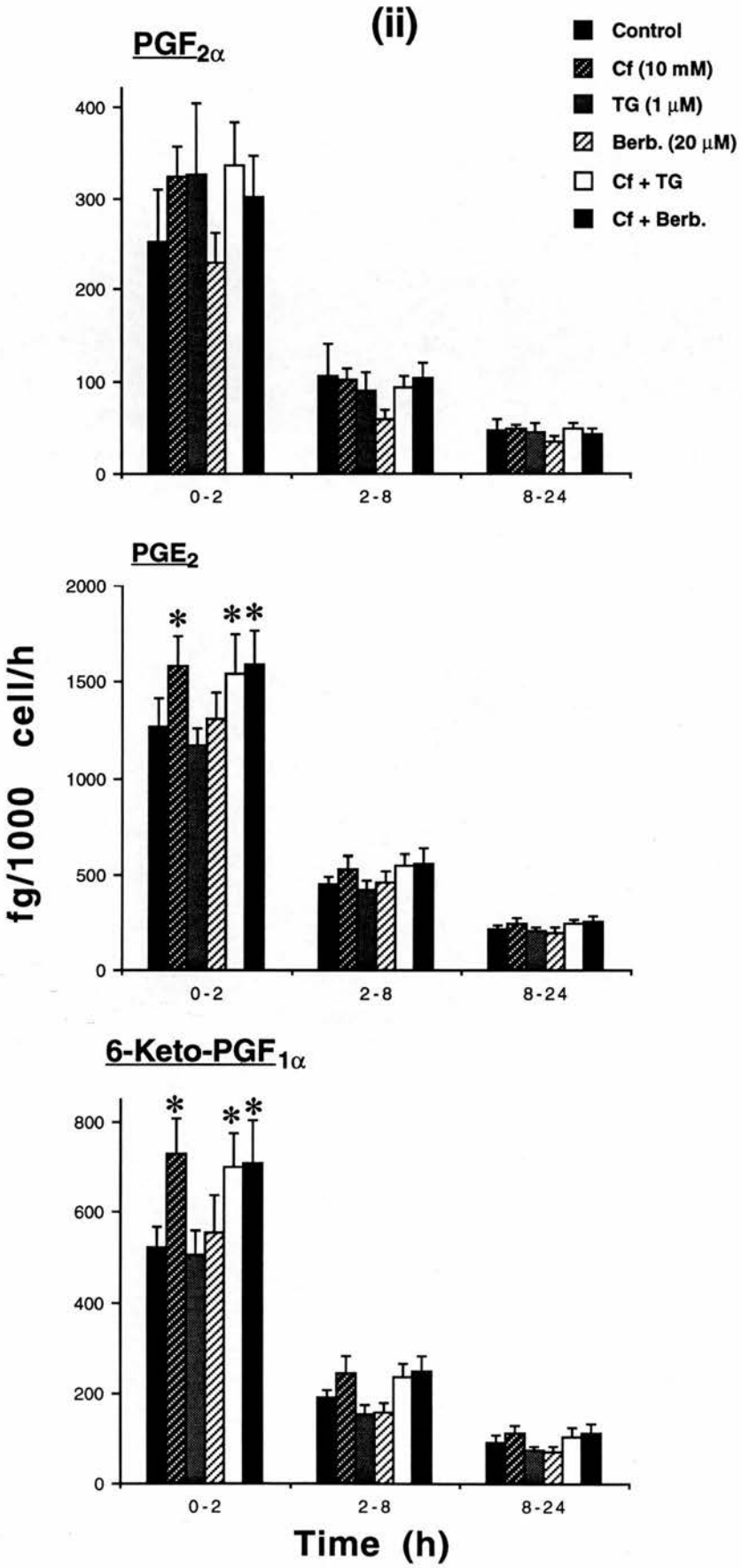
Figure 3.1.6.1. Mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 7 endometrial (i) epithelial and (ii) stromal cells in culture. Cells were treated with nothing (control), caffeine (Cf), dantrolene (DN), ryanodine (RY), Cf + DN or Cf + RY for 2, 6 and 16 h. ✻ Significantly ($p<0.05$) higher than the corresponding control value. † Significantly ($p<0.05$) lower than the corresponding value. ◆ Significantly ($p<0.05$) higher than the value obtained by Cf and/or DN alone for the same PG during the same culture period.

1.9- to 2.0-fold after 2 and 8 h of culture. Dantrolene plus caffeine significantly ($p<0.05$, $n=4$) stimulated the outputs of PGE_2 by up to 2.4-fold and 6-keto-$PGF_{1\alpha}$ by up to 3.5-fold from epithelial cells after 2, 8 and 24 h of culture (Figure 3.1.6.1). Dantrolene significantly ($p<0.05$, $n=4$) stimulated the outputs of PGE_2 by up to 2-fold and 6-keto-$PGF_{1\alpha}$ by up to 4-fold after 2, 8 and 24 h from stromal cells. Dantrolene (300 μM) plus caffeine (10 mM) caused a significant ($p<0.05$, $n=4$) increase in the outputs of $PGF_{2\alpha}$ by 2.3- to 3.4-fold, PGE_2 by 2.1- to 2.8-fold and 6-keto-$PGF_{1\alpha}$ by 4.9- to 7-fold after 2, 8 and 24 h from stromal cells (Figure 3.1.6.1). Dantrolene (300 μM) plus caffeine (10 mM)-induced increases in the outputs of PGE_2 after 2 and 24 h, and of 6-keto-$PGF_{1\alpha}$ after 24 h from stromal cells were significantly ($p<0.05$, $n=4$) greater than caffeine alone- and/or dantrolene alone-induced increases in the outputs of these prostaglandins from stromal cells after the same time periods of treatment (Figure 3.1.6.1).

Ryanodine (20 μM) alone had no significant effect on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial or stromal cells in culture (Figure 3.1.6.1). Caffeine plus RY had no effects on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial or stromal cells, except for a significant ($p<0.05$, $n=4$) inhibitory effect on $PGF_{2\alpha}$ output from epithelial cells after 24 h of culture, and a significant ($p<0.05$, $n=4$) stimulatory effect on 6-keto-$PGF_{1\alpha}$ from stromal cells after 2 h of culture (Figure 3.1.6.1). Ryanodine did not significantly modify the responses produced by caffeine alone.

In experiment 2, caffeine (10 mM) significantly ($p<0.05$, $n=4$) decreased the outputs of $PGF_{2\alpha}$ after 8 and 24 h, and 6-keto-$PGF_{1\alpha}$ after 8 h from epithelial cells in culture, an effect similar to that seen in experiment 1 (Figure 3.1.6.2). Caffeine (10 mM) also significantly ($p<0.05$, $n=4$) increased the outputs of PGE_2 by 1.3-fold and 6-keto-$PGF_{1\alpha}$ by 1.4-fold from stromal cells after 2 h of culture (Figure 3.1.6.2). Caffeine alone had no effect on PGE_2 output from epithelial cells.

Thapsigargin (1 μM) alone significantly ($p<0.05$, $n=4$) increased the output of $PGF_{2\alpha}$ from epithelial cells after 2 h, an effect which was lost by 8 h, and was reversed to a significant inhibition ($p<0.05$, $n=4$) after 24 h of cell culture (Figure 3.1.6.2). Thapsigargin had no effect on $PGF_{2\alpha}$ output from stromal cells, or on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial and stromal cells. Thapsigargin (1 μM) plus caffeine (10 mM) significantly ($p<0.05$, $n=4$) decreased the output of $PGF_{2\alpha}$ from epithelial cells after 8 and 24 h, but this response was not significantly different from caffeine or thapsigargin alone. Caffeine plus thapsigargin had no effect on $PGF_{2\alpha}$ output from epithelial cells after 2 h, so the presence of caffeine significantly ($p<0.05$, $n=4$) inhibited the stimulatory action of thapsigargin (Figure 3.1.6.2). Caffeine plus thapsigargin significantly ($p<0.05$, $n=4$) increased the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ by up to 1.4-fold from stromal cells after 2 h of culture. These effects are probably entirely due to the actions of caffeine, since caffeine alone produced the same effects whereas thapsigargin alone failed to do so (Figure 3.1.6.2). Caffeine plus


(ii)
Control
Cf (10 mM)
TG (1 μM)
Berb. (20 μM)
Cf + TG
Cf + Berb.
PGF2α
PGE2
6-Keto-PGF1α
fg/1000 cell/h
0
100
200
300
400
500
1000
1500
2000
600
800
*
**
0-2
2-8
8-24
Time (h)

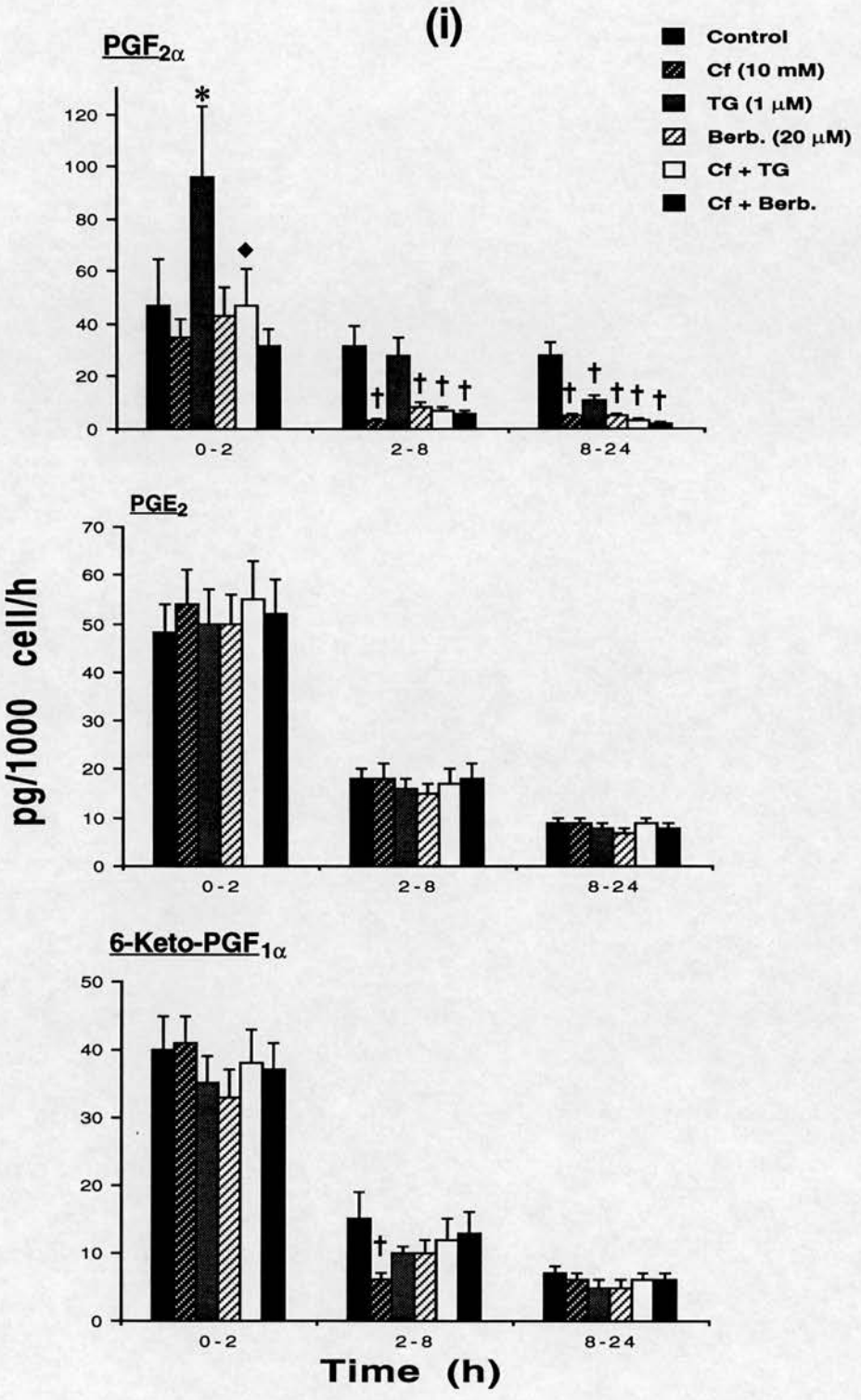


Figure 3.1.6.2. Mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured day 7 (i) epithelial and (ii) stromal cells. Cells were either untreated (control) or treated with caffeine (Cf), thapsigargin (TG), berberine (Berb), Cf +TG or Cf+Berb. for 2, 6 and 16 h. * Significantly ($p<0.05$) higher than the corresponding control value. † Significantly ($p<0.05$) lower than the corresponding control value. ◆ Significantly ($p<0.05$) lower than TG alone treatment during the same period of culture.

thapsigargin had no effect on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial cells and of $PGF_{2\alpha}$ from stromal cells (Figure 3.1.6.2).

Berberine (20 μM) alone significantly ($p<0.05$, $n=4$) decreased the output of $PGF_{2\alpha}$ from epithelial cells after 8 and 24 h culture (Figure 3.1.6.2). Berberine (20 μM) plus caffeine (10 mM) significantly ($p<0.05$, $n=4$) decreased the output of $PGF_{2\alpha}$ form epithelial cells after 8 and 24 h, and significantly ($p<0.05$, $n=4$) increased the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ by up to 1.4-fold from stromal cells after 2 h of culture (Figure 3.1.6.2). These stimulatory effects are probably due to the actions of caffeine, as caffeine alone produced the same effects whereas berberine alone failed to do so. Berberine alone or together with caffeine had no effect on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial cells, and on $PGF_{2\alpha}$ output from stromal cells (Figure 3.1.6.2).

Discussion:

Ryanodine (RY) alone did not affect the outputs of prostaglandins from the cultured endometrial cells. This is in agreement with the results obtained in earlier experiments (see Sections 3.1.2, 3.1.3 & 3.1.4). Ryanodine did not affect the actions of caffeine on the outputs of prostaglandins from the epithelial and stromal cells. These results indicate that caffeine-induced changes in the outputs of prostaglandins from the endometrial cells is not mediated by the activation of a RY-sensitive receptor. Hence, it seems that in the guinea-pig endometrium, RY and a RYR-channel is not involved in prostaglandin production. However, dantrolene, a RY-like modulator of calcium release from RYR-channel, stimulated

prostaglandin outputs from both epithelial and stromal cells in culture. Dantrolene also blocked the inhibitory effect of caffeine on the output of $PGF_{2\alpha}$ from epithelial cells, and potentiated caffeine-induced PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the cultured stromal cells. Dantrolene inhibits RY-binding and RY-induced calcium release in longitudinal muscle of the intestine of the guinea-pig (Kuemmerle *et al.*, 1994), but is ineffective as regards RY-binding to the rat cortical membrane (Smith & Nahroski, 1993). It has been shown that the skeletal muscle type RYR is more sensitive to dantrolene than cardiac type. Thus, these findings suggest that either there is a skeletal type RYR-like protein which is modulated by dantrolene or dantrolene is exerting a non-specific effect on the outputs of prostaglandins from the guinea-pig uterus. The latter explanation seems more likely since RY has no effect on PG output from endometrial cells.

The effects of thapsigargin on $PGF_{2\alpha}$ output from epithelial cells were similar to the action of A23187 (see Section 3.1.4). Thapsigargin stimulated the output of $PGF_{2\alpha}$ from epithelial cells after 2 h and inhibited $PGF_{2\alpha}$ output after 24 of cell culture. Thapsigargin releases calcium from internal pools (Herchuelz & Lebrun, 1993; Wegner, *et al.*, 1994; Toescu & Petersen, 1994; Diarra & Sauve, 1992; Jancezewski & Lakatta, 1993) and inhibits the caffeine-induced rise in intracellular calcium in chick ciliary ganglion cells (Sorimachi, 1993). Hence, it is possible that the stimulatory effect of thapsigargin on $PGF_{2\alpha}$ output may be due to its ability to release calcium from an internal store. Its inhibitory effect on $PGF_{2\alpha}$ output from epithelial cells may be due to inhibition of the endoplasmic reticulum Ca^{2+}/ATPase and subsequent depletion of an internal calcium pool and, in turn, lack of calcium

to maintain basal PG output. Thapsigargin did not prevent the increases in the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ induced by caffeine during the first 2 h of culture, indicating that, if this stimulatory action of caffeine is dependent upon the release of intracellular calcium, the calcium pool which is involved in the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ is not thapsigargin sensitive. On the other hand, caffeine inhibited thapsigargin-induced rise in $PGF_{2\alpha}$ output from epithelial cells after 2 h of culture. This finding indicates that either the inhibitory effect of caffeine on $PGF_{2\alpha}$ output has overcome the stimulatory effect of thapsigargin, or caffeine inhibited a thapsigargin-induced rise in free intracellular calcium by interfering possibly with Ca^{2+}/ATPase of the endoplasmic reticulum. However, more studies are required to elucidate further the above hypotheses.

It is thought that berberine inhibits caffeine-induced contractions by inhibiting calcium release from the sarcoplasmic reticulum (Chiou *et al.*, 1991). In the present study, berberine alone inhibited only the output of $PGF_{2\alpha}$ from epithelial cells after 8 and 24 h of cell culture. Thus, $PGF_{2\alpha}$ output from epithelial cells may dependent upon the release of intracellular calcium. The effects of caffeine on the outputs of prostaglandins from endometrial cells were not affected by berberine. Thus, an inhibitor of calcium release from the endoplasmic reticulum did not block the actions of caffeine. In summary, it was found that the caffeine-induced changes in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from endometrial cells in culture are modulated by dantrolene, but not by RY, thapsigargin or berberine in the concentrations used.

3.1.7 The Effects of Forskolin (An Activator of Adenyl Cyclase) on The Outputs of Prostaglandins From Endometrial Tissue and Endometrial Cell Cultures of Day 7 Guinea-Pig Uterus.

Introduction:

In the previous experiments (see Sections 3.1.1, 3.1.2 & 3.1.3), it was shown that caffeine and theophylline stimulated prostaglandin production from the guinea-pig uterine horn superfused *in vitro* and from endometrial tissue in culture. However, caffeine showed a stimulatory and an inhibitory effect in the outputs of prostaglandin from epithelial and stromal cells in culture (see Sections 3.1.4 to 3.1.6). Caffeine and theophylline are phosphodiesterase (PDE) inhibitors. It is possible that these compounds exert their inhibitory effect on PG output from the endometrial cells by inhibiting PDE activity and, as a result, by increasing the amount of cAMP. This increase in cAMP level may result in a decrease in intracellular calcium level and hence inhibiting PLA_2 activity. However, an increase in cAMP level did not affect prostaglandin synthesis by and release from the guinea-pig uterine horns superfused *in vitro* (Poyser, 1987a). Experiments carried out so far in the present study have indicated that prostaglandin synthesis and release from guinea-pig uterus due to various treatments, including caffeine, depends on the experimental protocol. To further elucidate a possible role for the involvement of cAMP in the inhibitory effects of caffeine, the effects of forskolin, which increases cAMP levels, were examined on endometrial tissue and cell cultures from the day 7 guinea-pig uterus.

Methods:

In experiment 1, epithelial and stromal cells from the endometrium of four day 7 guinea-pigs were isolated and cultured as described in Section 2.1.5. Culture medium was changed every 3 days and on the sixth day, cells in culture were treated in duplicate. Two wells containing each cell type were untreated (controls), and two wells from each cell type were treated with forskolin (10 μM). This concentration of forskolin is known to raise cAMP levels in guinea-pig endometrium and myometrium homogenates (Poyser, 1987a).

In experiment 2, tissue preparation and culture set up was as described in Section 2.1.3. Six tissue culture dishes were prepared from the endometrium obtained from each guinea-pig. Four of these petri dishes were left untreated (acted as controls), and the remaining dishes were treated in duplicate with forskolin (10 μM). In both experiments, culture medium was changed after 2, 8, and 24 h of culture. Samples of culture medium were stored at -20°C before radioimmunoassay, without extraction, for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$.

A concentrated solution of forskolin was prepared in DMSO and stored at -20°C. The appropriate concentration of forskolin was prepared by diluting 160-fold with cell culture medium or tissue culture medium solutions for use in experiments 1 and 2, respectively.

Statistical tests:

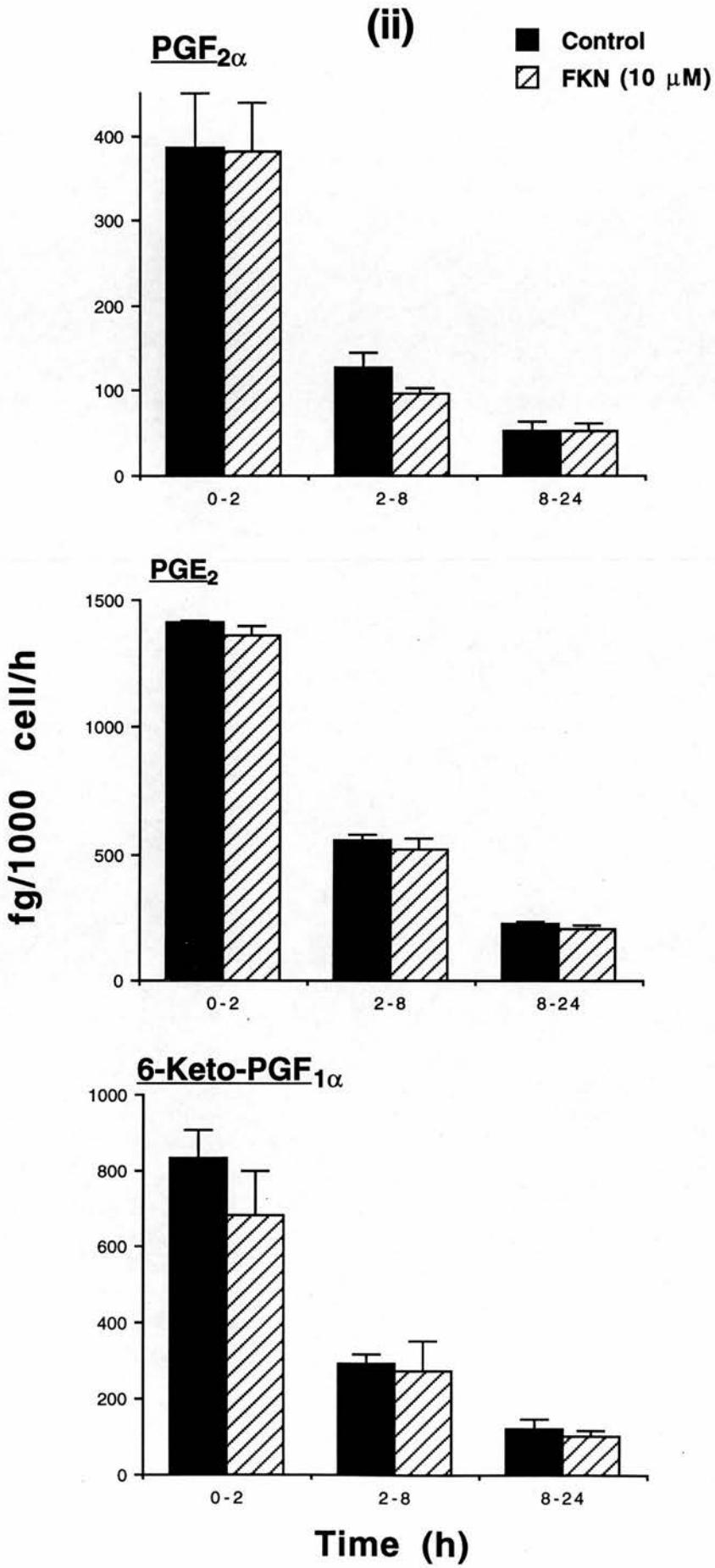
The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

Forskolin had no inhibitory effect on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from both epithelial and stromal cells after 2, 8 and 24 h of culture. In fact, forskolin significantly ($p<0.05$) increased $PGF_{2\alpha}$ output from epithelial cells after 8 h of cell culture (Figure 3.1.7.1). Forskolin had no effects in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the guinea-pig endometrium in culture (Figure 3.1.7.2).

Discussion:

In the guinea-pig, an increase in the endometrial cAMP level by forskolin is not associated with a change in the basal outputs of prostaglandins from the guinea-pig uterus superfused *in vitro* (Poyser, 1987a). In the present study, forskolin stimulated $PGF_{2\alpha}$ output from the epithelial cells after 8 h of cell culture, suggesting a role for cAMP in the PG output from the guinea-pig uterus. If cAMP were involved in the uterine PG synthesis and release, it would be expected that forskolin exerts an inhibitory effect rather than a stimulatory effect, since increase in cAMP concentration reduce intracellular free calcium level. However, forskolin had no inhibitory effect on the outputs of any of the 3 PGs measured from the cultured endometrium, indicating that cAMP is not involved in the regulation of endometrial prostaglandin synthesis and release. Thus, it is unlikely that cAMP is involved in the stimulation of $PGF_{2\alpha}$ output from epithelial cells, and that this stimulatory effect of forskolin may be due to some other non-specific action on


(ii)
Control
FKN (10 μM)
PGF2α
400
300
200
100
0
0-2
2-8
8-24
PGE2
1500
1000
500
0
0-2
2-8
8-24
fg/1000 cell/h
6-Keto-PGF1α
1000
800
600
400
200
0
0-2
2-8
8-24
Time (h)

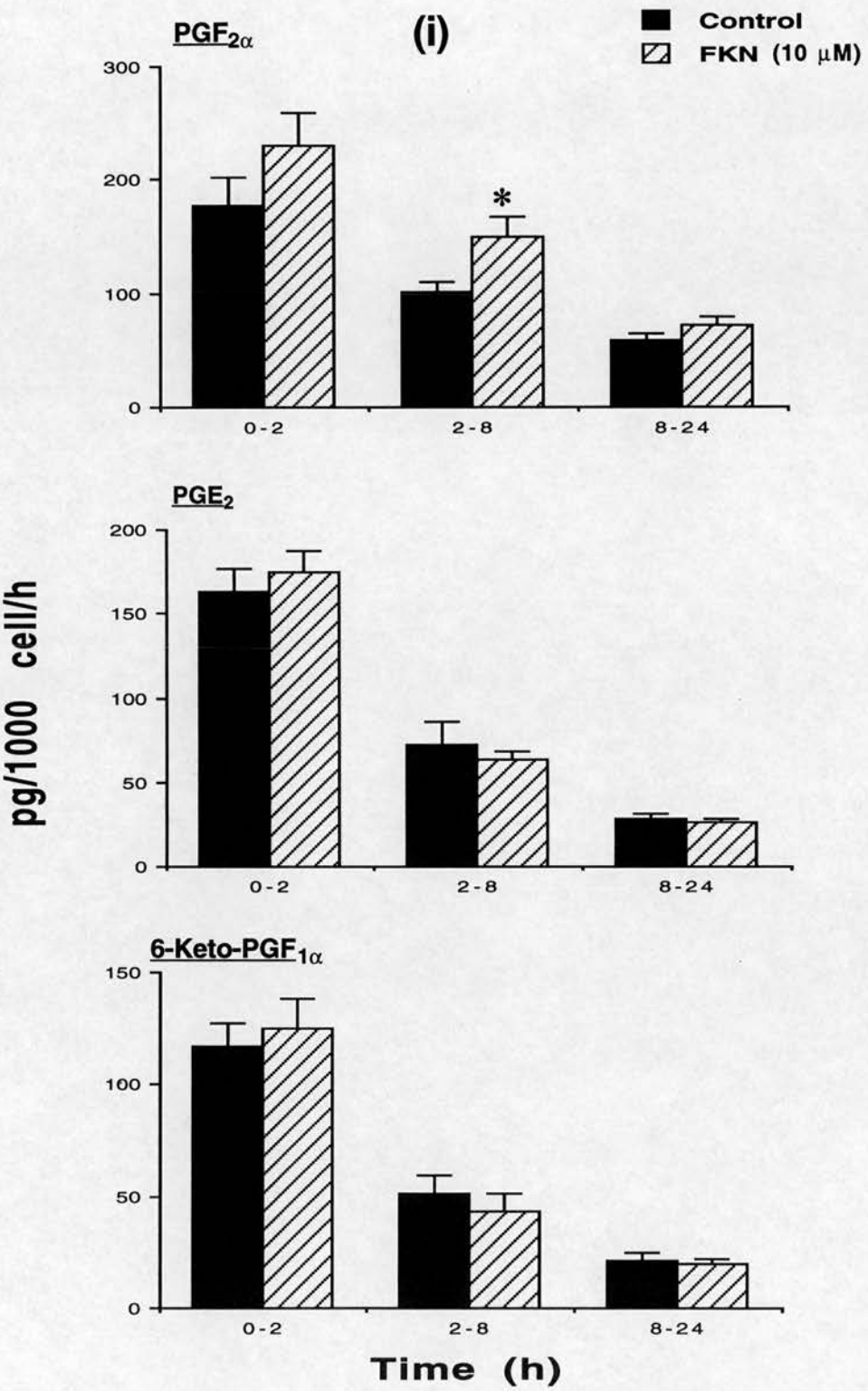


Figure 3.1.7.1. Effects of forskolin (FKN) on mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured (i) epithelial and (ii) stromal cells obtained from the day 7 guinea-pig endometrium.

* Significantly ($p<0.05$) increased by forskolin treatment.

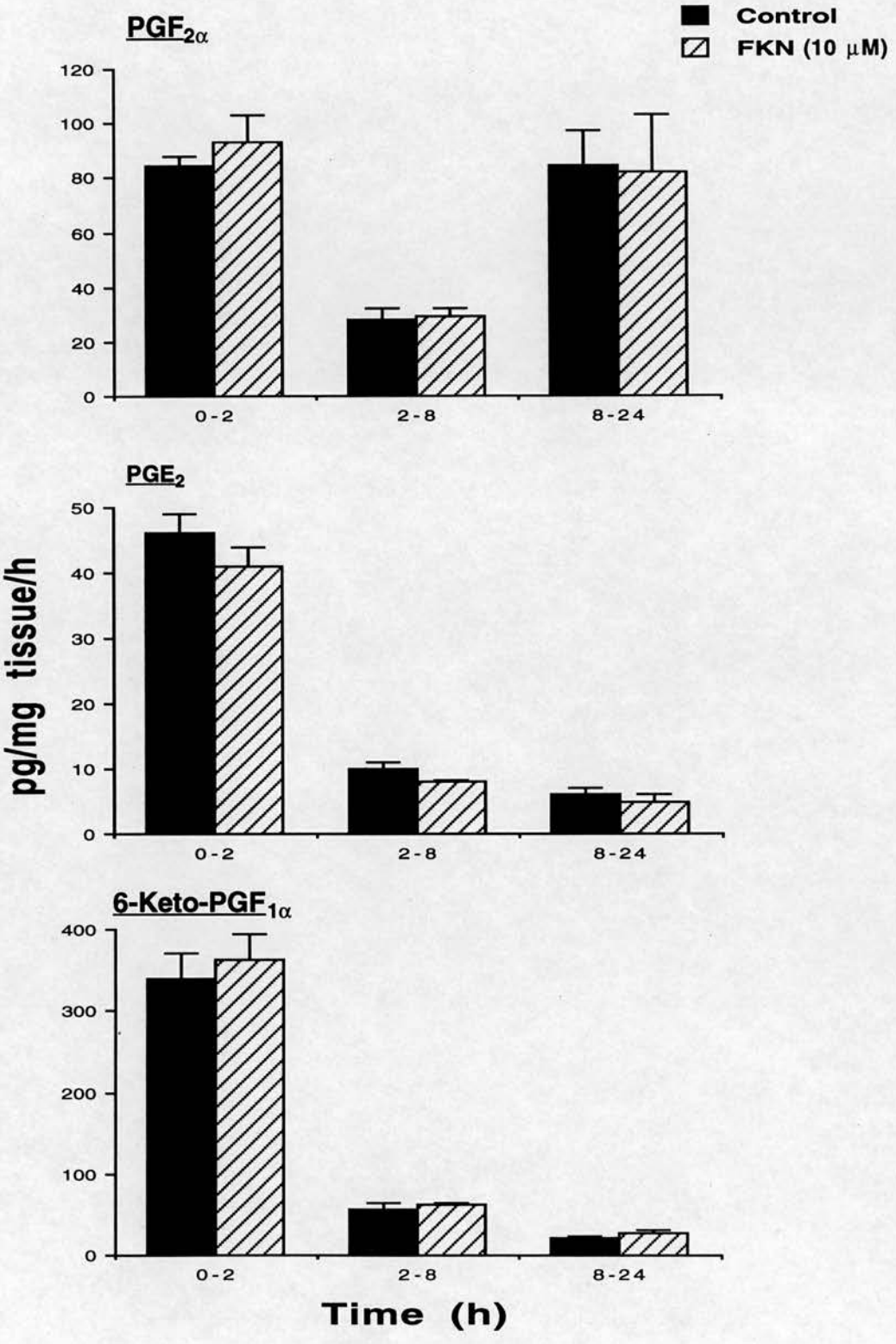


Figure 3.1.7.2. Effects of forskolin (FKN) on mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 7 guinea-pig endometrium in culture.

epithelial cells. However, a rise in cAMP level is associated with an increase in ovarian prostaglandin production (Zar *et al.*, 1977).

3.1.8 Effects of Methylxanthines, Ryanodine (RY), Phospholipase (PL) A_2 (PLA_2) and Platelet Activating Factor (PAF) on the Intracellular Free Calcium Concentration ($[Ca^{2+}]_i$) of Endometrial Epithelial and Stromal Cells From The Guinea-Pig Uterus.

Introduction:

Mobilisation of intracellular Ca^{2+} is mediated by two major mechanisms. Surface receptor activation stimulates phosphoinositide metabolism resulting in production of inositol trisphosphate (IP_3), a second messenger for releasing Ca^{2+} from endoplasmic reticulum (Sterb *et al.*, 1983; Berridge, 1984; see Berridge & Irvine, 1989; see Berridge, 1993). The second mechanism for calcium mobilisation is calcium-induced calcium-release (CICR). This mechanism which mediates RYR is best characterised in skeletal and cardiac muscles and has been shown to be modulated by substances such as caffeine and RY, (Komori & Bolton, 1989; Lee, 1993; Stauderman, *et al.*, 1991) as well as theophylline (Pessah *et al.*, 1993; Donoso *et al.*, 1994). Other agents such as histamine (Diarra & Sauve, 1992; Stauderman *et al.*, 1991), calcium ionophore (A23187) (Zimanyi *et al.*, 1992), muscarinic receptor agonist such as methacholine (Sorimachi, *et al.*, 1992), and thapsigargin (Chen & Van Breemen, 1993) have been shown to release calcium from one or more internal calcium stores, resulting an increase in free cytosolic calcium levels within the cells. Since results obtained from the previous experiments in this thesis have suggested that mobilisation of calcium from an internal store may be responsible for the stimulatory effects of caffeine,

theophylline and PLA_2 on PG outputs from guinea-pig uterus, this experiment was designed to further investigate the above hypothesis by measuring the internal calcium levels in the presence of these substances using a calcium-sensitive dye (fura-2 AM).

Methods:

For detailed methods of epithelial and stromal cell isolation and preparation see Section 2.1.5, and for loading of cells with fura-2/AM and intracellular calcium measurements see Section 2.1.6. Briefly, cells were loaded with 2 μM fura-2 acetoxymethyl ester (fura-2/AM) by incubating for 30 min at room temperature (25°C). Cells were then washed, re-suspended in appropriate fresh Krebs solution buffered with 20 mM HEPES, pH 7.4 and left to stand for a further 30 min to allow hydrolysis of the intracellular fura-2/AM. After 30 min the cells were washed, re-suspended in appropriate fresh Krebs solution, and transferred to a quartz cuvette maintained at 37°C. The cells were continuously stirred in the cuvette. Fluorescence was measured in a Shimadzo (type RF-5000) spectrofluorophotometer at 505 nm with alternate excitation at 340 nm and 380 nm. The intracellular free calcium concentration ($[Ca^{2+}]_i$) was then calculated using the equation of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = (k_d) \times (b) \times [R - R_{min}]/[R_{max} - R]$$

and the resultant experimental data of percentage change of $[Ca^{2+}]_i$ versus agonist concentration was obtained, where R is the ratio of fluorescence due to excitation

at 340 nm to that at 380 nm. R_{max} and R_{min} are the maximal and minimal fluorescence ratios of fura-2 obtained in Krebs solution containing saturated levels of calcium with a calcium ionophore, ionomycin (10 μM), and EGTA (30 mM), respectively. The K_d value for the fura-2-Ca^{2+}-complex was assumed to be 225 nM at 37°C (Buchan & Martin, 1991; Grynkiewicz *et al.*, 1985), and b is the ratio of the 380 nm signals in Ca^{2+}-free and Ca^{2+}-containing Krebs solution.

Solutions of caffeine, theophylline and PLA_2 were freshly made up in calcium-free Krebs solution prior to use. Concentrated solutions of RY, PAF and ionomycin in ethanol were prepared and stored at -20°C. The appropriate concentration of these compounds were then prepared by diluting at least 10-fold in calcium-free Krebs solution.

Statistical tests:

The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

In the presence of extracellular calcium, caffeine (10 & 20 mM) significantly ($p<0.05$, $n=3$) lowered the basal intracellular calcium concentration ($[Ca^{2+}]_i$) of stromal cells obtained from day 15 guinea-pig endometrium by up to 65.3% but had no effect on $[Ca^{2+}]_i$ in day 7 stromal cells or in day 7 and day 15 epithelial cells (Figure 3.1.8.1). In the absence of extracellular calcium, caffeine (10 & 20

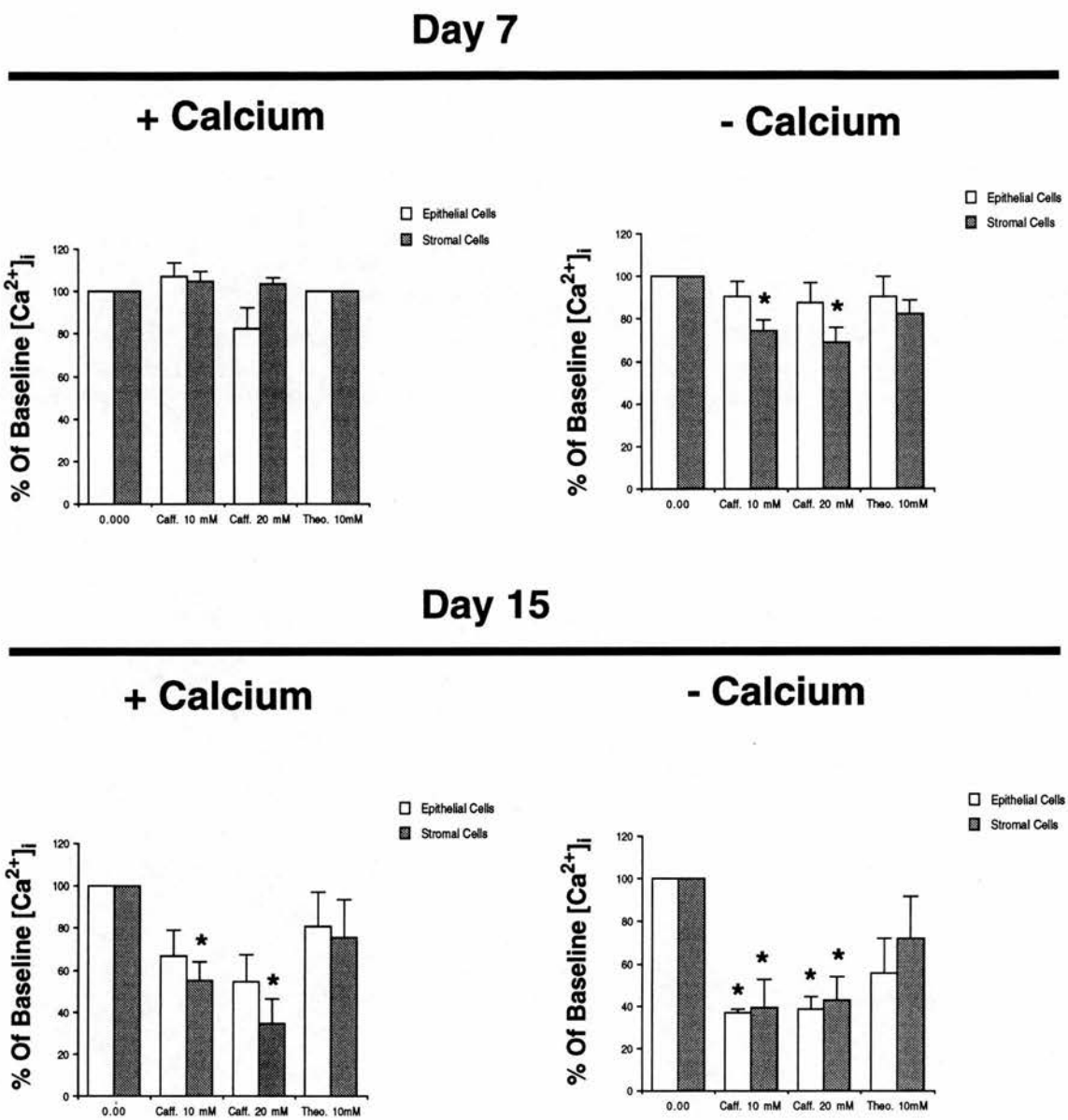


Figure. 3.1.8.1. Effects of caffeine (Caff.) (10 & 20 mM) and theophylline (Theo.) (10 mM) on changes in $[Ca^{2+}]_i$ of epithelial and stromal cells of day 7 and day 15 guinea-pig endometrium in the presence (+) or absence (-) of extracellular calcium.

* Significantly ($P< 0.05$, $n = 3$) lower than baseline.

mM) significantly ($p<0.05$, $n=3$) reduced $[Ca^{2+}]_i$ in day 7 and day 15 stromal cells, and in day 15 (but not day 7) epithelial cells, by 25.4% to 63.3% (Figure 3.1.8.1). Theophylline (10 mM) had no significant effect on $[Ca^{2+}]_i$ in day 7 or 15 epithelial and stromal cells in the presence or absence of extracellular calcium (Figure 3.1.8.1). Cumulative increasing concentrations of ryanodine (RY; 1 nM to 1 mM) had no effect on $[Ca^{2+}]_i$ in day 7 and day 15 epithelial and stromal cells in the presence or absence of extracellular calcium (Figure 3.1.8.2).

Cumulative increasing concentrations of PLA_2 (1 to 10 U/ml) caused significant changes in $[Ca^{2+}]_i$ in day 7 and day 15 epithelial and stromal cells in the presence or absence of extracellular calcium. In the presence of extracellular calcium, PLA_2 (1 to 10 U/ml) significantly ($p<0.05$, $n=3$) increased $[Ca^{2+}]_i$ in day 7 and day 15 stromal and epithelial cells by 21.9- to 72.1-fold (Figure 3.1.8.3). In the absence of extracellular calcium, PLA_2 (1 to 10 U/ml) significantly ($p<0.05$, $n=3$) increased $[Ca^{2+}]_i$ in day 15 stromal cells, and day 7 and day 15 epithelial cells by 2.8- to 6.1-fold. PLA_2 also increased $[Ca^{2+}]_i$ in day 7 stromal cells but these increases were not statistically significant (Figure 3.1.8.3). In the presence or absence of extracellular calcium, PLA_2 (4 to 10 U/ml) was significantly ($p<0.05$, $n=3$) more effective in increasing $[Ca^{2+}]_i$ of epithelial cells than of stromal cells from day 15 endometrium (Figure 3.1.8.3).

Platelet activating factor (PAF) (2 to 20 μM) significantly ($p<0.05$, $n=3$) increased $[Ca^{2+}]_i$ in day 7 and day 15 epithelial and stromal cells by 5.4- to 69.2-fold in the presence of extracellular calcium, and in day 15 (but not day 7) epithelial and stromal cells by 13.2- to 13.4-fold in the absence of extracellular

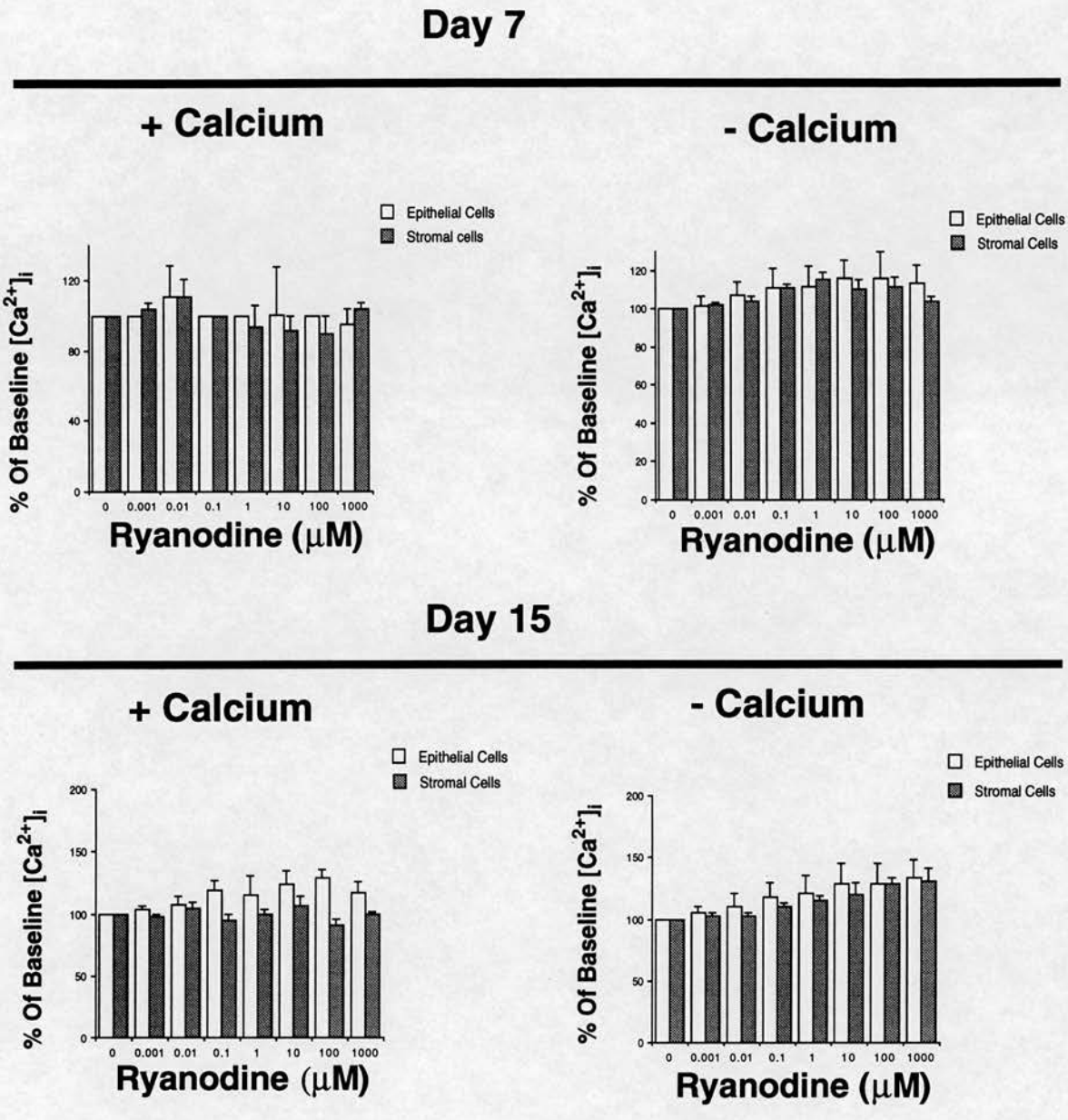


Figure. 3.1.8.2. Effects of cumulative increasing concentrations of ryanodine (1 nM to 1 mM) on changes in $[Ca^{2+}]_i$ of epithelial and stromal cells of day 7 and day 15 guinea-pig endometrium in the presence (+) or absence (-) of extracellular calcium.

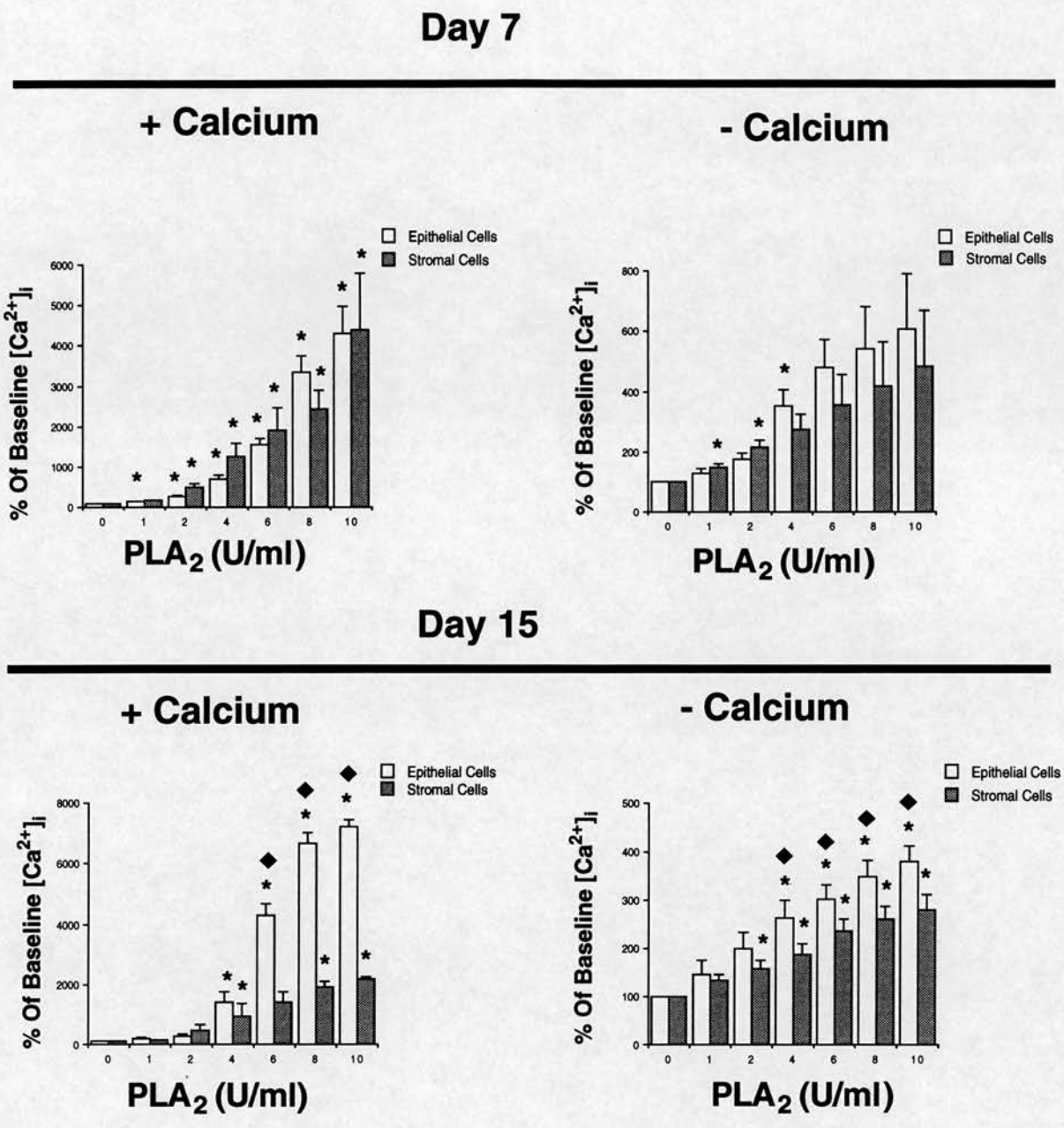


Figure.3.1.8.3. Effects of cumulative increasing concentrations of PLA_2 (1 to 10 U/ml) on changes in $[Ca^{2+}]_i$ of epithelial and stromal cells of day 7 and day 15 guinea-pig endometrium in the presence (+) or absence (-) of extracellular calcium.

* Significantly ($P<0.05$, $n = 3$) higher than baseline.

◆ Significantly ($p<0.05$, $n=3$) higher than the corresponding value obtained for stromal cells at the same concentration of PLA_2.

calcium (Figure 3.1.8.4). In the presence of extracellular calcium, PAF (4 to 20 μM) was significantly ($p<0.05$, $n=3$) more effective in increasing $[Ca^{2+}]_i$ of stromal cells than of epithelial cells from day 7 endometrium (Figure 3.1.8.4). In the presence of extracellular calcium, PAF (20 μM) was also significantly ($p<0.05$, $n=3$) more potent in increasing $[Ca^{2+}]_i$ of stromal cells than of epithelial cells from day 15 guinea-pig endometrium.

Discussion:

Caffeine and ryanodine interact with a RYR-channel on the membrane of sarcoplasmic reticulum (SR) and release calcium (Chen & Cheung, 1992; McPherson & Campbell, 1993a, b; Zahradink & Palade, 1993). As a result the concentration of cytosolic free calcium increases, and this increase is thought to be responsible for many cellular mechanisms including the activation of endometrial PLA_2 in the human (Bonney, 1985), rat (Dey, 1982) and guinea-pig (Downing & Poyser, 1983). The results obtained from Sections 3.1.1 and 3.1.2 suggested that caffeine may increase the intracellular calcium by interacting with a novel receptor in the membrane of the endoplasmic reticulum, thereby releasing calcium from an internal pool and consequently stimulating prostaglandin production by the guinea-pig uterus. However, in this study, caffeine and theophylline failed to increase intracellular calcium concentrations in day 7 and day 15 epithelial and stromal cells. In fact, caffeine and, to a lesser extent, theophylline decreased the basal free intracellular calcium concentration. These findings indicate that the caffeine- and theophylline-induced stimulations of prostaglandin production by guinea-pig uterus

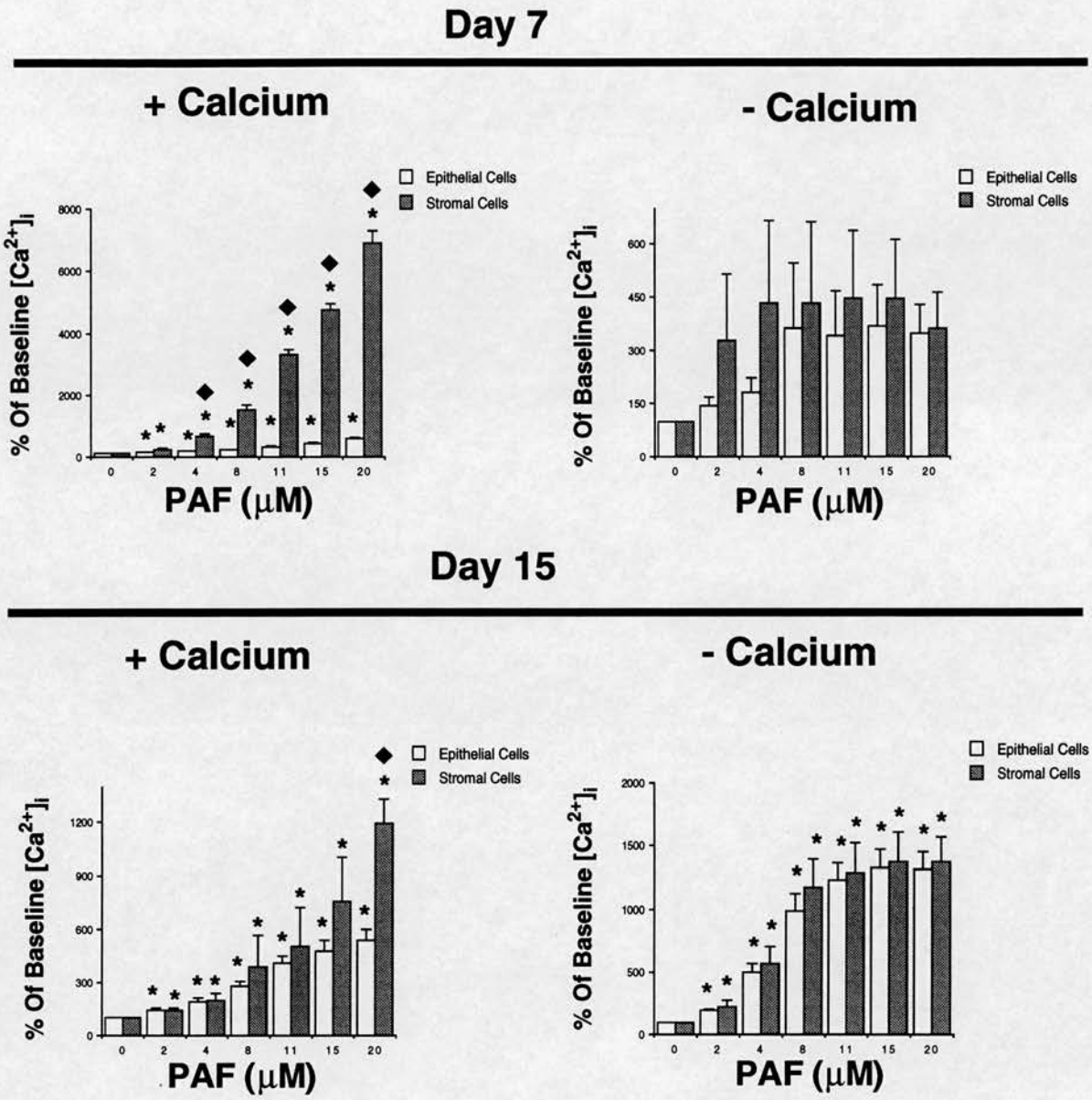


Figure.3.1.8.4. Effects of cumulative increasing concentrations of PAF (2 to 20 μM) on changes in $[Ca^{2+}]_i$ of epithelial and stromal cells of day 7 and day 15 guinea-pig endometrium in the presence (+) or absence (-) of extracellular calcium.

* Significantly ($P< 0.05$, $n = 3$) higher than baseline. ◆ Significantly ($p<0.05$, $n=3$) higher than the corresponding value obtained for epithelial cells at the same concentration of PAF.

are not associated with an increase in $[Ca^{2+}]_i$ in endometrial epithelial and stromal cells. However, the decrease in $PGF_{2\alpha}$ output from epithelial cells in culture induced by caffeine may be due to a decrease in intracellular free calcium concentration produced by caffeine.

Ryanodine did not affect PG output from guinea-pig uterus in any experimental protocol. Ryanodine also did not increase the intracellular calcium concentration in day 7 or day 15 guinea-pig epithelial or stromal cells. These findings indicate that there does not appear to be any RYR type 1, RYR type 2 or RYR type 3 present in the guinea-pig epithelial and stromal cells.

PLA_2 has been shown to stimulate PG output from guinea-pig uterus superfused *in vitro* (Poyser, 1991; Poyser & Ferguson, 1993) and from cultured endometrial epithelial and stromal cells (see Section 3.1.5). In the present study, PLA_2 significantly increased the intracellular calcium concentration in both epithelial and stromal cells in the presence or absence of extracellular calcium. These finding indicate that the increases in PG output from guinea-pig uterus produced by PLA_2 are associated with an increase in intracellular calcium concentration in endometrial epithelial and stromal cells, and that this increase in intracellular calcium concentration is due, at least in part, to the release of intracellular calcium.

PAF has been shown to stimulate PG output in the presence or absence of extracellular calcium from the guinea-pig uterus superfused *in vitro* (Norman & Poyser, 1992). In the present study, PAF dose-dependently increased the intracellular calcium concentration in both epithelial and stromal cells obtained from the day 7 and day 15 guinea-pig endometrium, suggesting mobilisation of

calcium from an internal store. This is in agreement with a recent report by Ahmed *et al.* (1994). Ahmed *et al.* (1994) have shown that PAF increases intracellular calcium concentration in human endometrial HEC-1B cell line in the presence or absence of calcium. These finding indicate that PAF-induced PG output from the guinea-pig uterus is associated with an increase in intracellular calcium concentration in endometrial and stromal cells, and that this increase in intracellular calcium concentration is due, at least in part, to the release of intracellular calcium. Nevertheless, Ahmed *et al.* (1994) have reported that the activation of PAF receptors in human endometrial HEC-1B cell line is linked to inositol lipid hydrolysis. They have shown that PAF dose-dependently increased IP_3 accumulation in human endometrial HEC-1B cell line. It is not known whether the increase in intracellular free calcium concentration induced by PAF in guinea-pig endometrial cells is due to an increase in the concentration of IP_3. The findings that PLA_2 was more effective in increasing $[Ca^{2+}]_i$ of epithelial cells whereas PAF was more potent on stromal cells indicates that PLA_2 may increase PG output from epithelial cells to a greater extent then from stromal cells whereas PAF may increase PG output from stromal cells to a greater extent than from epithelial cells.

3.2 THE EFFECTS OF CAFFEINE AND RYANODINE ON PROSTAGLANDIN SYNTHESIS BY AND RELEASE FROM THE RAT MESENTERIC VASCULAR BED.

Introduction:

From the experiments carried out so far, it was concluded that there are no ryanodine receptors of type 1, type 2 or type 3 in guinea-pig endometrium. However, the existence of RYR in cardiac muscle is well established (see McPherson & Campell, 1993a; Kijima *et al.*, 1993; see Sorrentino & Volpe, 1993). Caffeine and RY have been reported to cause contractions in vascular smooth muscles as well as cardiac muscles. These effects are thought to be mediated by activation of the cardiac type RYR, resulting in an increase in free cytosolic calcium level (Gyorke & Fill, 1993; see McPherson & Campell, 1993a; Jino *et al.*, 1994; Ezzaher *et al.*, 1992; Hisayama *et al.*, 1990). Considering these reports and finding from the experiments carried out so far in this study, it became necessary to design an experiment to examine whether a non-uterine tissue would produce prostaglandins in response to caffeine and/or ryanodine.

Methods:

The preparation of tissue and experimental set up were as described in Section 2.1.1. The mesenteric vascular bed was dissected out and cleaned from any connective tissues according to the procedure described by McGregor (1965) and by Lennon & Poyser (1986). After an initial equilibrium period of 30 min, samples

of perfusate were collected for periods of 1 min and stored at -20°C before being assayed. After an initial equilibrium period of 30 min, samples of perfusate were collected for periods of 1 min, according to the nature of the experiment, and stored at -20°C before being assayed. In experiment 1 (n=6), increasing caffeine doses (1 μg to 1 mg) were injected into the perfusing McEwans solution at 15 min intervals. In experiment 2 (n=4), 1 mg caffeine at 15 min intervals was injected into the perfusing McEwans solution on three successive occasions. In experiment 3 (n=7), 10 μg and 50 μg RY were injected into the perfusing McEwans solution with an interval of 3 min between each dose. In experiments 1 and 2, samples of perfusate were collected from 2 min before to 6 min after, and at 9 and 13 min after caffeine administration. In experiment three, samples of perfusate were collected from 1 min before the first dose to 2 min after the second dose of RY (i.e. for 6 min altogether). The amounts of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ in the samples of perfusate were measured by radioimmunoassay without extraction.

A solution of caffeine was prepared freshly in McEwans solution prior to use. A concentrated solution of RY was prepared in ethanol and stored at -20°C. The appropriate concentration of RY was by diluting 200-fold prepared in McEwans solution.

Statistical tests:

The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

The major prostaglandin released from the mesenteric vascular bed of the rat was 6-keto-$PGF_{1\alpha}$; the basal outputs of PGE_2 and $PGF_{2\alpha}$ were 50-70% lower than that of 6-keto-$PGF_{1\alpha}$. Caffeine significantly ($p<0.05$) increased the outputs of 6-keto-$PGF_{1\alpha}$ at doses between 1 μg and 1 mg by 1.7- to 3.3-fold. The maximum effect was obtained with 5 μg caffeine (Figure 3.2.1). Caffeine at doses of 100 μg and 1 mg significantly ($p<0.05$, $n=6$) increased the outputs of PGE_2 and $PGF_{2\alpha}$ between 1.3- to 2.4-fold. The maximum increase in PG output due to caffeine usually occurred within the first minute of administration, and this increase in PG output lasted for at least 2 min (Figure 3.2.1). Caffeine (1 mg) administered three times at 15 min intervals significantly ($p<0.05$, $n=4$) increased the output of 6-keto-$PGF_{1\alpha}$ between 2.4- and 3.6-fold following each dose of caffeine. However, the outputs of PGE_2 and $PGF_{2\alpha}$ were also increased between 1.4- to 2.3-fold, but this increases were not significant (Figure 3.2.2).

Ryanodine (10 μg) increased the outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ by 1.3- to 1.6-fold, but these increases were statistically significant ($p<0.05$, $n=7$) only for PGE_2 and $PGF_{2\alpha}$. Ryanodine (50 μg) significantly ($p<0.05$, $n=7$) increased the outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ by 2.8-, 2.7- and 2.0-fold, respectively (Figure 3.2.3).

Discussion:

Since prostaglandins are not stored in tissues, their release is immediately preceded by their synthesis. The rate-limiting step in PG synthesis is the release of

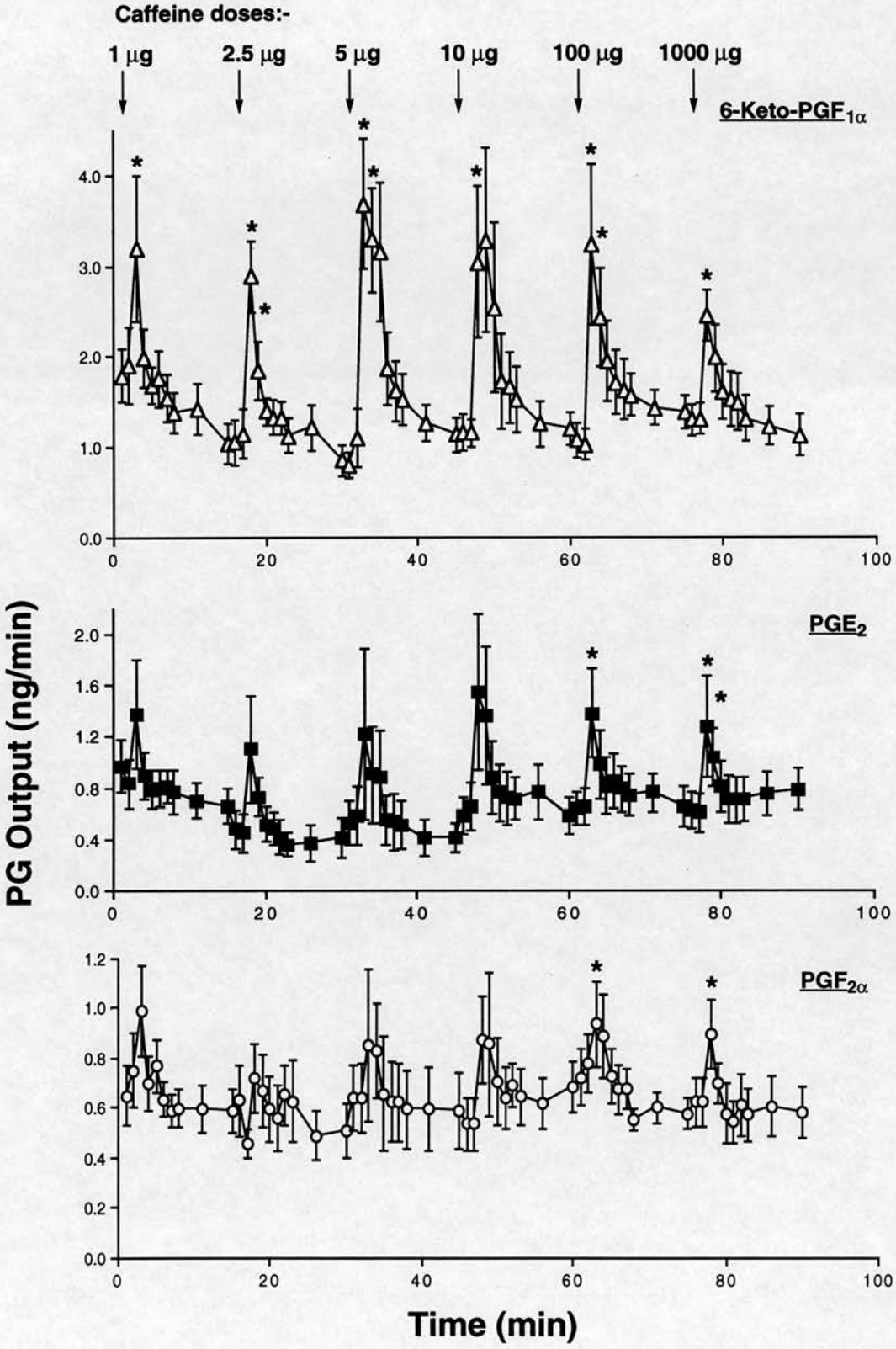


Figure 3.2.1. Effects of caffeine on mean (±SEM, n=6) outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ from the perfused mesenteric vascular bed of the rat. * Significantly ($p<0.05$) increased by caffeine.

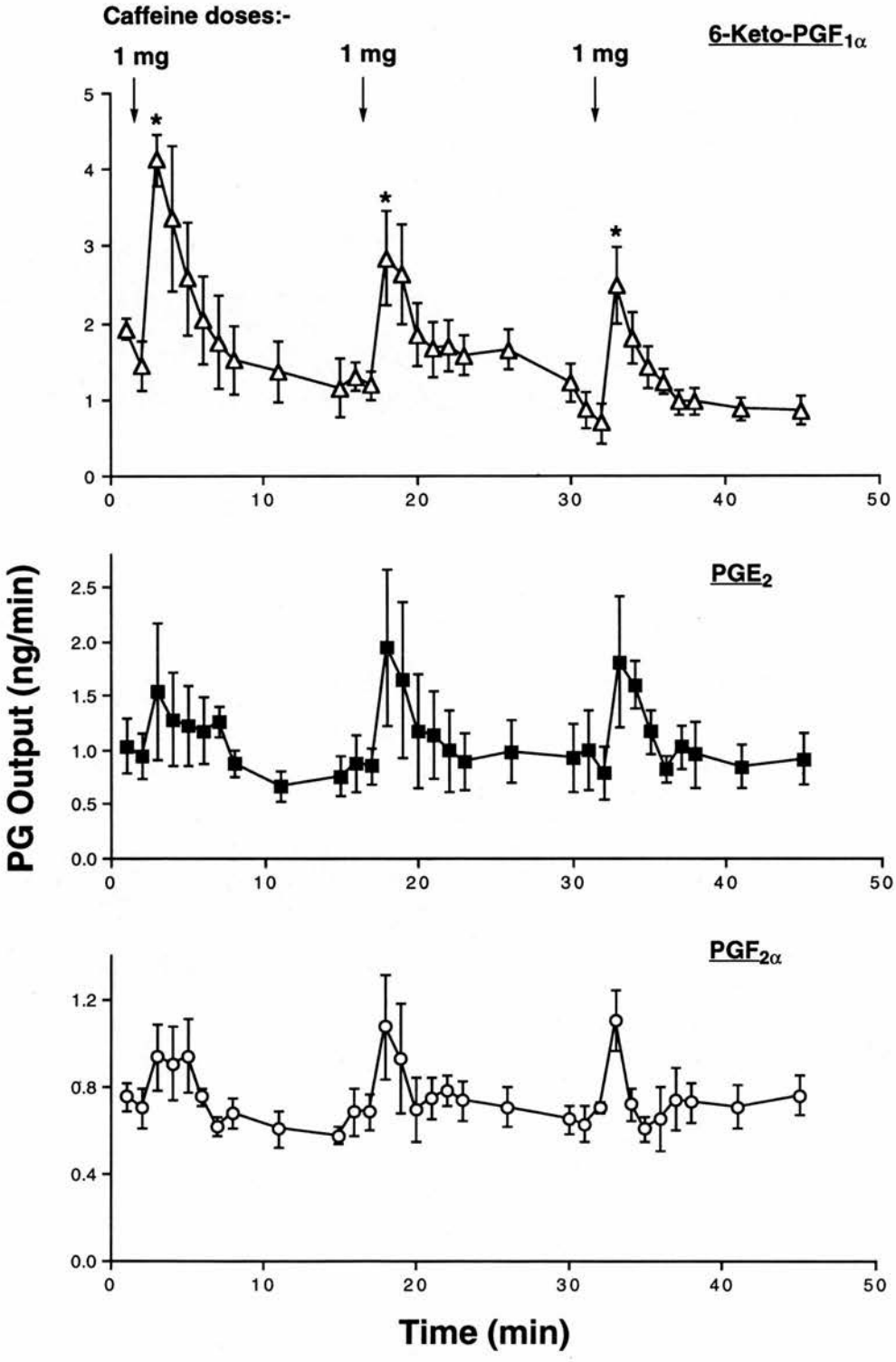


Figure 3.2.2. Effects of a high dose of caffeine (1 mg) administered three times on means (±SEM, n=4) outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ from the perfused mesenteric vascular bed of the rat.
* Significantly ($p<0.05$) increased by caffeine.

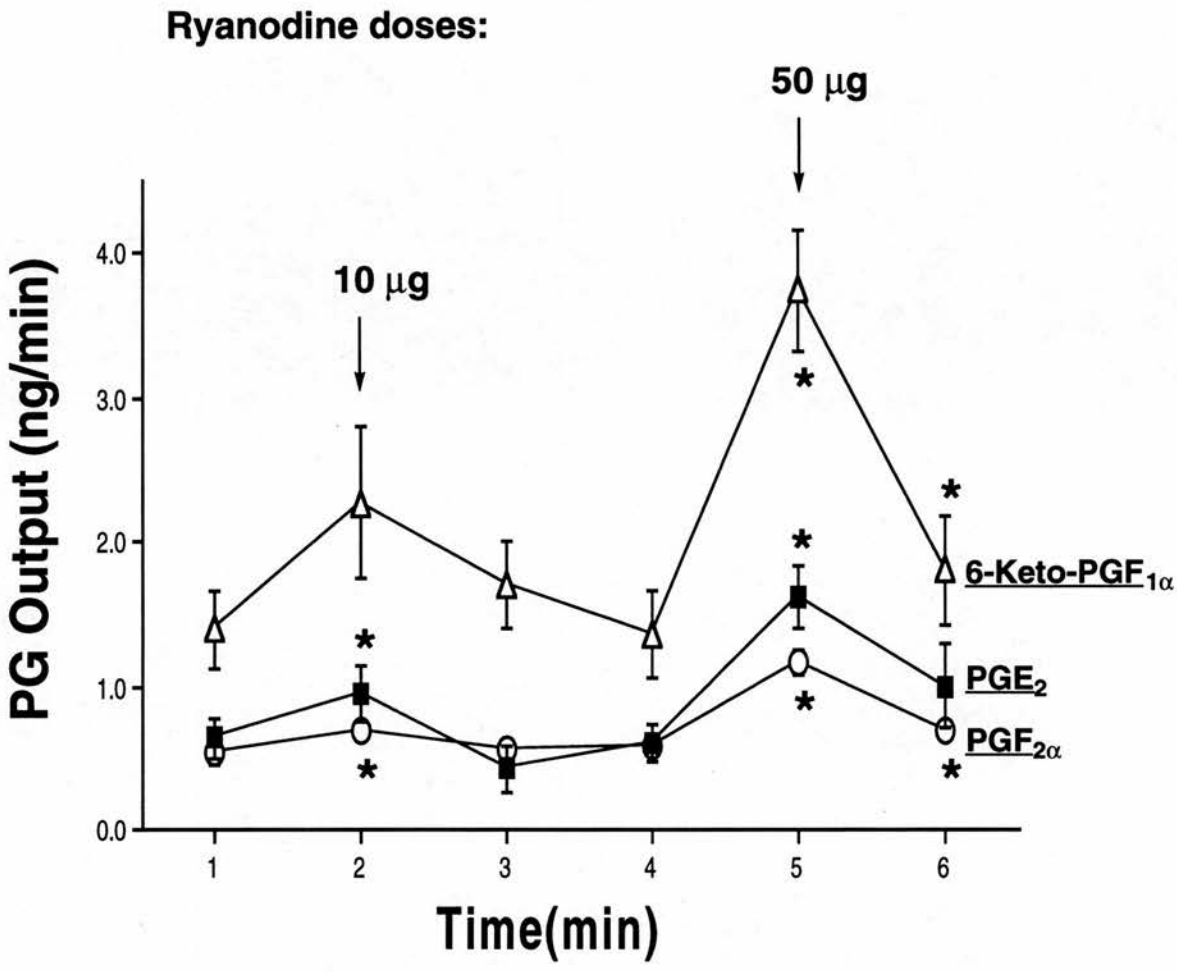


Fig.3.2.3. Effects of ryanodine on means (±SEM, n = 7) outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ from the perfused mesenteric vascular bed of the rat.
* Significantly ($P<0.05$) increased by ryanodine.

arachidonic acid from phospholipids (Ning & Poyser, 1984), usually by the action of PLA_2 (Dey *et al.*, 1982; Bonney, 1985). This enzyme is activated by calcium (Downing & Poyser, 1983), so the compounds which stimulate PG synthesis and release usually have to increase the intracellular free calcium concentration. Caffeine and RY have been shown to release calcium from cardiac muscle as well as from vascular smooth muscle (Gyorke & Fill, 1993; Hisayama *et al.*, 1990; Komori & Bolton, 1989; Watanabe *et al.*, 1992; Zhang *et al.*, 1993 and Chen & Van Breemen, 1993; Donoso *et al.*, 1994), by activating a RYR. Ryanodine and caffeine both released PGI_2 (measured as 6-keto-$PGF_{1\alpha}$), $PGF_{2\alpha}$ and PGE_2 from the perfused rat mesenteric vascular bed. After allowances are made for ryanodine's higher molecular weight, caffeine and RY were of similar potency. Hence, caffeine and RY may be initiating vascular PG synthesis and release by the same mechanism, i.e. through releasing intracellular calcium by activating a RYR.

This experiment has shown that caffeine stimulates prostaglandin production by the perfused mesenteric vascular bed as well as from the superfused guinea-pig uterus, indicating that the PG-releasing effect of caffeine is not unique to the uterus. However, in contrast to uterus where ryanodine had no effect, ryanodine stimulated PG production by the rat mesenteric vascular bed. This finding suggests that, if caffeine and ryanodine are stimulating PG synthesis through the same mechanism in blood vessels, then caffeine probably stimulates prostaglandin synthesis in the uterus by a different mechanism.

3.3 THE EFFECTS OF THE PLA_2 ACTIVATOR (MELITTIN), PLA_2 INHIBITOR (ARISTOLOCHIC ACID), STEROIDS (OESTRADIOL & PROGESTERONE), AND PROTEIN SYNTHESIS INHIBITORS (CYCLOHEXIMIDE, ACTINOMYCIN D & PUROMYCIN) ON THE OUTPUTS OF PROSTAGLANDINS FROM ENDOMETRIAL CELLS OF THE DAY 7 GUINEA-PIG UTERUS.

Introduction:

In the guinea-pig, oestradiol acting on a progesterone-primed uterus is the physiological stimulus for the increase in the output of $PGF_{2\alpha}$ at the end of the oestrous cycle (Poyser, 1983b). It has been shown that many actions of oestradiol on the uterus are mediated through increased protein synthesis (see Brenner & West, 1975). Previous experiments have shown that protein synthesis inhibitors, such as actinomycin D, cycloheximide and puromycin, inhibit prostaglandin output from the guinea-pig uterus (Poyser & Riley, 1987; Riley & Poyser, 1989) and increase the length of the oestrous cycle (Poyser, 1979). In the guinea-pig, increased prostaglandin synthesis is associated with an increase in PLA_2 activity (Downing & Poyser, 1983). Melittin, an activator of PLA_2, stimulates prostaglandin output from the guinea-pig uterus (Johnson & Poyser, 1991). Aristolochic acid, an inhibitor of PLA_2, prevents oxytocin-induced $PGF_{2\alpha}$ release from the sheep uterus (Lee & Silvia, 1994). Considering these reports the effects of protein synthesis inhibitors, oestradiol 17-β, progesterone, melittin and

aristolochic acid on the outputs of prostaglandins from guinea-pig endometrial cells in culture were investigated.

Methods:

Epithelial and stromal cells from day 7 guinea-pig endometrium were isolated and cultured as described in Section 2.1.5. Culture medium was changed every 3 days and on the sixth day cultured cells were treated in duplicate. In experiment 1, two wells containing each cell type were untreated (controls), and the remaining cells were treated with oestradiol 17-β (3.7 μM), progesterone (3.7 μM), melittin (2 μg/ml), or aristolochic acid (50 μM). In experiment 2, two wells containing each cell type were untreated (controls), and the remaining cells were treated with actinomycin D (10 & 50 μg/ml), cycloheximide (10 & 50 μg/ml), or puromycin (10 & 50 μg/ml).

In both experiments, culture medium was changed after 2, 8, and 24 h of culture. Samples of culture medium were stored at -20°C before radioimmunoassay, without extraction, for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$.

The concentrations of the oestradiol and progesterone used here are at the higher end of the concentrations which have been shown to effect PG output from the guinea-pig endometrium in culture (Riley & Poyser, 1987a). Melittin (2 μg/ml) has been shown to increase prostaglandin output from the guinea-pig uterus superfused *in vitro* (Johnson & Poyser, 1991). Aristolochic acid at concentrations of 10, 50 and 100 μM has been shown to completely inhibit prostaglandin output from the guinea-pig endometrium in culture (Norman S.J. & Poyser N.L., unpublished

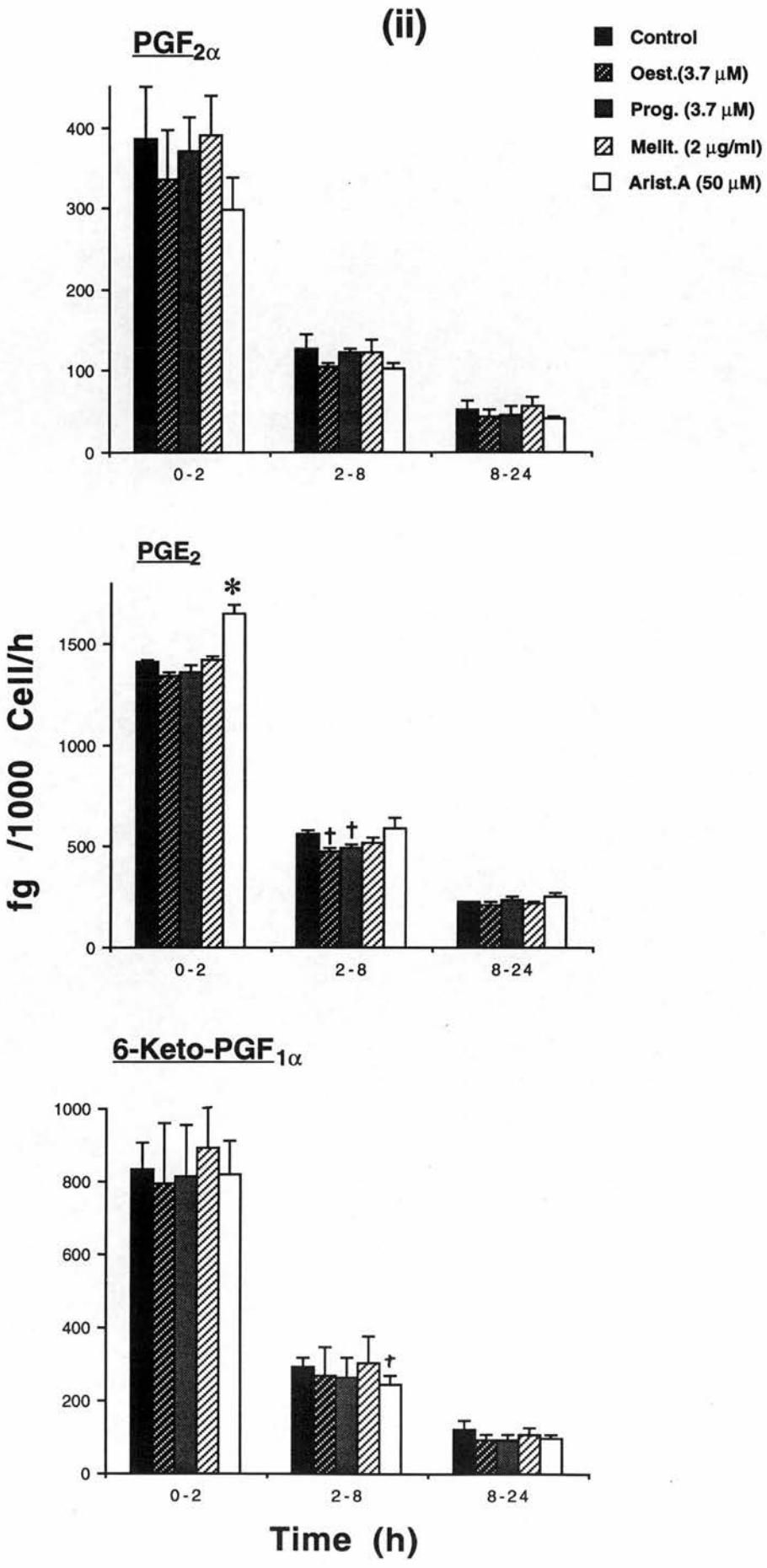
observations). Lee and Silvia (1994) have also reported that 10 μM aristolochic acid blocked ovine endometrial $PGF_{2\alpha}$ production stimulated by oxytocin, ALF^{-4} or melittin. Protein synthesis inhibitors at concentrations used in this set of experiments have been reported to inhibit PG output from the guinea-pig endometrium in culture (Poyser & Riley, 1987; Riley & Poyser, 1989).

Statistical tests:

The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

Results:

In experiment 1, oestradiol 17-β and progesterone significantly ($p<0.05$, $n=4$) decreased the outputs of $PGF_{2\alpha}$ from epithelial cells and PGE_2 from stromal cells after 8 h of cell culture (Figure 3.3.1). Oestradiol 17-β also significantly ($p<0.05$, $n=4$) decreased the outputs of $PGF_{2\alpha}$ from epithelial cells after 24 h of cell culture. Aristolochic acid, a PLA_2 inhibitor, significantly ($p<0.05$, $n=4$) inhibited the outputs of $PGF_{2\alpha}$ from epithelial cells after 8 and 24 h (Figure 3.3.1). Aristolochic acid had no significant effect on the outputs of $PGF_{2\alpha}$ from stromal cells and of 6-keto-$PGF_{1\alpha}$ from epithelial cells, but it significantly ($p<0.05$, $n=4$) decreased 6-keto-$PGF_{1\alpha}$ output from stromal cells after 8 h of culture. Surprisingly, aristolochic acid significantly ($p<0.05$, $n=4$) increased the output of PGE_2 from stromal cells after 2 h of culture (Figure 3.3.1). Melittin, a PLA_2 activator, significantly ($p<0.05$, $n=4$) increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ by up to


(ii)
Control
Oest.(3.7 μM)
Prog. (3.7 μM)
Melit. (2 μg/ml)
Arist.A (50 μM)
PGF2α
400
300
200
100
0
0-2
2-8
8-24
PGE2
*
†
†
1500
1000
500
0
fg /1000 Cell/h
6-Keto-PGF1α
†
1000
800
600
400
200
0
Time (h)

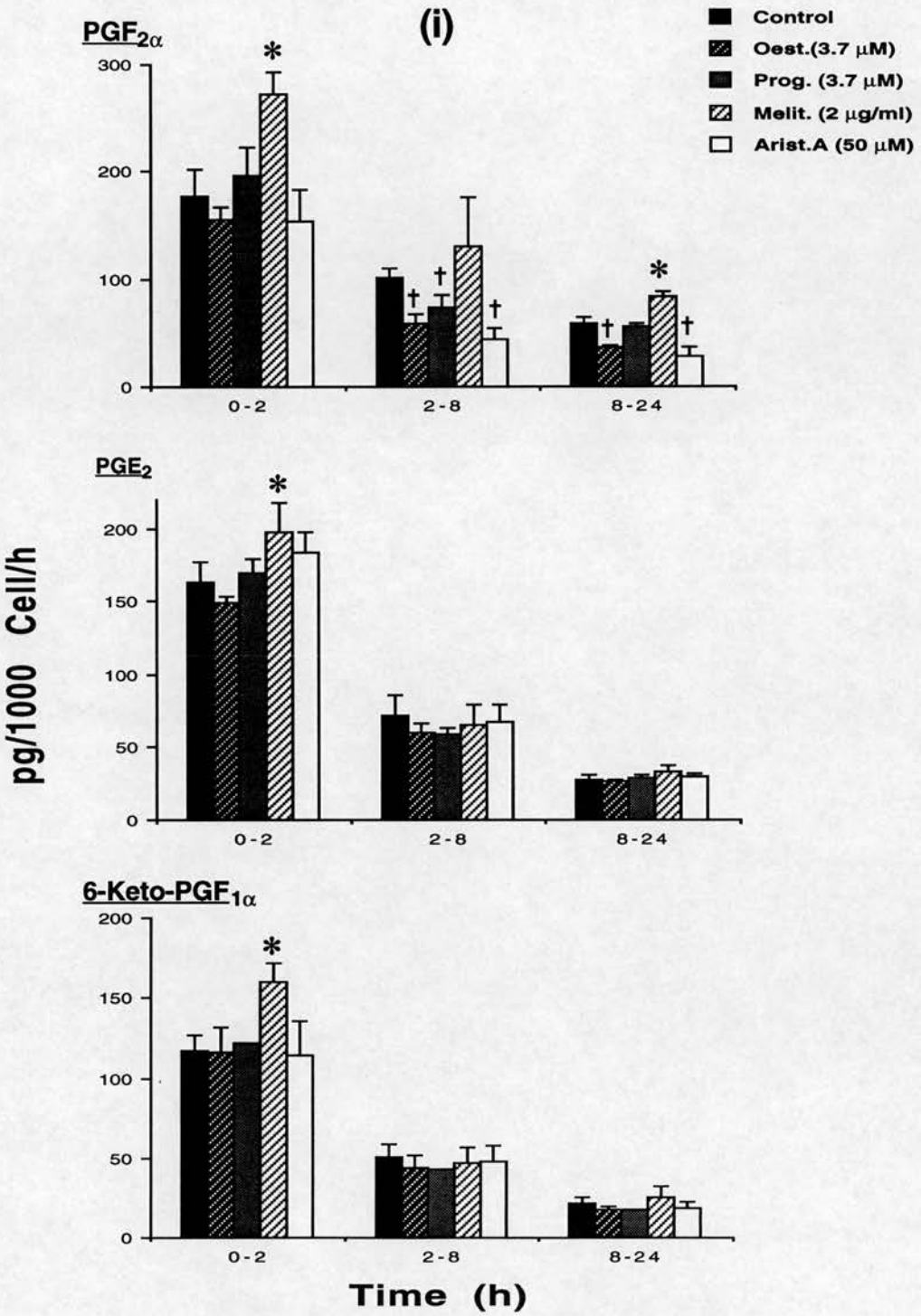
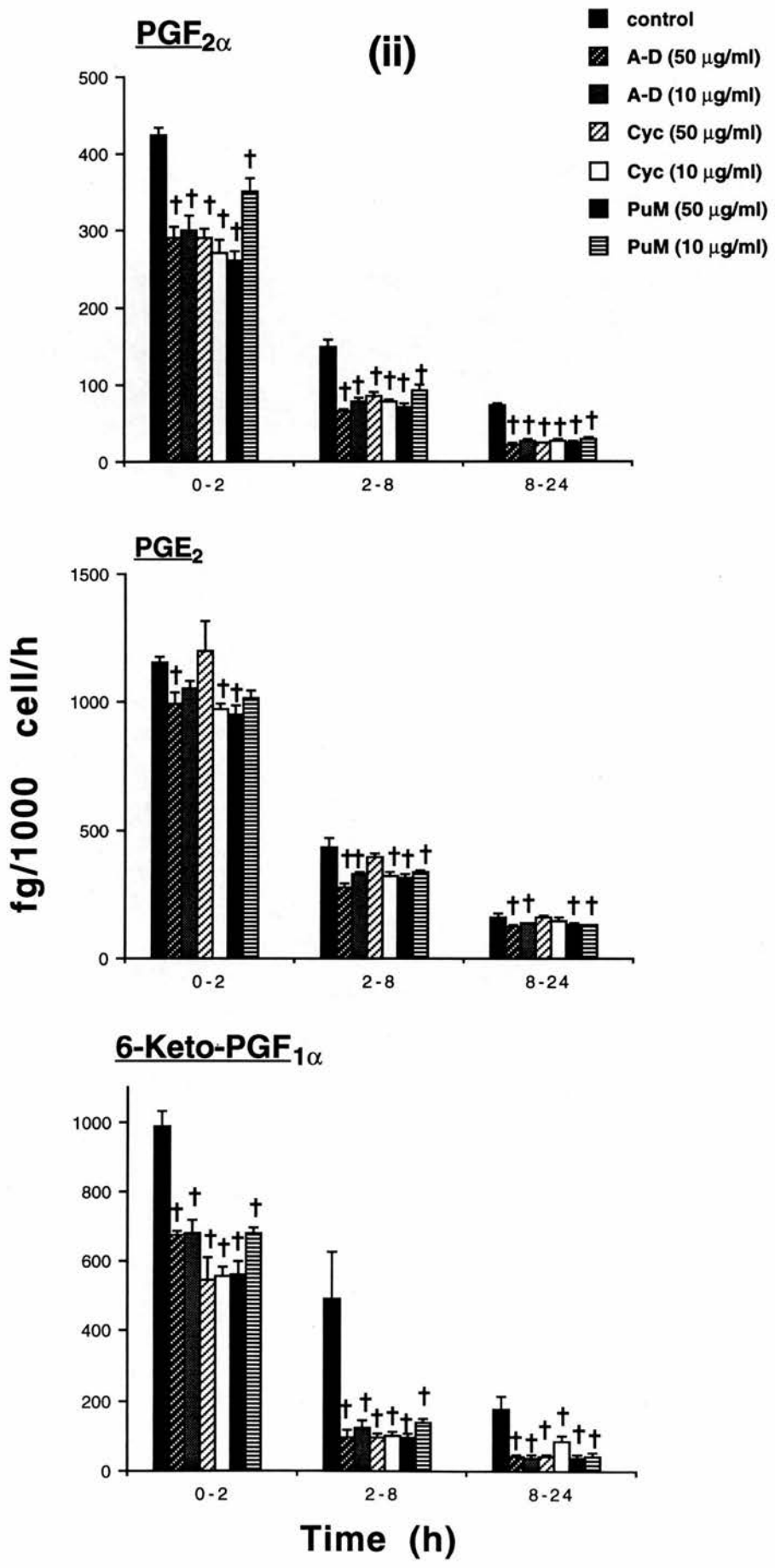


Figure 3.3.1. Mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured day 7 (i) epithelial and (ii) stromal cells. Cells were either untreated (control) or treated with oestardiol 17-β (Oest), progesterone (Prog), melittin (Melit) or aristolochic aicd (Arist.A) for 2, 6 and 16 h. * Significantly ($p<0.05$) higher than the corresponding control value. † Significantly ($p<0.05$) lower than the corresponding control value.

1.5-fold from epithelial cells after 2 of culture (Figure 3.3.1). Melittin also significantly ($p<0.05$, $n=4$) increased $PGF_{2\alpha}$ output from epithelial cells by 1.3-fold after 24 h of culture. Melittin had no effects on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from stromal cells (Figure 3.3.1).

In experiment 2, after 2 h of treatment with cycloheximide (10 & 50 μg/ml) or puromycin (10 & 50 μg/ml), a significant ($p<0.05$, $n=4$) inhibition in the output of $PGF_{2\alpha}$ from epithelial cells was apparent (Figure 3.3.2). This inhibition became greater as the length of treatment was increased. By 8 and 24 h, actinomycin D (10 & 50 μg/ml), cycloheximide (10 & 50 μg/ml) and puromycin (10 & 50 μg/ml) significantly ($p<0.05$, $n=4$) inhibited $PGF_{2\alpha}$ output from epithelial cells. Actinomycin D (10 & 50 μg/ml), cycloheximide (10 & 50 μg/ml) and puromycin (10 & 50 μg/ml) significantly ($p<0.03$, $n=4$) inhibited $PGF_{2\alpha}$ output from stromal cells after 2, 8 and 24 h of cell culture. This inhibition was greater after 8 and 24 h compared to that after 2 h (Figure 3.3.2).

The inhibition of PGE_2 output by protein synthesis inhibitors was somewhat selective. Cycloheximide (10 μg/ml) after 2 h, actinomycin D (10 & 50 μg/ml) and puromycin (50 μg/ml) after 8 h, and puromycin (10 μg/ml) after 24 h caused a significant ($p<0.05$, $n=4$) inhibition in the output of PGE_2 from epithelial cells (Figure 3.3.2). PGE_2 output from stromal cells was significantly ($p<0.05$, $n=4$) inhibited by actinomycin D (50 μg/ml) and puromycin (50 μg/ml) after 2, 8, and 24 h, by actinomycin D (10 μg/ml) and puromycin (10 μg/ml) after 8 and 24 h, and by cycloheximide (10 μg/ml) after 2 and 8 h of cell culture (Figure 3.3.2).


(ii)
control
A-D (50 μg/ml)
A-D (10 μg/ml)
Cyc (50 μg/ml)
Cyc (10 μg/ml)
PuM (50 μg/ml)
PuM (10 μg/ml)
PGF2α
PGE2
6-Keto-PGF1α
fg/1000 cell/h
0-2
2-8
8-24
Time (h)

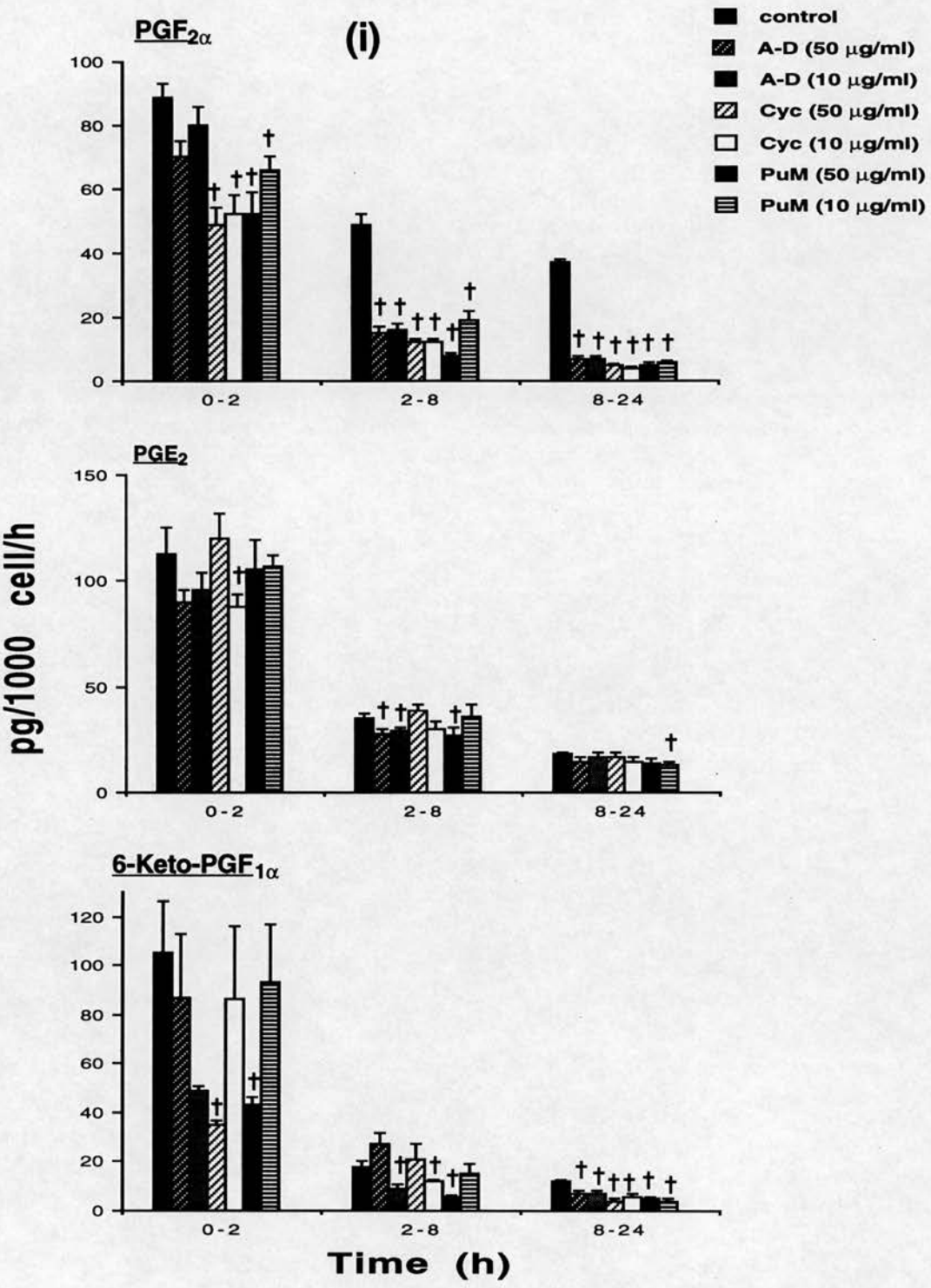


Figure 3.3.2. Mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured day 7 (i) epithelial and (ii) stromal cells. Cells were either untreated (control) or treated with actinomycin D (A-D; 10 & 50 μg/ml), cycloheximide (Cys; 10 & 50 μg/ml) or puromycin (PuM; 10 & 50 μg/ml) for 2, 6 and 16 h.

† Significantly ($p<0.05$) lower than the corresponding control value.

Cycloheximide (50 μg//ml) and puromycin (50 μg/ml) after 2 h, actinomycin D (10 μg/ml), cycloheximide (10 μg/ml) and puromycin (50 μg/ml) after 8 h of cell culture significantly ($p<0.05$, $n=4$) inhibited the output of 6-keto-$PGF_{1\alpha}$ from epithelial cells (Figure 3.3.2). By 24 h, all protein synthesis inhibitors at all concentrations caused a significant ($p<0.05$, $n=4$) inhibition of 6-keto-$PGF_{1\alpha}$ output from epithelial cells in culture. For stromal cells, 6-keto-$PGF_{1\alpha}$ output was significantly ($p<0.05$, $n=4$) inhibited by all protein synthesis inhibitors at all concentrations used after 2, 8 and 24 h (Figure 3.3.2).

Discussion:

Oestradiol 17-β and progesterone inhibited the outputs of $PGF_{2\alpha}$ and PGE_2 from epithelial and stromal cells, respectively. This is in agreement with a report which has shown that oestradiol 17-β and progesterone inhibit PG output from guinea-pig endometrium in culture (Riley and Poyser, 1987a), but not with the findings of Leaver and Seawright (1982) who reported that oestradiol stimulated PG output from guinea-pig endometrium in culture. Furthermore, it has been also reported that progesterone suppresses $PGF_{2\alpha}$ and PGE release from epithelial cells of human proliferative endometrium (Mitchell & Smith, 1992), and from epithelial and stromal cells of pig endometrium (Zhang & Davis, 1991). Considering these reports and the findings from the present study, progesterone *in vitro* clearly exerts an inhibitory effect on endometrial cells with respect to PG output in several species. The effect of oestradiol *in vitro* seems to vary among species. Oestradiol *in vitro* inhibits PG output from guinea-pig endometrium and endometrial cells,

whereas it stimulates $PGF_{2\alpha}$ output from human endometrium *in vitro* (Abel & Baird 1980; Schatz *et al.*, 1985).

Melittin has been shown to increase prostaglandin output from the guinea-pig uterus superfused *in vitro* (Poyser & Johnson, 1991). In this study melittin stimulated the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured epithelial but not from stromal cells. The stimulatory effect of melittin was the greatest on $PGF_{2\alpha}$ output indicating that the endogenous PLA_2 activated by melittin is directed towards the synthesis and release of mainly $PGF_{2\alpha}$. The ineffectiveness of melittin on PG output from stromal cells suggests that melittin selectively activates epithelial PLA_2 but not stromal PLA_2 in the guinea-pig endometrium.

Aristolochic acid inhibited the outputs of $PGF_{2\alpha}$ and, to a lesser extent 6-keto-$PGF_{1\alpha}$, from the cultured epithelial but not from the stromal cells, indicating that aristolochic acid inhibited an endogenous PLA_2 which is present in epithelial cells and is responsible for the release of mainly $PGF_{2\alpha}$ but not the release of PGE_2. Surprisingly, aristolochic acid stimulated the output of PGE_2 from stromal cells in culture. However, it is unlikely that the stimulatory effect of aristolochic acid on PGE_2 output is mediated via PLA_2 mechanism.

The intrauterine administration of actinomycin D to guinea-pig on day 10 inhibits the output of $PGF_{2\alpha}$ from the day 15 uterus superfused *in vitro* by 80 to 85% (Poyser & Riley, 1987). In the present study, all three protein synthesis inhibitors were more effective in inhibiting the output of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ than the output of PGE_2 from endometrial cells in culture. After 2 h of cell culture, cycloheximide and puromycin were more effective than actinomycin D. These

findings are in agreement with a previous report (Poyser & Riley, 1987) who showed that these 3 protein synthesis inhibitors inhibit PG output from guinea-pig endometrium in culture.

The finding that the output of $PGF_{2\alpha}$ was affected more than the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ by these protein synthesis inhibitors indicate that $PGF_{2\alpha}$ synthesis is more dependent upon increased protein synthesis.

Overall these findings indicate that prostaglandin synthesis and release, in particular $PGF_{2\alpha}$ from the cultured guinea-pig endometrial cells is affected by protein synthesis inhibitors, ovarian steroids and modulators of PLA_2 activity.

3.4 INVESTIGATIONS OF THE EFFECT OF A NOVEL PROSTAGLANDIN H SYNTHASE-2 INHIBITOR (NS-398) ON PG PRODUCTION FROM THE GUINEA-PIG UTERUS: A COMPARATIVE STUDY WITH INDOMETHACIN.

Introduction:

It is well-documented that $PGF_{2\alpha}$ output from the guinea-pig uterus increases towards the end of the oestrous cycle. The synthesis of $PGF_{2\alpha}$ involves the action of prostaglandin H synthase (PGHS) on arachidonic acid. Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, have been shown to inhibit prostaglandin synthesis by the PGHS enzyme (Miyamato *et al.*, 1974; Rome & Lands, 1975; Miyamato *et al.*, 1976). In the guinea-pig, indomethacin increases the length of the oestrous cycle (Horton & Poyser, 1973) by inhibiting $PGF_{2\alpha}$ synthesis (Poyser & Horton, 1975). In the guinea-pig, the endometrial PGHS concentration increases after day 11 of the cycle (Poyser, 1983a). Recently, it has been shown that there are two isoforms of prostaglandin H synthase, namely PGHS-1 and PGHS-2. PGHS-1 is a constitutive enzyme and PGHS-2 is an inducible enzyme (Maier *et al.*, 1990; Fu *et al.*, 1990; Sirois & Richards, 1992; Wimsatt *et al.*, 1993). It is widely believed that NSAID drugs exert their therapeutic effects by inhibiting PGHS-2 and their side-effects by inhibiting PGHS-1.

A new NSAID, NS-389, (Futaki *et al.*, 1993) has been shown to have an anti-inflammatory potency equal to that of indomethacin with no side-effects such as

gastric ulceration (Futaki *et al.*, 1993; Arai *et al.*, 1993). It is also reported that NS-398 selectively inhibits PGHS-2 activity *in vitro* without affecting PGHS-1 activity (Futaki *et al.*, 1994). In this set of experiments, the effects of NS-398 on prostaglandin production by day 7 and day 15 guinea-pig endometrial homogenates, by day 7 endometrium and myometrium in culture, and by cultured epithelial and stromal cells obtained from day 7 guinea-pig endometrium were investigated. Poyser (1987a) has shown that indomethacin at similar concentrations used here inhibits $PGF_{2\alpha}$ synthesis from guinea-pig uterus *in vitro*.

3.4.1 The Effect of NS-398 on Prostaglandin Synthesis by Homogenates of the Day 7 and Day 15 Guinea-Pig Endometrium.

Methods:

Endometrium from the uterus of four day 7 and four day 15 guinea-pigs was obtained as described in Section 2.1.1. Tissue homogenisation was as described in Section 2.1.4. Briefly, both uterine horns were removed from each animal, and the endometrium was separated from the myometrium and divided into 5 approximately equal amounts. Tissues were blotted dry, weighed, and chopped finely with scissors, and then homogenised in 5 ml Krebs solution containing either 100 µl ethanol (control) or treated with indomethacin (14 & 28 µM) or NS-398 (16 & 32 µM). The homogeniser was then washed with a further 5 ml Krebs solution containing the same treatment. The homogenate and the washings (total volume 10 ml) were then transferred to a 25 ml conical flask, and incubated for 60 min at

37°C in a shaking (230 oscillations/min) Grant Water bath. Prostaglandins were extracted from the homogenates as described in Section 2.1.1 except that 2 volumes of 20 ml ethyl acetate were used. The residue obtained from evaporation of the ethyl acetate fractions was redissolved in 4 ml of ethyl acetate. The samples were then stored at -20°C. Concentrated solutions of indomethacin and NS-398 were prepared in ethanol and stored at -20°C. The appropriate concentrations of indomethacin and NS-398 in Krebs solutions were prepared by adding up either 50 µl (in case of 14 µM indomethacin and 16 µM NS-398) or 100 µl (in case of 28 µM indomethacin and 32 µM NS-398) of the ethanolic stock solutions to 10 ml Krebs solution. The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ in the samples were measured by radioimmunoassay as described in Section 2.1.8.

Results:

Homogenates of day 15 guinea-pig endometrium synthesised 3.0-, 1.7- and 1.4-fold more $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ than homogenates of day 7 guinea-pig endometrium, respectively (Figure 3.4.1.1). Indomethacin (14 & 28 µM) significantly ($p<0.05$, $n=4$) reduced the amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ produced by both day 7 and day 15 guinea-pig endometrial homogenates (Figure 3.4.1.1). There were no significant differences between the potency of indomethacin 14 µM and indomethacin 28 µM in inhibiting production of the same PG by guinea-pig endometrium homogenates obtained from the same day of the oestrous cycle.

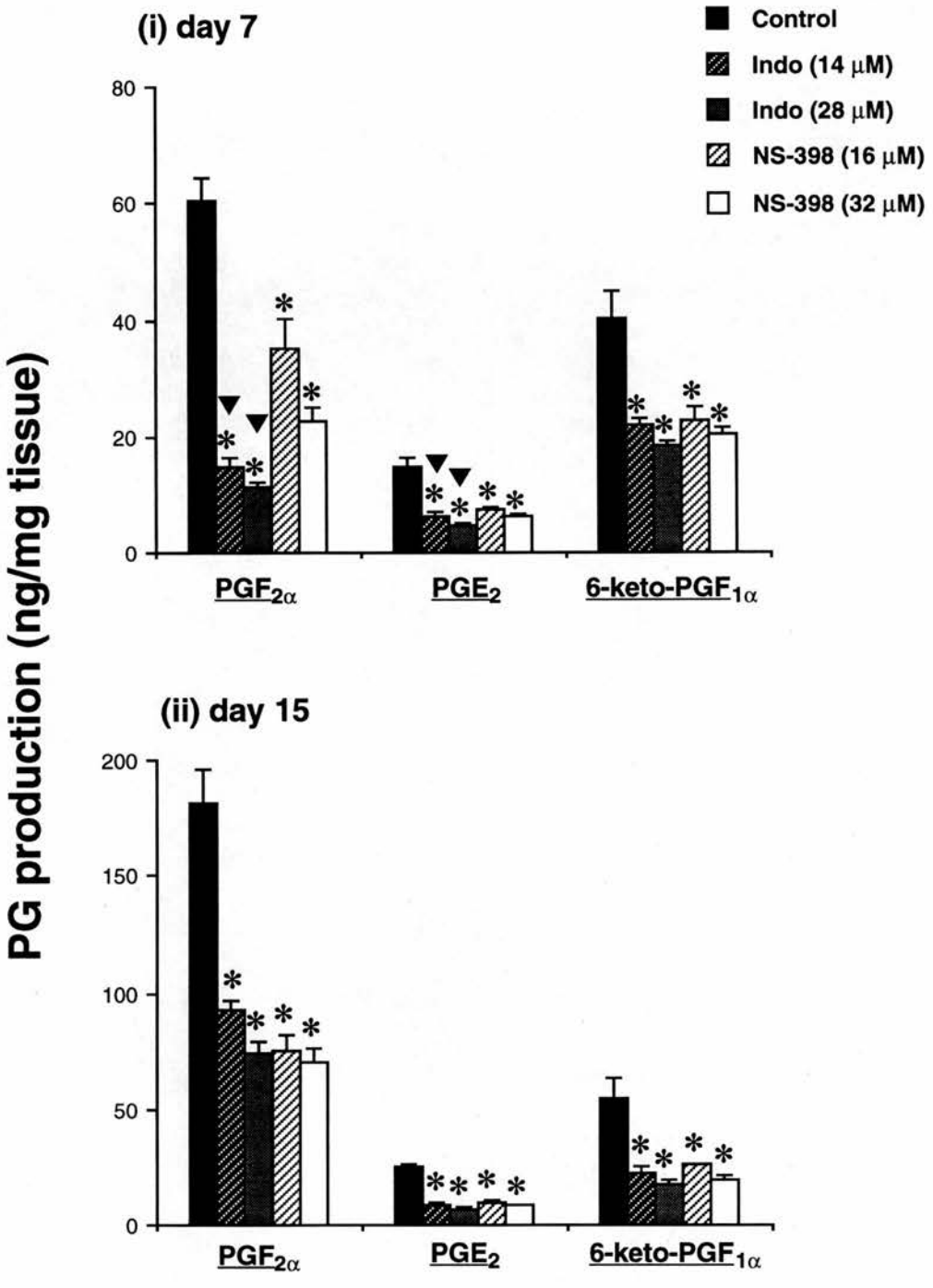


Figure 3.4.1.1. Effects of indomethacin (Indo; 14 & 28 μM) and NS-398 (16 & 32 μM) on the mean (±SEM, n=4) production of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ by endometrium homogenates of (i) day 7 and (ii) day 15 guinea-pig uterus.

* Significantly ($p<0.05$) inhibited by indomethacin or NS-398 treatment.

▼ Significantly ($p<0.05$) lower than the corresponding value obtained by NS-398 treatment.

NS-398 (16 & 32 μM) significantly ($p<0.05$, $n=4$) reduced the amount of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ produced from both day 7 and day 15 guinea-pig endometrial homogenates (Figure 3.4.1.1). There was no significant difference in the potency of NS-398 (16 μM) and NS-398 (32 μM) in inhibiting production of the same PG by guinea-pig endometrium homogenates obtained from the same day of the oestrous cycle. Indomethacin at an approximately similar concentration was significantly ($p<0.05$, $n=4$) more potent than NS-398 in inhibiting $PGF_{2\alpha}$ and PGE_2 production by homogenates of day 7 but not of day 15 guinea-pig endometrium (Figure 3.4.1.1).

3.4.2 The Effect of NS-398 on Prostaglandin Synthesis by and Release From the Day 7 Guinea-Pig Endometrium and Myometrium in Culture.

Methods:

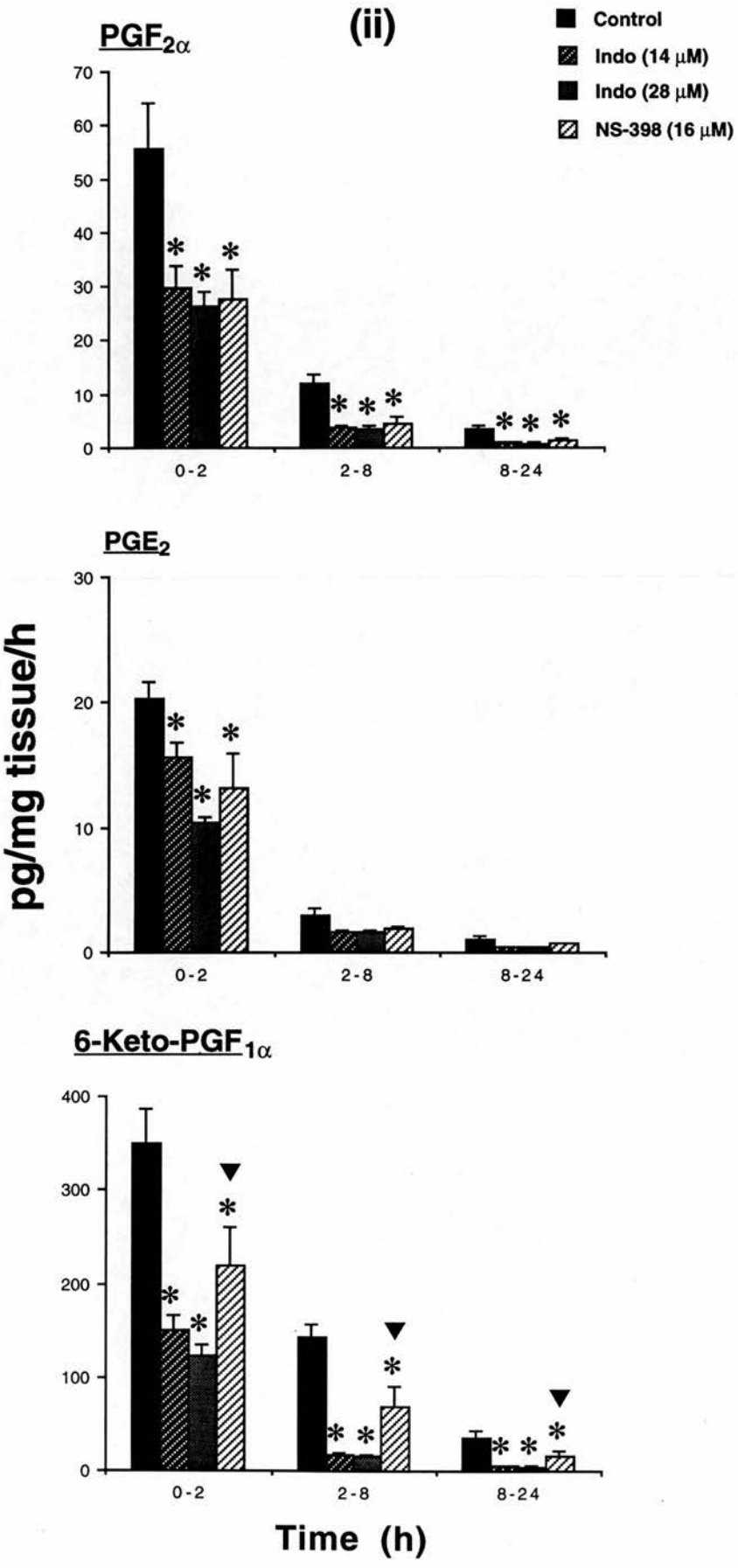
The preparation of guinea-pig endometrial and myometrial tissue and the culture set-up was as described in Section 2.1.3. From the endometrium and myometrium obtained from each guinea-pig, ten tissue culture dishes were prepared. Four of these tissue culture dishes were left untreated (controls), and the remaining dishes were treated in duplicate with indomethacin (14 μM), indomethacin (28 μM) or NS-398 (16 μM). In order to minimise the effect of the vehicle ethanol (20 μl) was added to the controls. Culture medium was changed after 2, 8, and 24 h of culture. The appropriate solutions of indomethacin and NS-398 were prepared by diluting up to 120-fold in tissue culture medium. Samples of culture medium were stored at

-20°C before radioimmunoassay, without extraction, for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$. Concentrated solutions of indomethacin and NS-398 were prepared in ethanol and stored at -20°C.

Results:

After 2 and 8 h of culture, the major prostaglandin release from both the endometrium and myometrium in culture was 6-keto-$PGF_{1\alpha}$. The outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from both endometrium and myometrium, and the output of $PGF_{2\alpha}$ from myometrium declined with time over 24 h of culture. However, the output of $PGF_{2\alpha}$ from endometrium declined between 2 and 8 h of culture but then increased between 8 and 24 h of culture (Figure 3.4.2.1). This is in agreement with the results obtained from the earlier experiments (see Section 3.1.3).

Indomethacin (14 & 28 μM) and NS-398 (16 μM) significantly ($p<0.05$, $n=4$) inhibited the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from both endometrium and myometrium, and the output of PGE_2 from endometrium after 2, 8 and 24 h of culture. The output of PGE_2 from myometrium was significantly ($p<0.05$, $n=4$) inhibited by indomethacin (14 & 28 μM) and NS-398 (16 μM) after 2 h but not after 8 or 24 h of culture (Figure 3.4.2.1). NS-398 was as effective as indomethacin at inhibiting the output of all 3 PGs from both day 7 and day 15 guinea-pig endometrium and myometrium in culture, except for 6-keto-$PGF_{1\alpha}$ output from the myometrium. Indomethacin at both concentrations was significantly ($p<0.05$, $n=4$) more potent than NS-398 in inhibiting the output of 6-keto-$PGF_{1\alpha}$ from myometrium after 2, 8 and 24 h of culture (Figure 3.4.2.1).


(ii)
Control
Indo (14 μM)
Indo (28 μM)
NS-398 (16 μM)
PGF2α
PGE2
6-Keto-PGF1α
pg/mg tissue/h
0-2
2-8
8-24
Time (h)

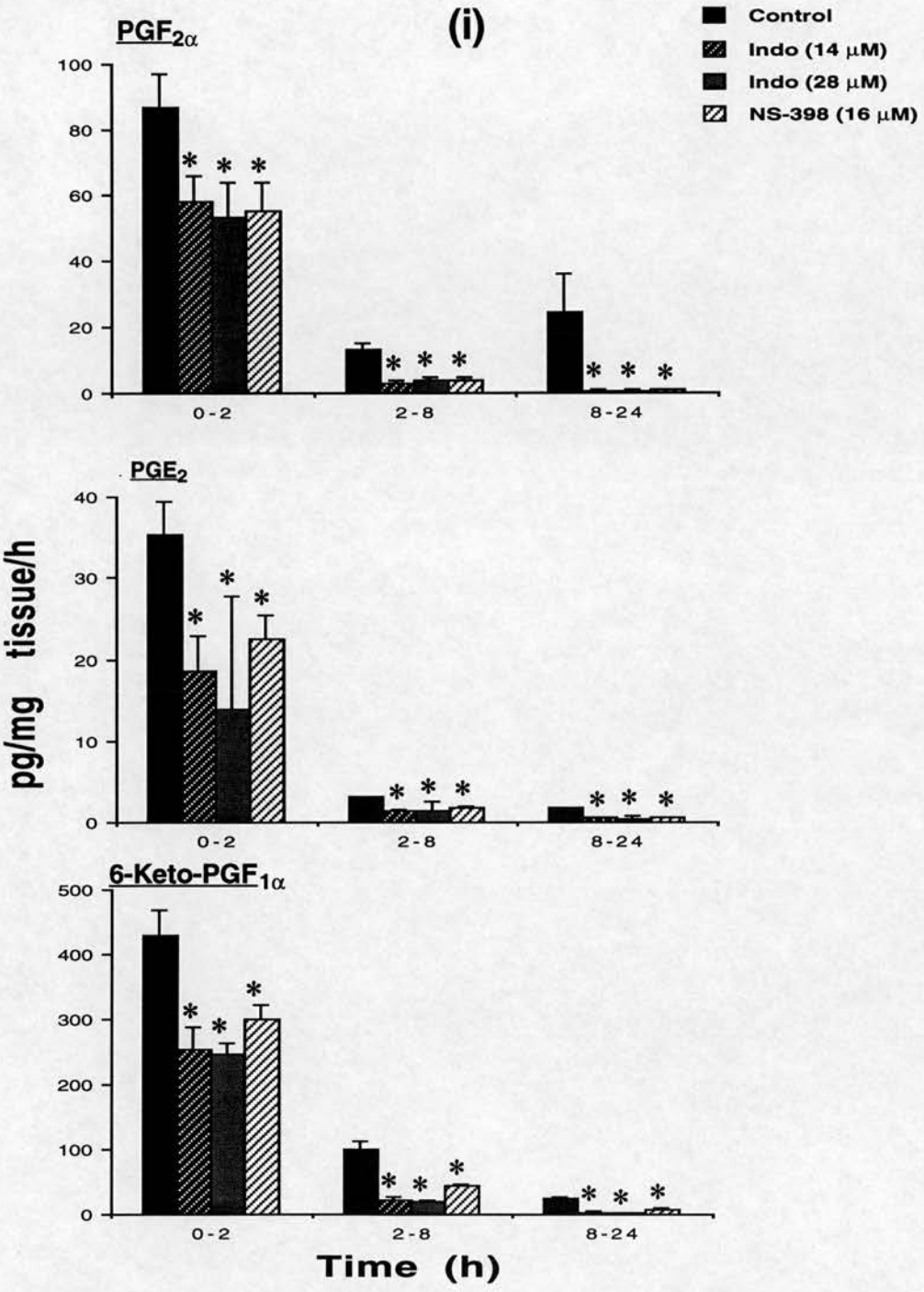


Figure 3.4.2.1. Effects of indomethacin (Indo; 14 and 28 μM) and NS-398 (16 μM) on mean (±SEM) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig (i) endometrium and (ii) myometrium in culture. * Significantly ($p<0.05$) reduced by indomethacin or NS-398 treatment. ▼ Significantly ($p<0.05$) lower than the corresponding control value but higher than the values obtained by indomethacin treatment during the same culture period.

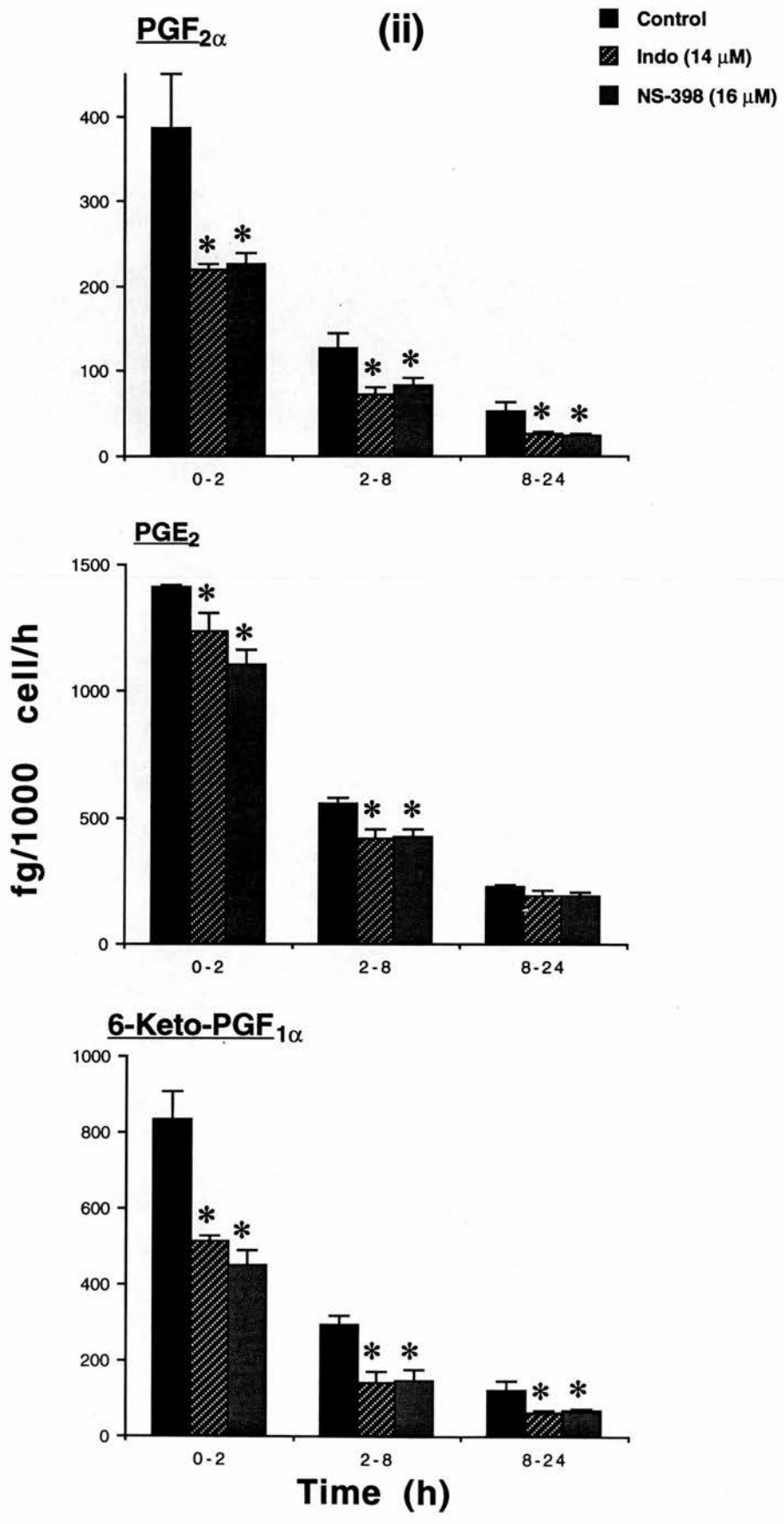
3.4.3 The Effect of NS-398 on Prostaglandin Synthesis by and Release From Cultured Epithelial and Stromal Cells Obtained From Day 7 Guinea-Pig Endometrium.

Methods:

Epithelial and stromal cell obtained from the endometrium of four day 7 guinea-pigs were isolated and cultured as described in Section 2.1.5. Culture medium was changed every 3 days and on the sixth day, cells in culture were treated in duplicate. Two wells containing each cell type were left untreated (controls), and the remaining wells of each cell type were treated in duplicate with indomethacin (14 μM) or NS-398 (16 μM). Concentrated solution of indomethacin and NS-398 were prepared in ethanol and stored at -20°C. The appropriate solutions of indomethacin and NS-398 were prepared by diluting 200-fold in cell culture solution. In order to minimise the vehicle effect, ethanol (20 μl) was added to the control cells. Culture medium was changed after 2, 8, and 24 h of culture. Samples of culture medium were stored at -20°C before radioimmunoassay, without extraction, for $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$.

Results:

For epithelial cells, indomethacin (14 μM) and NS-398 (16 μM) significantly ($p<0.05$, $n=4$) inhibited the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ after 2, 8 and 24 h of culture (Figure 3.4.**3.1**). For stromal cells, indomethacin (14 μM) and NS-398 (16 μM) significantly ($p<0.05$, $n=4$) inhibited the outputs of $PGF_{2\alpha}$ and


(ii)
Control
Indo (14 μM)
NS-398 (16 μM)
PGF2α
PGE2
6-Keto-PGF1α
fg/1000 cell/h
0
100
200
300
400
500
600
800
1000
1500
0-2
2-8
8-24
Time (h)

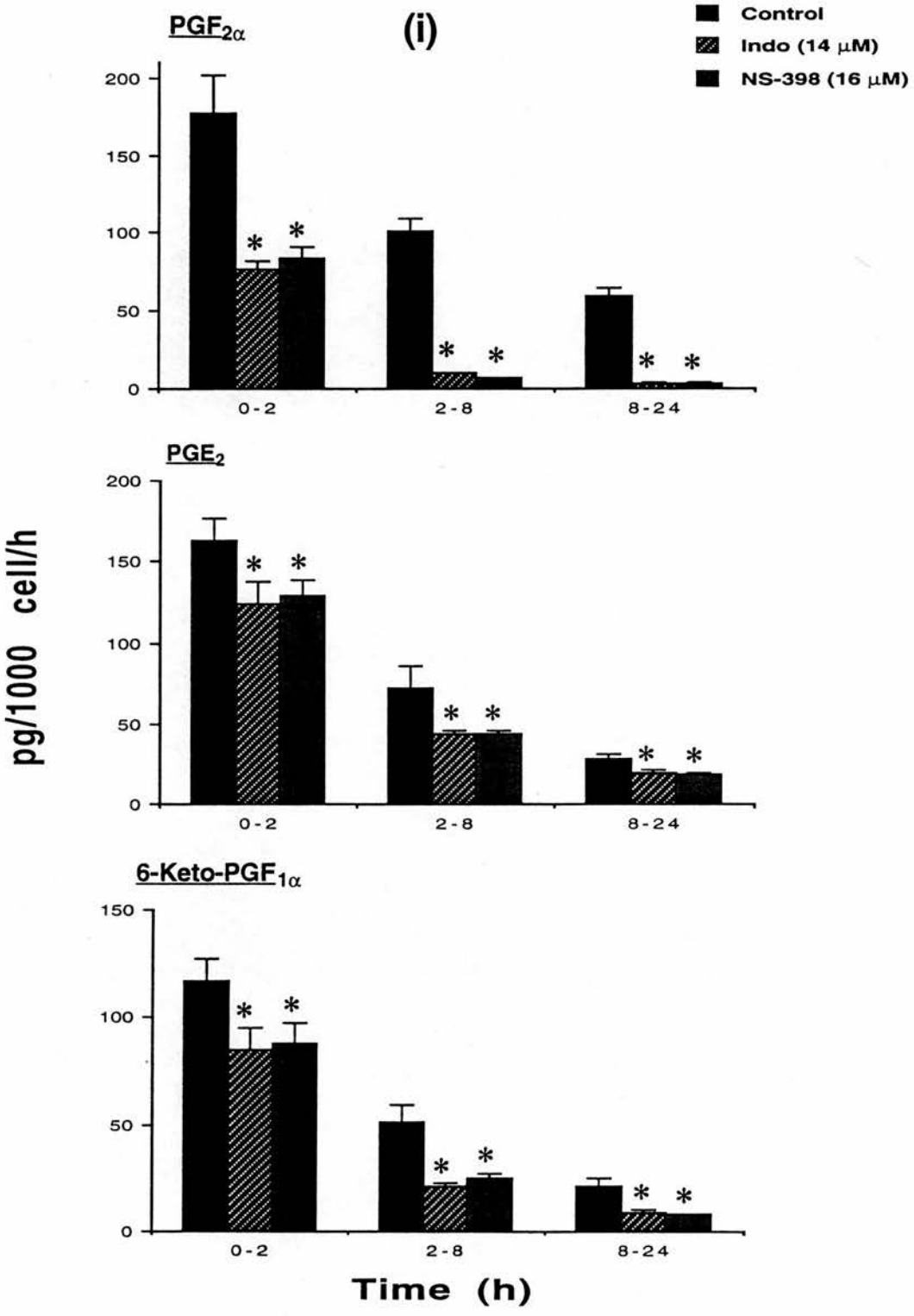


Figure 3.4.3.1. Effects of indomethacin (Indo) and NS-398 on mean (±SEM, n=4) outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig (i) epithelial and (ii) stromal cells in culture. * Significantly ($p<0.05$) inhibited by indomethacin or NS-398 treatment.

6-keto-$PGF_{1\alpha}$ after 2, 8 and 24 h, and PGE_2 output after 2 and 8 h but not after 24 h of culture. There was no difference between the potencies of indomethacin and NS-398 in inhibiting the outputs of all 3 PGs from the epithelial and stromal cells in culture.

Discussion:

The inhibitory effect of indomethacin on prostaglandin synthesis by the guinea-pig uterus seen in this study is in agreement with previous reports (Poyser, 1985a). Indomethacin inhibits prostaglandin synthesis by inhibiting the prostaglandin H synthase (PGHS) enzyme (Miyamato *et al.*, 1974; Miyamato *et al.*, 1976; Rome & Lands, 1975). Recently, two isoforms of PGHS, namely PGHS-1 and PGHS-2 have been identified: PGHS-1, which is constitutively expressed in many cell types and tissues, and PGHS-2, which is an inducible form (Maier, *et al.*, 1990; Fu *et al.*, 1990; Wong & Richards, 1991; Sirois & Richards, 1992; Wimsatt *et al.*, 1993). PGHS-2 but not PGHS-1 has been induced by interleukin-1α in human dermal fibroblasts (Seibert & Masferrer, 1994), by lipopolysaccharide in rat alveolar macrophages (Lee *et al.*, 1992) and by hCG in granulosa cells of rat preovulatory follicles (Sirois & Richards, 1992). The induction of PGHS-2 in ovine cotyledonary tissue during late gestation has also been reported (Wimsatt *et al.*, 1993). Indomethacin is a non-selective PGHS inhibitor, that is it inhibits both PGHS-1 and PGHS-2 activities (see Seibert & Masferrer, 1994).

NS-398 inhibited prostaglandin production by the day 7 and day 15 guinea-pig endometrial homogenates, by day 7 endometrium and myometrium in culture, and

by cultured epithelial and stromal cells obtained from day 7 guinea-pig endometrium. It has been shown that NS-398 selectively inhibits PGHS-2 activity *in vitro* without affecting PGHS-1 activity (Futaki *et al.*, 1994, Masferrer *et al.*, 1994). Hence, it is likely that NS-398 inhibits PGHS-2 activity in the guinea-pig endometrium and myometrium. This implies that the guinea-pig uterus (both the endometrium and myometrium) contains functionally active PGHS-2. It is not known that whether PGHS-1 is also present in the guinea-pig uterus. A selective PGHS-1 inhibitor would be a useful tool to investigate whether guinea-pig uterus contains functionally active PGHS-1 as well as PGHS-2 at both stages of the oestrous cycle examined here. The finding that indomethacin and NS-398 had similar potencies in inhibiting PG production by day 7 endometrium and myometrium (except for 6-keto-$PGF_{1\alpha}$ output from myometrium) in culture, by cultured epithelial and stromal cells obtained from the day 7 guinea-pig endometrium, and by day 15 guinea-pig endometrium homogenates indicates that both indomethacin and NS-398 were inhibiting PGHS-2 activity in these systems. Nevertheless, indomethacin at an approximately similar concentration was significantly more potent than NS-398 in inhibiting the synthesis of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ by day 7 guinea-pig endometrial homogenates and the output of 6-keto-$PGF_{1\alpha}$ from the myometrium in culture. This suggests that indomethacin apart from inhibiting PGHS-2, inhibits some other enzyme responsible for $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ production by day 7 guinea-pig endometrium homogenates and 6-keto-$PGF_{1\alpha}$ by day 7 myometrium in culture. Overall, this study indicates that PGHS-2

is present in the guinea-pig uterus, and that PGHS-2 is the predominant prostaglandin-forming enzyme in the guinea-pig uterus.

3.5 DETECTION OF PROSTAGLANDIN H SYNTHASE-2 (PGHS-2) IN THE GUINEA-PIG ENDOMETRIUM.

Introduction:

It has been shown in various tissue that at least two different PGHS isoforms exist, denoted as PGHS-1 (Mr 69 kDa) and PGHS-2 (Mr 72 kDa). In the previous experiment (see Section 3.4), NS-398 (a selective inhibitor of PGHS-2) inhibited prostaglandin production by the guinea-pig uterus, implicating the presence of PGHS-2 enzyme in the guinea-pig endometrium. As a result, in this experiment, immunoblotting was used to detect the presence of PGHS-2 in endometrium from the day 6 and day 17 guinea-pig uterus.

Methods:

(a) Immunoblotting:

Soluble cell extracts from guinea-pig endometrium were prepared as described in Section 2.1.7.1. In summary, endometrial tissue from guinea-pigs on days 6 and 17 of the oestrous cycle was prepared as described in Section 2.1.4. All tissues were homogenised in PE buffer (10 mM potassium phosphate, pH 6.8, and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane-sulfonate (CHAPS). The homogenates were centrifuged for 5 min at 30000 *g* at 4°C. The supernatant was subjected to the total protein assay as described in Section 2.1.7.2. A known amount of protein (70 μg/ml) was subjected to a one

dimensional SDS-PAGE immunoblotting procedure as described in Section 2.1.7.3. Proteins were precipitated by a chloroform/methanol technique. The pellet was re-suspended in 10 µl of sample (Laemmli) buffer (see Section 2.2.7.7.1 for composition) and boiled for 10 min. Molecular weight markers (MWM) were diluted (1:5) with Laemmli buffer and boiled for 10 min. Samples and MWM were allowed to cool and then loaded directly onto a 10%-15% SDS gels *via* a sample applicator 8/1 as follows. Lanes 1, 3, 5 and 7 were loaded with 1 µl of Molecular Weight Markers (MWM), lanes 2 and 4 with 1 µl of day 6 protein samples, and lanes 6 and 8 with 1 µl of day 17 protein samples. Gels were then run on a Pharmacia PhastSystem (separation & control unit) as described in Section 2.1.7.3.

(b) Western Blotting:

Western blotting was as described in Section 2.1.7.3. Briefly, "Transfer Membrane" of equal size to a SDS gel was soaked in methanol for 30 s, washing with MQ water, followed by soaking in Transfer Buffer (see Section 2.2.7.7.2 for composition) at pH 8.2 until use. After the gels were run, they were then transferred on to a "Transfer Membrane" and run on a Pharmacia PhastSystem (separation & control unit) as described in Section 2.1.7.3. The blotting took 15 min after which it was removed from the machine. The blotting paper was washed with MQ water 5 times, placed in 10 ml of 5% BSA in Tris Buffer Saline (TBS) (see Section 2.2.7.7.3 for composition) solution at pH 7.6, and was shaken gently for 3 h. The 5% BSA was acting as the blocking agent in order to reduce the background by blocking the sites on the Transfer Membrane. After 3 h, this

solution was discarded and replaced with primary antibody (PGHS-2) (1:10, PGHS-2:MQ water). The "Transfer Membrane" was incubated with primary antibody at 4°C overnight on a shaking plate. The following morning, the primary antibody was discarded. The "Transfer Membrane" was washed 5 times with 3 ml of TBS containing 1% Tween-20 (TTBS; 1% BSA & 0.1% Tween-20, diluted 1:10 with MQ water) for 20 s, and then three times with 10 ml TTBS for 5 min each time. The TTBS solution was discarded and the "Transfer Membrane" was incubated with conjugated secondary antibody (bionated peroxidase labelled anti-rabbit antibody) (5 µl of secondary antibody in 5 ml of TTBS + 2 drops of solution A & 2 drops of solution B from Elite ABC Kit Vectastain) for 90 min. The "Transfer Membrane" was then washed 3 times with 10 ml TTBS for 5 min each time, and incubated with peroxidase substrate kit (DAB Kit) for 10 min until colour was developed. The "Transfer Membrane" was then washed under running tap water five times and allowed to dry at room temperature.

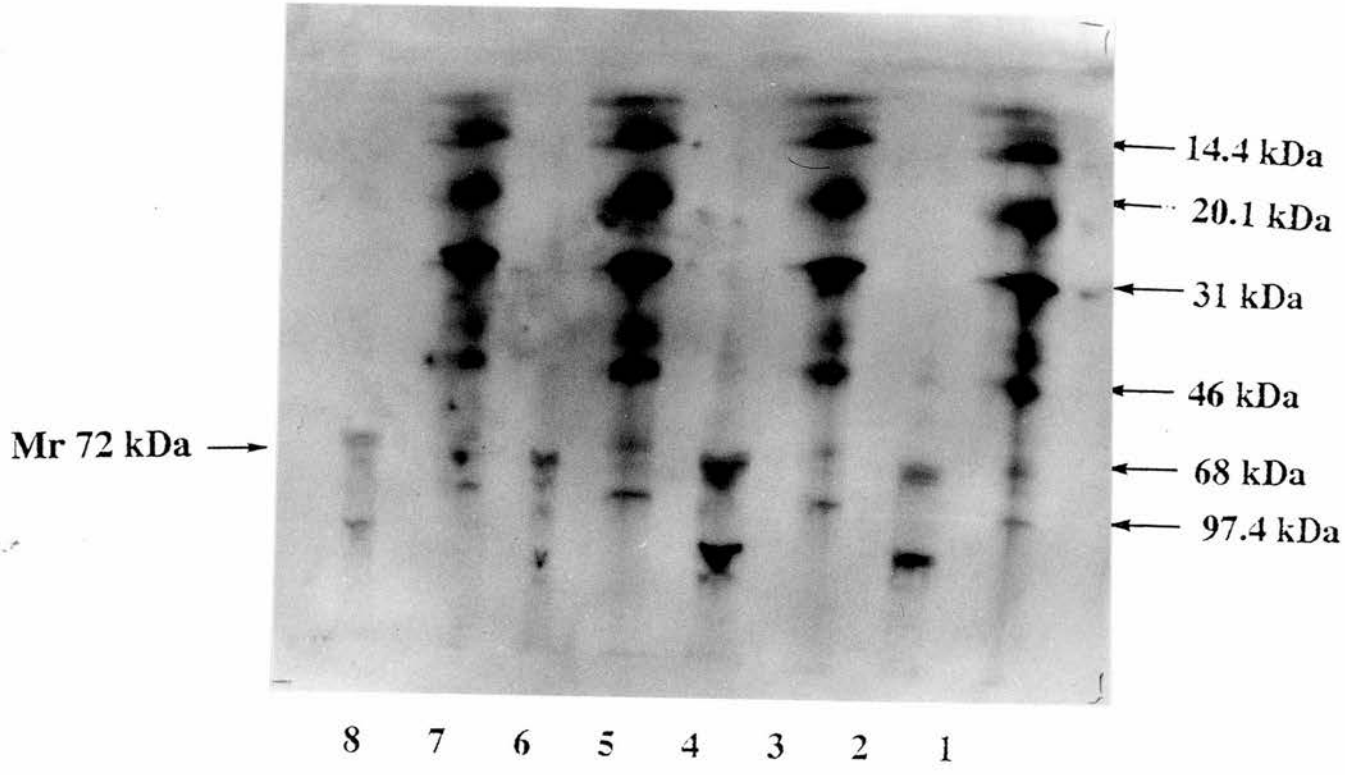
Results:

Western blotting analyses show that PGHS-2 (Mr 72 kDa) was present in the soluble extracts of guinea-pig endometrium obtained on both days 6 and 17 of the oestrous cycle (Figure 3.5.1).

Discussion:

Interleukin-1α (IL-1α) induces the synthesis of PGHS-2, as well as the synthesis and release of $PGF_{2\alpha}$ in response to stimulation by exogenous arachidonic acid in

Figure 3.5.1. Immunodetection of PGHS-2 in day 6 and day 17 guinea-pig endometrium. Soluble cell extract proteins (7 μg) were resolved by one-dimentional SDS-PAGE and electrophoretically transferred to nitro-cellulose filters. Prostaglandin H synthase-2 was detected by a polyclonal rabbit IgG antibody raised against murine PGHS-2 (Cascade Biochem Ltd, Berkshire, England). Molecular Weight Marker (lanes 1, 3, 5 and 7), Day 6 endometrium (lanes 2 & 4) and Day 17 endometrium (lanes 6 & 8).



human cell line (ECV304) (Risimaki *et al.*, 1994). However, IL-1α did not increase PGHS-1 mRNA. In rat alveolar macrophages, lipopolysaccharide (LPS) induced selective expression of the PGHS-2 without affecting PGHS-1 synthesis (Lee *et al.*, 1992). Dexamethasone inhibits PGHS-2 mRNA expression and PG output (Masferrer *et al.*, 1994), indicating that PG output is associated with increased in PGHS-2 concentration.

The presence of PGHS-2 in the soluble extracts of guinea-pig endometrium obtained from day 6 (a day of low PG output) and day 17 (a day of high PG output) indicates that increased output of $PGF_{2\alpha}$ from guinea-pig uterus at the end of the oestrous cycle dose not depend upon the "inducible" form of PGHS (i.e. PGHS-2) being synthesised specifically at the end of the oestrous cycle.

Protein inhibitors, such as actinomycin D, cycloheximide and puromycin, inhibit PG output from the guinea-pig endometrium in culture (Riley & Poyser, 1989) and from cultured guinea-pig endometrial cells (see Section 3.3.2). However, it has been reported that IL-1α-stimulated PGHS-2 mRNA expression is potentiated by cycloheximide, pactamycin and puromycin, although it is inhibited by actinomycin D (Risimaki *et al.*, 1994). These finding suggests that either (a) inhibition of PG output from guinea-pig uterus by protein inhibitors is not due to inhibition of PGHS-2 synthesis, (b) the PGHS-2 present in the guinea-pig uterus is different from the one reported by Risimaki *et al.* (1994) in human cell line (ECV304) or (c) cycloheximide and puromycin having non-specific effect on human cell line (ECV304) in the presence of interleukin-1α.

It has been reported that PGHS-2, but not PGHS-1, level and activity in cotyledons of sheep increases during late gestation, indicating that PGHS-2, rather than PGHS-1, is associated with the increased PG synthesis in this tissue (Wimsatt *et al.*, 1993). However, Salamonsen and Findlay (1990) reported that, in the sheep, conceptus-induced changes in $PGF_{2\alpha}$ output do not occur via changes in the concentration or cellular localisation of PG synthase, but rather the activity of the enzyme is modified. A similar hypothesis has also been stated by Keirse *et al.* (1977) who reported that, in human, the conceptus-induced changes in $PGF_{2\alpha}$ release do not occur via changes in the concentration or cellular localisation of PGHS, but rather the activity of the enzyme is modified. This may also be the case in the guinea-pig uterus, such that, oestradiol acting on a progesterone-primed uterus modifies the activity of the PGHS-2 present in the uterus (by controlling arachidonic acid release) as well as stimulating further PGHS-2 synthesis.

SECTION FOUR

4.1 GENERAL DISCUSSION

The basal output of $PGF_{2\alpha}$ was approximately 14-fold higher from day 15 guinea-pig uterus than from day 7 guinea-pig uterus superfused *in vitro* (see Section 3.1.1). It is well-documented that, in the guinea-pig uterus, the endometrium is the major site of production of the uterine luteolytic hormone, i.e. $PGF_{2\alpha}$, (Poyser, 1983a). When guinea-pig endometrium was cultured, the basal $PGF_{2\alpha}$ output was approximately 20-fold higher on day 15 guinea-pig than on day 7 (see Section 3.1.3). There was little or no difference between day 7 and day 15 in the basal outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from tissues obtained from the guinea-pig uterus (see Sections 3.1.1 & 3.1.3).

The basal outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 7 guinea-pig uterus superfused *in vitro* were similar. However, the output of $PGF_{2\alpha}$ was up to 20-fold higher than the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 15 guinea-pig uterus superfused *in vitro* (see Section 3.1.1). The same pattern of PG output from the day 15 uterus was also seen in culture. The basal amount of $PGF_{2\alpha}$ produced from day 15 guinea-pig endometrium in culture was up to 14-fold higher than the amounts of PGE_2 and 6-keto-$PGF_{1\alpha}$ produced (see Section 3.1.3). When endometrium was homogenized and incubated, $PGF_{2\alpha}$ was the major PG synthesized by both day 7 and day 15 guinea-pig endometrium. Between days 7 and 15, $PGF_{2\alpha}$ production significantly increased 3-fold, but PGE_2 and 6-keto-

$PGF_{1\alpha}$ production showed no changes (see Section 3.4.1). These findings are in agreement with previous reports (Poyser, 1979; Leaver & Seawright, 1982; Poyser, 1983a, b; Poyser & Brydon, 1983; Riley & Poyser, 1987a; Leckie & Poyser, 1990a, b), and reflect the higher $PGF_{2\alpha}$ concentrations observed in guinea-pig uterine venous plasma that occur towards the end of the oestrous cycle (Blatchley *et al.*, 1972).

It was mentioned earlier that the endometrium is the major site of $PGF_{2\alpha}$ synthesis; however, the endometrium consists of two major cell types which produce prostaglandin, namely epithelial and stromal cells (Schatz & Gurpide, 1983; Schatz *et al.*, 1985; Fortier *et al.*, 1988; Watson *et al.*, 1992). There are conflicting reports as to which of the above cell types (i.e. epithelial and stromal cells) is the main site for prostaglandin production. Gal *et al.* (1982) have reported that, in human, endometrial stromal cells are the major cell type producing prostaglandin. PGE_2 was the major PG produced, followed by $PGF_{2\alpha}$ (Gal *et al.*, 1982). This report was confirmed by Casey *et al.* (1985). However, other studies indicate that epithelial cells produce more $PGF_{2\alpha}$ than stromal cells (Schatz *et al.* 1985; Smith & Kelly, 1988). Smith and Kelly (1988) reported that $PGF_{2\alpha}$ was the major PG produced by both epithelial and stromal cells followed by PGE_2.

Isolated endometrial epithelial cells from ewes produced more $PGF_{2\alpha}$ than the stromal cells, whereas PGE_2 output from the stromal cells was greater than from the epithelial cells (Cherny & Findlay, 1990; Kim & Fortier, 1995). A similar result was reported regarding prostaglandin production by endometrial cells of the cow. It was reported that, in the cow, $PGF_{2\alpha}$ output from epithelial cells is about

13-fold higher than from stromal cells, while PGE_2 output from stromal cells is about 3-fold greater than from epithelial cells (Fortier *et al.*, 1988). In the mare, however, it was reported that there was no significant difference in PGF production between epithelial and stromal cells at 2 and 8 h, but, by 24 h of culture, epithelial cells produced significantly more PGF than stromal cells (Watson *et al.*, 1992).

In the studies presented in this thesis, guinea-pig endometrial epithelial cells produced significantly more prostaglandin than stromal cells (see Sections 3.1.4 to 3.1.6). Even after 2 h of culture, the basal outputs of $PGF_{2\alpha}$, PGE_2, and 6-keto-$PGF_{1\alpha}$ were 200-, 170-, and 150-fold greater from epithelial cells than from stromal cells obtained from day 7 guinea-pig endometrium. Similarly, the basal outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ were 800-, 330- and 310-fold greater from epithelial cells than from stromal cells obtained from day 15 guinea-pig endometrium. These findings clearly indicates that, in the guinea-pig, epithelial cells are the major site of prostaglandin production by the endometrium. This suggests that, with regard to prostaglandin production, guinea-pig endometrial cells behave similar to human endometrial cells as it has been shown that epithelial cells are the major prostaglandin producing cells in the human endometrium (Schatz & Gurpide, 1983; Schatz *et al.* 1985; Smith & Kelly, 1988). Although there were no significant differences among the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial cells obtained from day 7 guinea-pig endometrium, stromal cells produced significantly more PGE_2 than $PGF_{2\alpha}$.

The basal output of $PGF_{2\alpha}$ from epithelial cells obtained from day 15 guinea-pig endometrium was significantly higher (4-fold) than from epithelial cells obtained from day 7 endometrium (see Section 3.1.4). There were no significant increases in the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial cells, and in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from stromal cells between days 7 and 15 guinea-pig endometrium. This indicates that, in the guinea-pig, the increase in uterine $PGF_{2\alpha}$ output seen on day 15 (i.e. at the end of the oestrous cycle) is due to an increase in $PGF_{2\alpha}$ synthesis by epithelial cells and not by stromal cells.

Caffeine significantly increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 and day 15 guinea-pig uterus superfused *in vitro*. Theophylline also significantly increased the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but not PGE_2 output from the day 7 guinea-pig uterus superfused *in vitro*. Caffeine and theophylline are phosphodiesterase (PDE) inhibitors. In many tissues their pharmacological effects are believed to be due to their inhibitory effects on the activity of PDE (see Daly 1993) which increases cAMP concentrations. In the guinea-pig, it has been reported that an increase in cAMP level does not lead to an increase in PG output from the endometrium (Poyser, 1987a). In the present study, in general, forskolin (which increases cAMP levels) did not increase the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 7 guinea-pig endometrium tissue in culture or from cultured epithelial (with the exception of $PGF_{2\alpha}$ after 8 h of culture) and stromal cells. These results indicates that the caffeine- and theophylline-induced rise in guinea-pig uterine PG output are probably not mediated via an inhibition of PDE activity.

The caffeine-induced increase in prostaglandin output from the guinea-pig uterus superfused *in vitro* was not affected by calmodulin inhibitors (W-7 & TFP) indicating that the caffeine effect is not mediated by calmodulin. In fact W-7 greatly potentiated the stimulatory effect of caffeine on $PGF_{2\alpha}$ output and, to a lesser extent, on PGE_2 output. Moreover, the caffeine-induced increase in prostaglandin production by the guinea-pig uterus superfused *in vitro* was not affected by the lack of extracellular calcium, suggesting that effect of caffeine does not depend on the presence of extracellular calcium. Since calcium is necessary for PG production by the guinea-pig uterus (Riley & Poyser, 1987b), it is possible that caffeine exerts its stimulatory effect on PG synthesis by releasing calcium from an internal calcium store. It is well documented that caffeine and theophylline release calcium from an intracellular calcium pool. Caffeine causes intracellular calcium release from vascular endothelial cells, skinned myocardial fibres of rat, isolated snail neurones and bovine adrenal chromaffin cells by acting on ryanodine receptors (RYR) type 1 and 2 (RYR-1 & RYR-2) (Zhang *et al.*, 1993; Su & Shang, 1993; Kostyuk & Kirischuk, 1993; Cheek *et al.*, 1993). This effect of caffeine is blocked by ruthenium red and high concentrations of RY (see Sorrentino & Volpe, 1993). TMB-8 (an intracellular calcium antagonist) completely abolished the caffeine-induced increase in the output of $PGF_{2\alpha}$, but had no effect on the caffeine-induced increases in outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$. This finding suggests that, in the guinea-pig, the stimulation of uterine $PGF_{2\alpha}$ synthesis and release by caffeine is dependent on intracellular calcium. The stimulation of guinea-pig uterine PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis and release by caffeine is

either not dependent upon intracellular calcium, or is dependent on an intracellular calcium store which is not affected by TMB-8. Ryanodine and ruthenium red did not affect the stimulatory action of caffeine on prostaglandin output from the guinea-pig uterus superfused *in vitro*. Ryanodine alone or in combination with caffeine had no effect on the outputs of prostaglandin from the day 7 guinea-pig uterus superfused *in vitro*, from the day 7 guinea-pig endometrium in culture, and from the day 7 and day 15 guinea-pig endometrial cells in culture. These results indicate that the caffeine-induced increases in the outputs of prostaglandins from uterine tissue are not mediated by the activation of RYR-1 or RYR-2. Another RYR type (RYR-3) has recently been identified in mink lung which binds RY but it is not activated by caffeine. Hence, it is unlikely that the stimulatory effect of caffeine on uterine PG production is mediated by the activation of RYR-3. It has been reported that rat liver hepatocytes contain an intracellular calcium pool which is not sensitive to RY or theophylline but is activated by caffeine (McNulty & Taylor, 1993). Thus, it is possible that the caffeine-sensitive calcium store present in hepatocytes is also present in the guinea-pig uterus, and is involved in the caffeine-induced stimulation of uterine PG output. However, theophylline also induced increases in PG output from the guinea-pig uterus, an effect which was not seen in hepatocytes (McNulty & Taylor, 1993). These findings suggest that the mechanism by which theophylline induces PG output from the uterus may be different from that of caffeine or, if caffeine and theophylline are acting through the same mechanism, then the caffeine effects on the uterus are different from the caffeine effect on hepatocytes.

Donoso *et al.* (1994) have investigated the effects of methylxanthines on changes in intracellular calcium concentrations in rat ventricular myocytes. Caffeine (10 mM) and theophylline (10 mM) produced a transient rise of $[Ca^{2+}]_i$ in rat cardiac myocytes (Donoso *et al.*, 1994). In the studies presented here, both caffeine and theophylline stimulated prostaglandin production from the day 7 guinea-pig uterus superfused *in vitro*. Thus, if caffeine and theophylline stimulate uterine PG output via a similar mechanism, and if these stimulatory effects of caffeine and theophylline involve the mobilization of intracellular calcium, then the effects of caffeine on the uterus and on the cardiac myocytes are possibly via a similar mechanism.

When changes in the free cytosolic calcium concentration in epithelial and stromal cells obtained from day 7 and day 15 guinea-pig endometrium were measured, caffeine or theophylline failed to increase the intracellular free calcium concentration. In fact, caffeine and, to a lesser extent, theophylline reduced the free cytosolic calcium concentrations in both day 7 and day 15 endometrial cells. These results may suggest that either the stimulatory effects of caffeine and theophylline on guinea-pig uterine prostaglandin synthesis and release are not mediated by an increase in cytosolic free calcium concentration, or it is possible that caffeine and theophylline release calcium from an internal calcium pool in the short term (i.e. the time taken to perform uterine superfusion between 1-2 h) but in the longer period of time required to prepare isolated cells, the effects of caffeine are reversed due to this longer time factor or to some other factors such as the processes involved in cell separation. But how do caffeine and theophylline

decrease the intracellular free calcium concentrations in epithelial and stromal cells? In general, calcium is moved between four different pools: extracellular milieu, cytoplasm, mitochondria, and a non-mitochondrial pool (endoplasmic (ER) and sarcoplasmic reticulum (SR)). A cell-specific, subcompartmentalization of these pools is likely to occur. The calcium transport system is composed of a variety of cytosolic and membrane bound proteins that bind calcium with high affinity. The most important of the former group are calmodulin and troponin C which, in addition to contributing to the buffering of cytosolic calcium, also function as cofactors in different calcium-dependent processes (see Thastrup, 1990). The true transport of calcium is carried out by membrane proteins such as channels (receptor or voltage operated), pumps (Ca^{2+}/ATPases), exchangers, and electrophoretic uniports. A decrease in intracellular free calcium concentration can be achieved by the appropriate interference with any of the above mentioned calcium-transport systems, as well as by chelating the free calcium (as is seen with EGTA or EDTA).

It has been reported that caffeine at concentrations ranging from 1 mM to 10 mM does not increase the intracellular free calcium concentration in Hela cells (Diarra & Sauve, 1992) which is similar to the results obtained in this study. In fact, Diarra and Sauve (1992) have postulated that caffeine causes a refilling of the histamine-related calcium pools in Hela cells and thereby lowering the intracellular free calcium concentration. This hypothesis was based on the observation that a calcium release in response to a second application of the agonist (histamine) was always measured in cells which were exposed to caffeine during the first

application of the agonist, but not in control condition (i.e. cells which were not exposed to caffeine during the first histamine application). It is possible that caffeine may have a similar effect in guinea-pig endometrial cells (i.e. induce calcium sequestration into non-mitochondrial calcium pools). It is also possible that caffeine and theophylline decrease the intracellular free calcium concentration by increasing calcium uptake by mitochondria. In order to achieve reduction of free cytosolic calcium levels, caffeine should (a) activate ER Ca^{2+}/ATPase, (b) inhibit mitochondrial Na^{+}/Ca^{2+} exchange, and/or (c) activate mitochondrial calcium uniport. Alternatively, caffeine could reduce the intracellular free calcium concentration by acting on Ca^{2+}/ATPase or the Na^{+}/Ca^{2+} exchange in the plasmalemma membrane resulting in a greater pumping of calcium to the extracellular matrix. One other possibility is that caffeine actually chelates the free intracellular calcium thus reducing the cytosolic free calcium concentration. The last effect is possible, since caffeine exerted its effect within 1 second and the effect was not reversed over the next 20 min. A similar effect was also seen by the addition of 10 mM EGTA. There have been no previous reports which have shown that caffeine actually decreases the basal concentration of the free calcium in the cytosol of any intact cell type. The report by Diarra and Sauve (1992) in which caffeine lowered the intracellular free calcium concentration examined the effect of caffeine on stimulated cells where the intracellular free calcium concentrations were already elevated up to 600 nM. In the present study the basal free cytosolic calcium concentrations in epithelial and stromal cells were between 70 to 120 nM. How caffeine lowered the intracellular calcium concentration below the basal levels

in epithelial and stromal cells from guinea-pig endometrium is not clear, and further study is required to examine the possibilities stated above.

Caffeine and theophylline increased the outputs of prostaglandins from guinea-pig endometrium tissue in culture as well as from the guinea-pig uterus superfused *in vitro*. However, both caffeine and theophylline despite increasing the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured epithelial and stromal cells, failed to increase $PGF_{2\alpha}$ output from cultured epithelial and stromal cells. In fact, both caffeine and theophylline inhibited $PGF_{2\alpha}$ output from epithelial and stromal cells in culture. TMB-8 alone or in combination with caffeine inhibited the output of $PGF_{2\alpha}$ from epithelial cells, indicating the involvement of intracellular calcium in $PGF_{2\alpha}$ synthesis by guinea-pig endometrial cells in culture. A similar effect on $PGF_{2\alpha}$ synthesis by cultured endometrial cells was also seen by berberine, which is thought to inhibit calcium release from the sarcoplasmic (endoplasmic) reticulum (Chiou *et al.*, 1991). Berberine also inhibited $PGF_{2\alpha}$ output from epithelial cells, indicating that $PGF_{2\alpha}$ output from epithelial cells may depend upon the release of intracellular calcium. This was further highlighted by the action of thapsigargin. It has been shown that thapsigargin release calcium from an internal pool (Herchuelz & Leburn, 1993, Wegner, *et al.*, 1994). In the present study, thapsigargin stimulated the output of $PGF_{2\alpha}$ from epithelial but not from stromal cells after 2 h of culture. Thus, it is possible that within the first 2 h of culture, thapsigargin induced the release of calcium from an internal store and, as a result, increased the output of $PGF_{2\alpha}$ from epithelial cells. However, after 8 and 24 h of culture, thapsigargin inhibited $PGF_{2\alpha}$ output from epithelial cells. The latter action of

thapsigargin may be due to its ability to inhibit the endoplasmic reticulum Ca^{2+}/ATPase (see Thastrup, 1990; Chen & van Breemen, 1993; Tanaka & Tashjian, 1993) and the subsequent depletion of calcium from pools in the endoplasmic reticulum which may, in turn, result in the lack of sufficient intracellular free calcium to maintain basal $PGF_{2\alpha}$ output. Thapsigargin did not prevent the stimulatory effect of caffeine on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from stromal cells after 2 h of culture indicating that, if the stimulatory effects of caffeine on PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis from stromal cells is dependent upon the release of intracellular calcium, this calcium pool is not thapsigargin sensitive. However, caffeine prevented the stimulatory effect of thapsigargin on $PGF_{2\alpha}$ output from epithelial cells after 2 h of culture, suggesting that any thapsigargin-induced increase in intracellular calcium is either sequestered (Diarra & Sauve, 1992) or chelated by caffeine.

Although caffeine and theophylline decrease $PGF_{2\alpha}$ output from isolated epithelial cells, the question is raised as to why caffeine and theophylline did not inhibit $PGF_{2\alpha}$ output from guinea-pig endometrium in culture? One possible explanation is that, to see a true effect of methylxanthines on the guinea-pig uterine PG output, it is necessary for the epithelial and stromal cells to be in direct contact with each other. The interaction between stromal and epithelial cells has been shown in the human endometrial cycle (Roberts *et al.*, 1988). Roberts *et al.* (1988) have reported four significant changes in human endometrium in the transition from the early proliferative phase (days 5 to 9) to the early secretory phases (days 15 to 19). These changes include: (1) an increase in the number and size of lamina densa

distribution, (2) an increase in the number and size of gap junctions, (3) an increase in the number of epithelial cells, and (4) an increase in close contacts between stromal and epithelial cells (Roberts *et al.*, 1988). If close contacts between guinea-pig epithelial and stromal cells are vital with regard to PG output due to the effects of stimulus, then it is possible that methylxanthines would have different effects on endometrial tissue and endometrial cells in culture.

Caffeine has also ability to interfere with protein synthesis. In the rat striatum, caffeine induces neurotensin and cholecystokinin mRNA synthesis (Schiffmann & Vanderhaeghen, 1993). However, in rat brain caffeine decreases glial cell numbers (Marret *et al.*, 1993). Hence, it is possible that, in the long term, caffeine inhibits $PGF_{2\alpha}$ output by inhibiting protein synthesis from the guinea-pig endometrial cells in culture, as protein synthesis inhibitor (actinomycin D, cycloheximide and puromycin) inhibited PG output from both epithelial and stromal cells in culture. However, caffeine did not inhibit $PGF_{2\alpha}$ output from day 7 guinea-pig endometrium in culture. In fact, caffeine stimulated $PGF_{2\alpha}$ output after 8 and 24 h of culture. It has been reported previously that actinomycin D, cycloheximide and puromycin inhibit the outputs of prostaglandin from guinea-pig endometrium in culture (Poyser & Riley, 1987; Riley & Poyser, 1989). Thus it appears unlikely that caffeine is inhibiting PG output in cultured epithelial and stromal cells by inhibiting protein synthesis. The inhibitory effects of actinomycin D, cycloheximide and puromycin on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from cultured epithelial and stromal cells obtained from day 7 guinea-pig endometrium is in agreement with previous reports which have shown that these

compounds inhibited prostaglandin production from the guinea-pig endometrium in culture by inhibiting protein synthesis (Poyser & Riley, 1987; Riley & Poyser, 1989). Thus the synthesis of prostaglandins, in particular $PGF_{2\alpha}$, from guinea-pig epithelial cells, is dependent upon the synthesis of fresh protein(s).

It has been shown that caffeine and ryanodine (RY) release calcium from cardiac muscle and from vascular smooth muscle (Komori & Bolton, 1989; Hisayama *et al.*, 1990; Donoso *et al.*, 1994). Caffeine and ryanodine both stimulated the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the rat mesenteric vascular bed. The PG-releasing effect of caffeine was similar to its effect on the guinea-pig uterus. However, the ryanodine effect was in contrast to its ineffectiveness on guinea-pig uterus. These findings suggest that, if caffeine and RY are stimulating prostaglandin production by the same mechanism in blood vessels, then caffeine is probably stimulating prostaglandin synthesis in the uterus by a different mechanism.

It has been reported that calmodulin inhibitors, in particular TFP, cause a transient rise in $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from guinea-pig uterus superfused *in vitro* (Poyser, 1985a). However, they inhibited A23187-induced increase in PG output from the guinea-pig uterus superfused *in vitro* (Poyser, 1985a, b). Furthermore, W-7 and TFP inhibited $PGF_{2\alpha}$ output from guinea-pig endometrium in culture (Riley & Poyser, 1987b). In the studies presented in this thesis, TFP but not W-7 caused a transient rise in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the guinea-pig uterus superfused *in vitro*. Moreover, both W-7 and TFP decreased the outputs of $PGF_{2\alpha}$ and, to a much lesser extent, 6-keto-$PGF_{1\alpha}$ from both epithelial and

stromal cells in culture. These findings indicate that calmodulin has a role in maintaining the basal outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but not of PGE_2, from the guinea-pig endometrial cells.

The stimulatory effect of A23187 on uterine PG output may be due to it causing a calcium influx through the cell membrane, which in turn, stimulates PLA_2 (Poyser, 1984; Poyser, 1987b). Bonney *et al.* (1987) have shown that there are two types of PLA_2 in human endometrium; PLA_2 type 1 is mainly present in epithelial cells and PLA_2 type 2 is mainly present in stromal cells. PLA_2 type 1 is calcium dependent whereas PLA_2 type 2 is calcium-independent (Bonney *et al.*, 1987). Consequently A23187 time-dependently stimulated arachidonic acid release from human epithelial but not from stromal cells in culture (Bonney *et al.*, 1991). However, in the present study, during 2 h of culture A23187 stimulated PG output by both epithelial and stromal cells obtained from day 7 guinea-pig endometrium indicating that the PLA_2 present in guinea-pig endometrial epithelial and stromal cells is similar to and may be the counterpart of human PLA_2 type 1. Although, A23187 showed a stimulatory effect during the first 2 h of culture, this stimulation was lost by 8 h, and A23187 inhibited PG output after 24 h of culture from both epithelial and stromal cells. It has been reported that the guinea-pig uterus superfused *in vitro* exhibits a partial refractoriness to repeated stimulation by A23187 (Poyser, 1991; Poyser & Ferguson, 1993). Thus this lack of stimulatory effect of A23187 on $PGF_{2\alpha}$ output from cultured endometrial cells after 8 h of culture may be due to this refractoriness effect. However, the phenomenon of refractoriness does not explain the inhibitory effects of A23187 on the output of prostaglandins after 24 h

of culture. How prolonged treatment with A23187 inhibits PG output merits further study.

Melittin has been shown to stimulate prostaglandin production by the guinea-pig uterus superfused *in vitro* (Johnson & Poyser, 1991) and to stimulate PLA_2 in mouse 3T3-4a fibroblasts (Shier, 1979). The effects of melittin on mouse 3T3-4a fibroblasts and on the guinea-pig uterus have been shown to be dependent upon the presence of calcium. The stimulatory effect of melittin on PG output from the guinea-pig uterus was partially inhibited by the lack of extracellular calcium, but was completely inhibited by TMB-8 (Johnson and Poyser, 1991). In mouse 3T3-4a fibroblasts, stimulation of PLA_2 by melittin was also dependent upon calcium, as the addition of EDTA to the culture medium prevented the rise in PG production by melittin (Shier, 1979). Johnson and Poyser (1991) have reported that melittin stimulates the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but not of PGE_2, from the day 7 guinea-pig uterus superfused *in vitro*. Melittin stimulated $PGF_{2\alpha}$ output, and to a much lesser extent, the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from epithelial but not from stromal cells. On the other hand, exogenous PLA_2 mainly increased the output of PGE_2, and to a lesser extent, the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from both epithelial and stromal cells in culture. These results suggest that there is a preferential stimulation of PGE_2 output by exogenous PLA_2, while melittin (a stimulator of endogenous PLA_2) stimulates mainly $PGF_{2\alpha}$ production. The activity of PLA_2 is the rate-limiting step in PG synthesis. It has been reported that formation of specific prostaglandin is influenced by substrate concentration. Flower *et al.* (1973) have shown that, in bovine seminal vesicles, PGE_2 formation was at

the maximum at a relatively low concentration of arachidonic acid. High concentration of arachidonic acid inhibited PGE_2 formation and stimulated $PGF_{2\alpha}$ formation to a maximal level (Flower *et al.*, 1973). Thus, it is possible that exogenous PLA_2 and melittin exert their effects on uterine PG output due to different levels of availability of arachidonic acid. The finding that melittin was ineffective in increasing PG output from stromal cells indicates that melittin has not stimulated the endogenous PLA_2 present in guinea-pig stromal cells suggesting that the PLA_2 present in stromal cells, which is possibly activated by A23187, is unresponsive to melittin. Interestingly, aristolochic acid (a PLA_2 inhibitor) inhibited only the output of $PGF_{2\alpha}$ from epithelial, but not from stromal cells, suggesting that the mechanism by which melittin and aristolochic acid modulate $PGF_{2\alpha}$ output from guinea-pig uterus is similar, and this mechanism is different from the mechanism by which exogenous PLA_2 stimulates PG outputs from the guinea-pig uterus. Also, the result with aristolochic acid indicates that $PGF_{2\alpha}$ synthesis in guinea-pig epithelial cells is dependent upon the activation of PLA_2 in these cells.

Previous studies have shown that PLA_2 is a calcium-dependent enzyme (Dey *et al.*, 1982; Downing & Poyser, 1983; Bonney, 1985). It has been shown that exogenous PLA_2 induces prostaglandin synthesis by and release form the guinea-pig uterus superfused *in vitro* in the presence (Poyser, 1987a; Poyser, 1991; Poyser & Ferguson, 1993) or absence (Scott, M., Scotland, R.A. & Poyser, N.L., unpublished observations) of extracellular calcium. Thus it seems that exogenous PLA_2 exerts its stimulatory effect on PG synthesis, at least in part, by releasing

intracellular calcium especially as TMB-8 inhibits the action of PLA_2 (Poyser, 1987a). Intracellular calcium measurements carried out in this thesis support this hypothesis. Exogenous PLA_2 dose-dependently increased the intracellular free calcium concentrations of guinea-pig epithelial and stromal cells. Platelet activating factor (PAF) has also been shown to stimulate PG output from guinea-pig uterus superfused *in vitro*, in the presence or absence of extracellular calcium (Norman & Poyser, 1992). PAF has also been shown to stimulate PGE_2 synthesis (Smith & Kelly, 1988) and calcium release from human endometrial cells in the presence or absence of extracellular calcium (Ahmed & Smith, 1992; Ahmed *et al.*, 1994). In the study presented in this thesis, PAF also dose-dependently increased intracellular calcium concentrations of epithelial and stromal cells in the presence or absence of extracellular calcium. In human endometrium, PAF stimulates PLC and thereby the formation of IP_3 and DAG (Barzaghi *et al.*, 1989; Ahmed & Smith, 1992; Ahmed *et al.*, 1994) and this generated IP_3 releases intracellular calcium from an IP_3-sensitive calcium store (Berridge, 1984; see Berridge & Irvine, 1989; see Berridge, 1993). If the effect of PAF on human and guinea-pig endometrial cells is via a similar mechanism, it is possible that PAF stimulates PG output from the guinea-pig uterus by stimulating IP_3 formation. This IP_3 then releases calcium from an IP_3-sensitive calcium store, resulting in the activation of PLA_2 and the subsequent synthesis of prostaglandin. However, it is not known whether the increase in intracellular free calcium released by PAF in guinea-pig endometrial cells is due to an increase in the formation of IP_3.

In the guinea-pig, oestradiol acting on a progesterone-primed uterus is the physiological stimulus for the increase in the output of $PGF_{2\alpha}$ at the end of the oestrous cycle (Poyser, 1983a, b). In the present study, oestradiol-17β and progesterone failed to increase prostaglandin outputs from cultured endometrial cells obtained from the day 7 guinea-pig uterus. In fact, oestradiol and progesterone inhibited the outputs of $PGF_{2\alpha}$ from epithelial cells and of PGE_2 from stromal cells. These inhibitory effects of oestradiol and progesterone are in agreement with previous studies regarding the effects of these two steroid or hormones on PG output from guinea-pig endometrium in culture (Riley & Poyser, 1987a). The finding that progesterone suppressed PG output from cultured guinea-pig endometrial cells in this study is in agreement with similar studies on human endometrial cells (Zhang & Davis, 1991; Mitchell & Smith, 1992). In the human, oestradiol stimulates $PGF_{2\alpha}$ output from endometrium *in vitro* (Abel & Baird, 1980; Schatz *et al.*, 1985). Thus, it seems that the effect of oestradiol *in vitro* differs among species, having an inhibitory effect on the output of $PGF_{2\alpha}$ from guinea-pig uterus but stimulating $PGF_{2\alpha}$ output from human endometrium (Abel & Baird, 1980; Schatz *et al.*, 1985). However, progesterone clearly exerts an inhibitory effect on endometrial cells with respect to PG output in several species. The inhibitory effect of progesterone on prostaglandin output from cultured guinea-pig endometrial cells may be due to its ability to inhibit arachidonic acid release, since it has been shown that progesterone inhibits arachidonic acid release from human endometrial epithelial cells (Wilson *et al.*, 1986).

It is well documented that $PGF_{2\alpha}$ output from the guinea-pig uterus increases towards the end of the oestrous cycle. The synthesis of $PGF_{2\alpha}$ involves the action of prostaglandin H synthase (PGHS) on arachidonic acid. Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), has been shown to inhibit PG synthesis by PGHS (Miyamato *et al.*, 1974; Rome & Land, 1975). In the guinea-pig, the endometrial PGHS concentration increases after day 11 of the cycle (Poyser, 1983a). Recently, two isoforms of PGHS, namely PGHS-1 and PGHS-2, have been identified: PGHS-1 is constitutively expressed in many cells types and tissues, and PGHS-2 is an inducible form (Maier *et al.*, 1990; Wong & Richards, 1991; Wimsatt *et al.*, 1993). In the present study, indomethacin (a non-selective NSAID) inhibited the production of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ by guinea-pig uterine tissues, an effect reported previously on guinea-pig uterus superfused *in vitro* (Poyser, 1985a). Indomethacin also inhibited PG production by endometrium homogenates, cultured endometrium and cultured endometrial cells. A novel PGHS-2 inhibitor (Futaki *et al.*, 1994; Masferrer *et al.*, 1994), NS-398, also inhibited prostaglandin production by day 7 and day 15 guinea-pig endometrial homogenates, by day 7 endometrium and myometrium in culture, and by cultured epithelial and stromal cells obtained from day 7 guinea-pig endometrium. These finding clearly indicate that the guinea-pig uterus (both the endometrium and myometrium) contains PGHS-2 at both stages of the oestrous cycle. This hypothesis was further supported by the finding that Western blotting analyses indicated the presence of PGHS-2 (Mr 72 kDa) (Maier *et al.*, 1990; Fu *et al.*, 1990; Wong & Richards, 1991) in the soluble extracts of guinea-pig endometrium

obtained on both the day 6 (a day of low PG output) and day 17 (a day of high PG output) of the oestrous cycle. Furthermore, NS-398 was as effective as indomethacin in inhibiting the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from guinea-pig uterus, suggesting that in the guinea-pig, PGHS-2 is the predominant PG-forming enzyme. However, it has been shown that the endometrial PGHS concentration increases after day 11 of the cycle (Poyser, 1983a). Thus, these findings indicating that, in the guinea-pig uterus, oestradiol acting on a progesterone-primed uterus stimulates further endometrial PGHS-2 synthesis.

In summary, the studies in this thesis have shown that, in the guinea-pig, epithelial cells in the endometrium are the major cell type responsible for the uterine prostaglandin synthesis. The effects of methylxanthines on $PGF_{2\alpha}$ but not on PGE_2 and 6-keto-$PGF_{1\alpha}$ production by the guinea-pig uterus depends on the experimental protocol. The effects of methylxanthines on PG synthesis by the guinea-pig uterus are not mediated by the activation of any of the known RY receptors. Although it was not possible to demonstrate a calcium releasing effect for methylxanthines, however, such an effect cannot be completely ruled out. The findings also suggest that the release of intracellular calcium as well as the activity of calmodulin are necessary to maintain basal $PGF_{2\alpha}$ output from cultured epithelial cells. It is proposed that the increased output of $PGF_{2\alpha}$ from the guinea-pig uterus at the end of the oestrous cycle is not associated with PGHS-2 solely being synthesized specifically at the end of the oestrous cycle. PGHS-2 is probably present in the endometrium throughout the cycle, and its concentration in the endometrium increases after day 11.

4.2 FUTURE WORK

In order to further elucidate the mechanisms of actions of methylxanthines on prostaglandin production by the guinea-pig uterus, it is necessary to evaluate possible effects of these compounds on protein synthesis by the guinea-pig endometrium, in particular by the endometrial cells. Binding studies using [^{3}H]RY should also be carried out so that the presence or absence of RYR can be determined. With regards to the inhibitory effect of methylxanthines on the free cytosolic calcium concentrations of epithelial and stromal cells, the effects of these compounds on calcium-transport systems should be investigated. This could be done by investigating the effects of caffeine and theophylline on Ca^{+2}/ATPases of endoplasmic reticulum and plasmalemma membrane, on the uniport transport of mitochondria, and on membrane calcium channels. Also, the possible importance of epithelial and stromal cell contact in guinea-pig endometrium in the regulation of endometrial $PGF_{2\alpha}$ synthesis should always be taken into consideration.

Present and previous studies have indicated that the release of intracellular calcium is necessary for increased $PGF_{2\alpha}$ synthesis by the guinea-pig endometrium (Leckie & Poyser, 1991a). Previous study (Ning & Poyser, 1984) have indicated that stimulation of the PI cycle is not involved. The present study has shown that calcium release through the activation of typical ryanodine receptor is probably also not involved. Consequently, future studies will need to investigate what other possible intracellular stores of calcium are involved in endometrial $PGF_{2\alpha}$ synthesis by the epithelial cells.

In order to determine whether changes in PGHS concentration observed at the end of the guinea-pig oestrous cycle, is due to an increase in the contents of PGHS-1 or PGHS-2, the effects of ovarian steroids (i.e. oestradiol and progesterone) should be studied on PGHS (i.e. PGHS-1 and PGHS-2) concentration of guinea-pig endometrium. Thus, it is necessary to develop an antibody specific to PGHS-1. The availability of specific PGHS-1 inhibitors would also help to determine the degree of importance of either PGHS-1 or PGHS-2 with respect to prostaglandin production at the early or late stages of the guinea-pig oestrous cycle.

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1. **Naderali, E.K. & Poyser, N.L.** (1993). Effects of caffeine on prostaglandin production by the guinea-pig uterus. *J. Reprod. Fertil.*, Abstract series Number **11**, P184 (Abstract)

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4. **Naderali, E.K. & Poyser, N.L.** (1994). The effects of caffeine on prostaglandin output from the perfused mesenteric vascular bed of the rat. *Prostaglandins Leukot. Essen. Fatty Acids,* **51**, 415-418.

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The effect of caffeine on prostaglandin output from the guinea-pig uterus

E.K. Naderali & [1]N.L. Poyser

Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ

1 Caffeine increased the outputs of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), PGE_2 and 6-keto-$PGF_{1\alpha}$ from the guinea-pig uterus on days 7 and 15 of the oestrous cycle. The effect on PGE_2 output depended on the age of the animals and was absent in younger guinea-pigs (<4 months). Theophylline also stimulated the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but not the output of PGE_2, from the day 7 guinea-pig uterus.

2 The stimulatory effects of caffeine on the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the guinea-pig uterus were not prevented by lack of extracellular calcium, ryanodine or ruthenium red (both inhibitors of calcium release via the ryanodine receptor), although the increase in $PGF_{2\alpha}$ output tended to be slower when extracellular calcium was absent. Also, ryanodine flattened and broadened the peak of increased $PGF_{2\alpha}$ release.

3 The calmodulin antagonists, W-7 and trifluoperazine, had no inhibitory effect on the caffeine-stimulated increases in uterine prostaglandin output. In fact, W-7 (but not trifluoperazine) greatly potentiated the action of caffeine on uterine $PGF_{2\alpha}$ output, but had little or no potentiating effect on the action of caffeine on uterine PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs.

4 TMB-8, an intracellular calcium antagonist, inhibited the increase in $PGF_{2\alpha}$ output produced by caffeine without preventing the increases in outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$.

5 These studies suggest that caffeine stimulates uterine $PGF_{2\alpha}$ synthesis and release by a mechanism dependent upon intracellular calcium, but this mechanism is not mediated by activation of any of the three well-characterized ryanodine receptors or by calmodulin. Furthermore, the increases in the synthesis and release of PGE_2 and 6-keto-$PGF_{1\alpha}$ in the guinea-pig uterus induced by caffeine appear to involve mechanism(s) different from that which stimulates $PGF_{2\alpha}$ production.

Keywords: Caffeine; prostaglandins; uterus; ryanodine; ruthenium red; TMB-8; trifluoperazine, W-7; theophylline

Introduction

Prostaglandins produced by the uterus are involved in several reproductive processes. In many non-primate mammalian species, increased prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production by the endometrium is responsible for terminating the life-span of the corpus luteum and thereby regulating the length of an oestrous cycle or a pseudopregnancy and, in some species, a pregnancy (see Horton & Poyser, 1976; Poyser, 1981). In women, there is an increase in the amounts of $PGF_{2\alpha}$ and, to a lesser extent, PGE_2 produced by the endometrium at menstruation (see Poyser, 1981). Since prostaglandins contract the uterus and affect the vasculature, it is assumed that they have a role in the menstrual process. An overproduction of $PGF_{2\alpha}$ by the endometrium is the probable cause of dysmenorrhoea (Lundström *et al.*, 1976), whereas an imbalance of uterine prostaglandin production in favour of prostacyclin (PGI_2) may be the cause of menorrhagia (Smith *et al.*, 1981). The reasons for these disorders of menstruation are unknown, but may be due to intrinsic factors, such as the higher than normal production of endogenous hormones which affect uterine prostaglandin synthesis (e.g. vasopressin; Åkerlund *et al.*, 1979), or to extrinsic factors (e.g. dietary substances).

Previous experiments in the rat, guinea-pig and man have shown that increased endometrial prostaglandin synthesis is associated with increased activity of phospholipase A_2, a calcium-dependent enzyme (Dey *et al.*, 1982; Downing & Poyser, 1983; Bonney, 1985). In the guinea-pig, it was considered that the activation of phospholipase A_2 by calcium (Downing & Poyser, 1983) leads to the release of arachidonic acid from phosphatidylcholine and phosphatidylethanolamine (Ning & Poyser, 1984) which, in turn, results in increased $PGF_{2\alpha}$ synthesis by and release from the endometrium. Subsequent studies in the guinea-pig indicated that increased uterine prostaglandin production is dependent upon the release of intracellular calcium (Riley & Poyser, 1987a; Leckie & Poyser, 1990; Johnson & Poyser, 1991). However, there is no increase in inositol turnover in the guinea-pig endometrium at the end of the oestrous cycle when $PGF_{2\alpha}$ synthesis is high (Ning & Poyser, 1984). This finding indicates that the stimulation of endometrial $PGF_{2\alpha}$ synthesis is not dependent on the generation of inositol-1,4,5-trisphosphate (IP_3) and, therefore, does not involve the release of calcium from an IP_3-sensitive store.

Studies on several cell types have indicated that only part of the calcium stored in the endoplasmic reticulum is IP_3-sensitive and there is another store of calcium which can be released by caffeine. This latter pool of calcium was first described in skeletal and cardiac muscle. The release of calcium from this pool is modulated by ryanodine, and the receptors involved are called ryanodine receptors. Initial studies indicated that there were two types of the receptor; Types 1 and 2 are found in skeletal and cardiac muscle, respectively. Ryanodine can cause an initial release of calcium and then blocks the action of caffeine. Ruthenium red inhibits the calcium-releasing action of caffeine at the level of the ryanodine receptor. Ryanodine receptor Types 1 and 2 are not exclusively confined to skeletal and cardiac muscle and are present in other tissues. In addition, it is now clear that there are more than two types of ryanodine receptor (see Sorrentino & Volpe, 1993). Consequently, since caffeine (a dietary substance) releases calcium from various cell types and since uterine prostaglandin production is calcium-dependent, the effect of caffeine on uterine prostaglandin production in the guinea-pig has been investigated. As the initial experiments showed that caffeine does stimulate uterine pro-

[1] Author for correspondence.

staglandin synthesis and release, further experiments were performed to investigate some of the mechanisms by which caffeine may exert this stimulatory effect.

Methods

Procedures

Fifty-two virgin, Dunkin-Hartley guinea-pigs (600–900 g) were examined daily and a vaginal smear was taken when the vagina was open. Day 1 of the cycle was taken as the day preceding the post-ovulatory influx of leucocytes when cornification was at a maximum. All guinea-pigs had exhibited at least two cycles of normal length (about 16–17 days) before being killed (by stunning and incising the neck) on day 7 or day 15 of the cycle. Each uterus was removed and separated into its two uterine horns. The uterine horns were weighed, 'opened' by cutting longitudinally and suspended in an organ bath with one end attached to a lever so that isometric contractions of the uterus were observed. Each uterine horn was superfused with Krebs solution (5 ml min^{-1}) at 37°C, as described by Poyser & Brydon (1983), and was superfused initially for a settling period of 60 min. Samples of superfusate were then collected for 10-min periods over the next 80 min (i.e. 8 samples per uterine horn) in experiments 1, 2 and 8, and over 100 min (i.e. 10 samples per uterine horn) in experiments 3 to 7. The uterine horns in each experiment were treated as described below.

Treatments

In expt 1, caffeine (10 mmol l^{-1}) was present in the Krebs solution superfusing one uterine horn from each of five animals on day 7 of the oestrous cycle and from five animals on day 15 of the cycle during the collection of samples 4 and 5. The other uterine horn from each animal was untreated. In expt 2, one uterine horn from each of five animals on day 7 of the cycle was superfused with normal Krebs solution and the other uterine horn was superfused with Krebs solution from which the calcium chloride had been omitted (calcium-depleted Krebs solution). Caffeine (10 mmol l^{-1}) was present in both types of Krebs solution during the collection of samples 4 and 5.

In experiments 3 to 7, both uterine horns from 4 or 5 guinea-pigs on day 7 of the cycle were superfused with normal Krebs solution, and caffeine (10 mmol l^{-1}) was present in the solution superfusing both uterine horns during the collection of samples 6 and 7. The following additions were made to the Krebs solution superfusing one uterine horn from each animal during the collection of samples 4 to 7: Expt 3: 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8; an intracellular calcium antagonist, Malagodi & Chiou, 1974; 150 μmol l^{-1}; $n = 5$); Expt 4: Ryanodine (2, 20 or 200 μmol l^{-1}; $n = 4$); Expt 5: Ruthenium red (10 or 100 μmol l^{-1}; $n = 4$); Expt 6: N-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide (W-7; a calmodulin antagonist, Hidaka *et al.*, 1978; 150 μmol l^{-1}; $n = 4$). Expt 7: Trifluoperazine (a calmodulin antagonist, Levin & Weiss, 1977; 100 μmol l^{-1}; $n = 4$).

In experiments 6 and 7, the concentrations of W-7 and trifluoperazine used had previously been shown to be sufficient to inhibit the increase in $PGF_{2\alpha}$ output induced by A23187 from the guinea-pig uterus (Poyser, 1985a,b).

In expt 8, both uterine horns from each of four animals on day 7 of the cycle were superfused with normal Krebs solution, and theophylline (10 mmol l^{-1}) was present in the Krebs solution superfusing one uterine horn from each animal during the collection of samples 4 and 5.

Solutions of each compound were freshly made up in Krebs solution before use, except for ryanodine. A concentrated solution of ryanodine was prepared in ethanol and stored at −20°C. The appropriate concentration of ryanodine in Krebs solution was prepared by adding up 0.25 ml of the ethanolic solution to 250 ml Krebs solution. Krebs solution superfusing the control uterine horn during the same time period contained an equivalent concentration of ethanol.

Assays

After collection, the pH of each sample was lowered to 4.0 with 1 M HCl and the prostaglandins were extracted by shaking twice with 50 ml ethyl acetate. The two ethyl acetate fractions were combined and evaporated to dryness on a rotary evaporator. The recoveries of $PGF_{2\alpha}$ and PGE_2 by this method are >90% and the recovery of 6-keto-$PGF_{1\alpha}$ by this method is >80% (Poyser & Scott, 1980; Swan & Poyser, 1983). Each dried extract was re-dissolved in 10 ml ethyl acetate and stored at −20°C. The amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ present in each sample were measured by radioimmunoassay using antibodies raised in this laboratory; the cross-reactivities have been reported elsewhere (Poyser, 1987). The intra-assay coefficients of variation were <12% for all three assays. The inter-assay coefficients of variation were 19.9%, 11.5% and 18.8% for the $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ assays, respectively. The detection limit was 20–40 pg per assay tube. Since prostaglandins are not stored in the guinea-pig uterus (Poyser, 1972), prostaglandin release into the superfusing fluid reflects fresh prostaglandin synthesis particularly as their release is inhibited by indomethacin (Poyser, 1985b).

Sources of material

Caffeine, ruthenium red, TMB-8, W-7, trifluoperazine and theophylline were purchased from Sigma Chemical Co., Poole, Dorset; ryanodine was purchased from Calbiochem-Novabiochem, Nottingham.

Statistical tests

Changes in the output of prostaglandins with time were analysed by Duncan's multiple range test. Other comparisons were made using Student's t test, or if the variances of the two groups were significantly different by the Variance ratio F test, by a modified t test for unequal variances (see Steel & Torrie, 1980).

Results

Caffeine significantly ($P<0.05$) increased the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the day 7 and day 15 guinea-pig uterus, and of PGE_2 from the day 15 uterus (Figure 1). Caffeine significantly ($P<0.05$) increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from day 7 guinea-pig uterine horns superfused with calcium-containing or calcium-depleted Krebs solution. However, the stimulatory effect of caffeine on $PGF_{2\alpha}$ output tended to be reduced during the first 10 min period of treatment in calcium-depleted Krebs solution compared to normal Krebs solution (Figure 2). TMB-8 caused small, but significant ($P<0.05$) increases in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus, an effect reported previously (Poyser, 1985a). However, TMB-8 prevented the increase in $PGF_{2\alpha}$ output produced by caffeine without preventing the increases in outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ induced by caffeine (Figure 3).

Ryanodine alone had no effects on the outputs of $PGF_{2\alpha}$ and PGE_2 from the day 7 guinea-pig uterus, although ryanodine (20 and 200 μmol l^{-1}) significantly ($P<0.05$) increased the output of 6-keto-$PGF_{1\alpha}$. Ryanodine (2 to 200 μmol l^{-1}) did not inhibit the increase in outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ induced by caffeine from the day 7 guinea-pig uterus (Figure 4). However, ryanodine (20 and 200 μmol l^{-1})

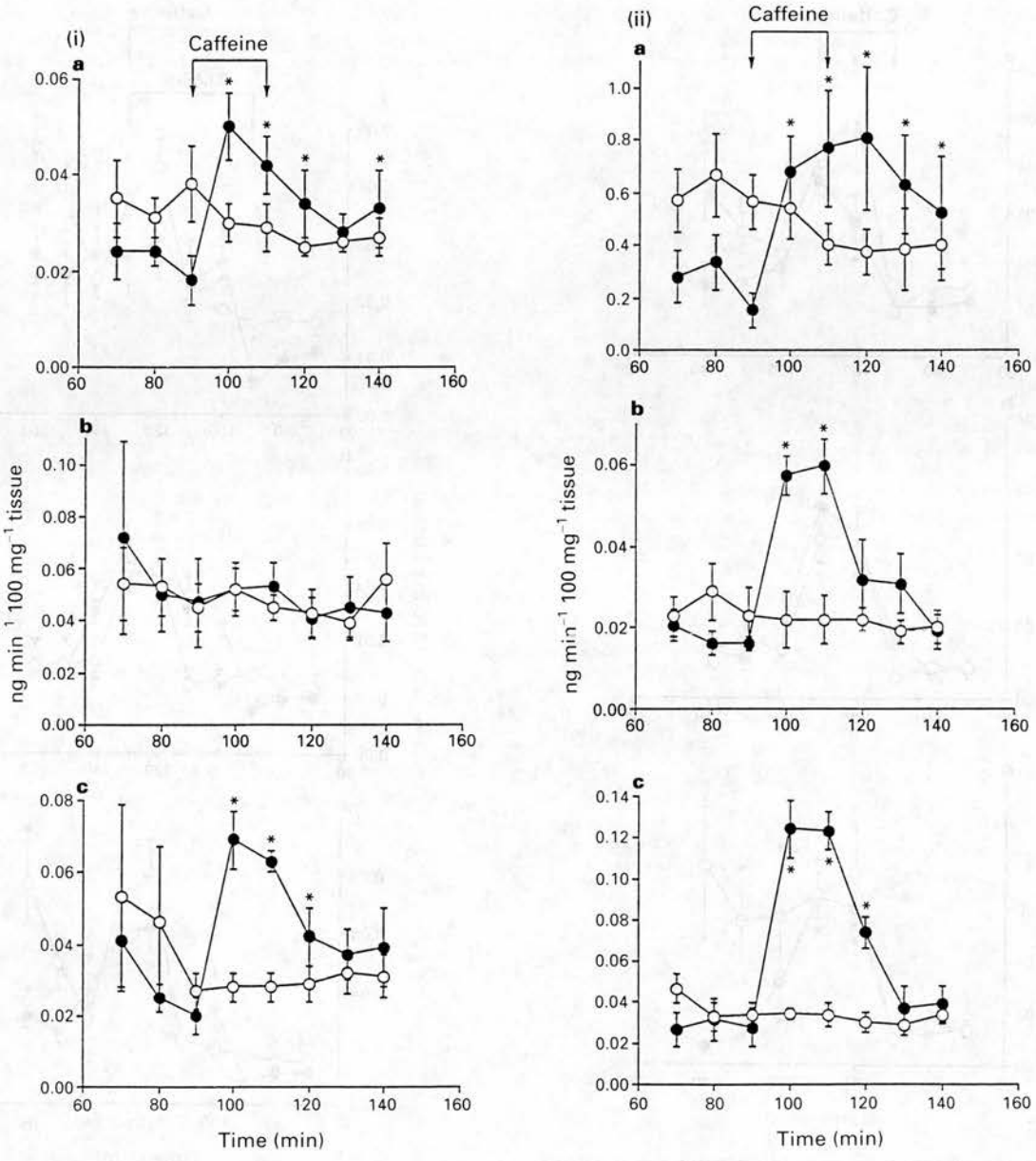

Figure 1 Mean (± s.e. mean, $n = 5$) outputs of (a) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), (b) PGE_2 and (c) 6-keto-$PGF_{1\alpha}$ from the (i) day 7 and (ii) day 15 guinea-pig uterus superfused *in vitro* in the presence (●) and absence (○) of caffeine. *Significantly ($P < 0.05$) higher than before caffeine treatment.

delayed both the increase and subsequent decrease in $PGF_{2\alpha}$ output produced by caffeine such that the 'peak of increased $PGF_{2\alpha}$ release' was flatter and broader. PGE_2 output was not affected by caffeine or ryanodine in this experiment (Figure 4).

In the absence of ruthenium red, caffeine increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus 2.3, 2.1 and 3.8 fold, respectively. In the presence of ruthenium red (10 μmol l^{-1}), caffeine increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ by 2.5, 1.9 and 3.8 fold, respectively. In the presence of ruthenium red (100 μmol l^{-1}), caffeine increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ 2.1, 2.3 and 3.9 fold, respectively. Therefore, ruthenium red at both concentrations used had no significant effect on the increases in outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ produced by caffeine from the day 7 guinea-pig uterus.

W-7 had no inhibitory effect on the increases in output of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ produced by caffeine from the day 7 guinea-pig uterus. In fact, W-7 caused a 3 fold potentiation of the increase in $PGF_{2\alpha}$ output induced by caffeine. W-7 also caused a much smaller potentiation of the increase in PGE_2 output and had no effect on the increase in 6-keto-$PGF_{1\alpha}$ output produced by caffeine from the day 7 guinea-pig uterus (Figure 5). Trifluoperazine produced small increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus, an effect noted previously (Poyser, 1985b). However, trifluoperazine had no effect on the increases in $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ produced by caffeine from the day 7 guinea-pig uterus. In this experiment, PGE_2 output from the uterus increased after the caffeine treatment had finished in both the control and trifluoperazine-treated uterine horns (Figure 5). Theophylline significantly ($P < 0.05$) increased the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus 3.6 and 4.3 fold, respectively. Theophylline had no significant effect on uterine PGE_2 output.

Caffeine, ryanodine (2, 20 or 200 μmol l^{-1}), ruthenium red (100 but not 10 μmol l^{-1}) and theophylline relaxed the superfused uterine horns and abolished any occasional spontaneous contractions. W-7 and TMB-8 caused an initial contraction of the uterus which lasted about 3 min. Following the

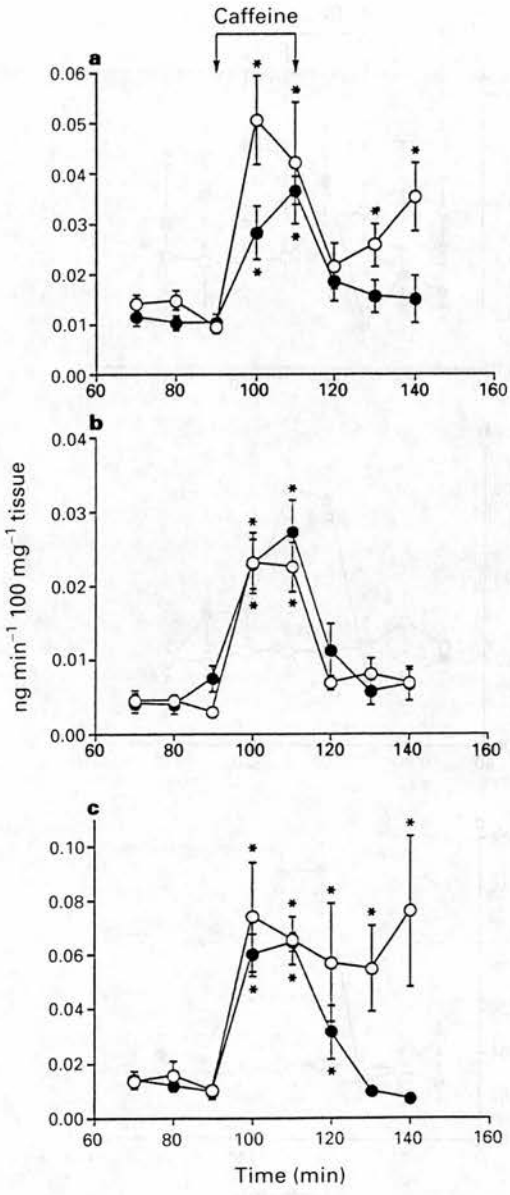

Figure 2 Effect of caffeine on the mean (± s.e. mean, $n = 5$) outputs of (a) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), (b) PGE_2 and (c) 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the presence of normal Krebs solution (○) and calcium-depleted Krebs solution (●). *Significantly ($P < 0.05$) higher than before caffeine treatment.

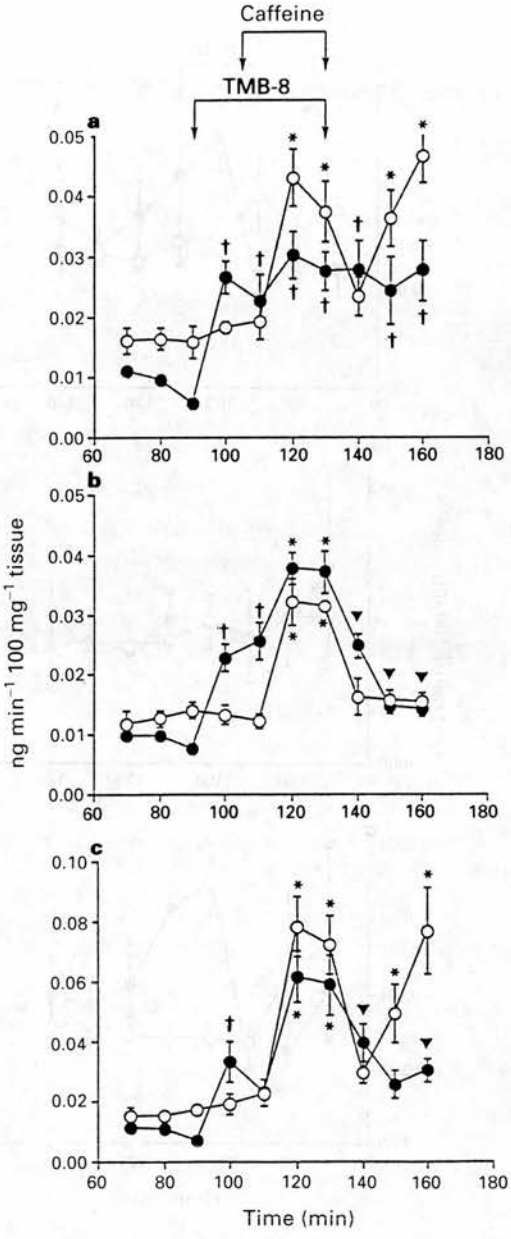

Figure 3 Effect of caffeine on the mean (± s.e. mean, $n = 5$) outputs of (a) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), (b) PGE_2 and (c) 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the presence (●) and absence (○) of TMB-8. †Significantly ($P < 0.05$) higher than before TMB-8 treatment alone. *Significantly ($P < 0.05$) increased by caffeine treatment. ▼Significantly ($P < 0.05$) lower following the end of caffeine treatment but significantly higher than before TMB-8 treatment.

subsequent relaxation, the uterine horns continued to exhibit the occasional spontaneous contraction. Trifluoperazine had no effect on the contractile state of the uterus.

Discussion

The basal outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus were typically low. However, by day 15 the output of $PGF_{2\alpha}$ from the uterus had increased approximately 20 fold whereas the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ showed little change. This is in agreement with previous findings (Poyser & Brydon, 1983). Studies in ovariectomized guinea-pigs have shown that oestradiol acting on a uterus which has been primed with progesterone for 10 days is the optimum stimulus for this selective increase in uterine $PGF_{2\alpha}$ synthesis and release (Poyser, 1983a). During the oestrous cycle, progesterone output from the ovary is increased after day 3 and oestradiol output from the ovary increases after day 10 (Joshi *et al.*, 1973). This increase in oestradiol output from the ovary precedes an increase in $PGF_{2\alpha}$ output from the uterus by 24 h (Blatchley *et al.*, 1972; Earthy *et al.*, 1975; Antonini *et al.*, 1976). Oestradiol acting on a progesterone-primed uterus appears to be the physiological stimulus for increased $PGF_{2\alpha}$ production by the guinea-pig uterus towards the end of the cycle, especially as treatment of guinea-pigs with a progesterone receptor antagonist or an oestrogen receptor antagonist from days 11 to 14 of the cycle prevents the increase in uterine $PGF_{2\alpha}$ output seen on day 15 (Poyser, 1993). Oxytocin has no stimulatory effect on uterine $PGF_{2\alpha}$ output in guinea-pigs (Poyser & Brydon, 1983; Riley & Poyser, 1987b). The guinea-pig uterus is a good animal model for the human uterus when studying the factors controlling prostaglandin production since oestradiol acting

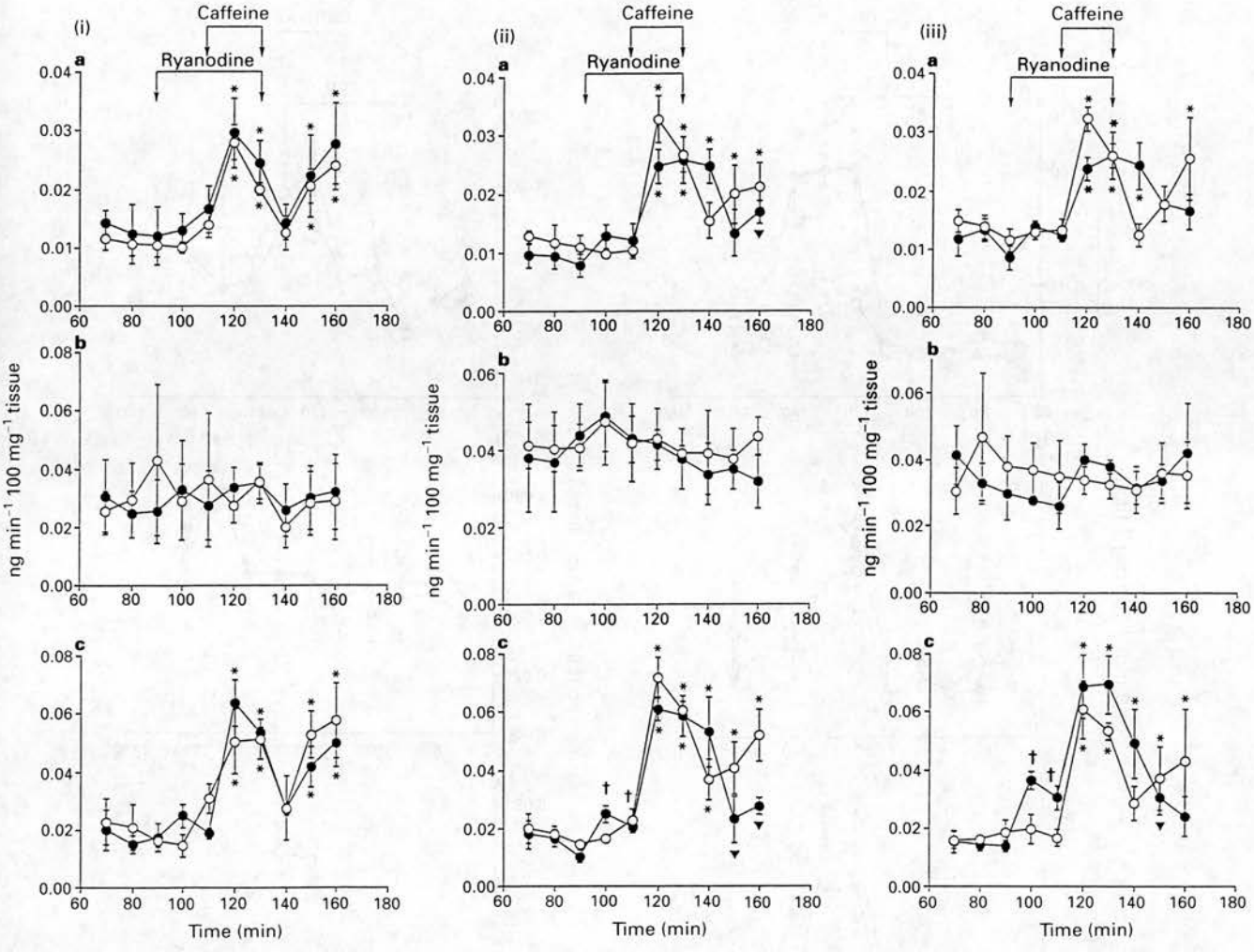

Figure 4 Effect of caffeine on the mean (± s.e. mean, $n = 4$) outputs of (a) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), (b) PGE_2 and (c) 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the absence (○) and presence of ryanodine (●) at concentrations of (i) 2 μmol l^{-1}, (ii) 20 μmol l^{-1} and (iii) 200 μmol l^{-1}. †Significantly ($P < 0.05$) higher than before ryanodine treatment. *Significantly ($P < 0.05$) higher than before caffeine treatment. ▼Significantly ($P < 0.05$) lower following the end of caffeine treatment but significantly higher than before ryanodine treatment.

on a progesterone-primed uterus is the optimum stimulus for increased $PGF_{2\alpha}$ production by the human uterus (Smith *et al.*, 1984), and oxytocin has only a weak stimulatory effect on $PGF_{2\alpha}$ production by the nonpregnant human uterus (Leaver & Richmond, 1984).

Caffeine caused 3 to 4 fold increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the guinea-pig uterus on days 7 and 15 of the oestrous cycle. $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ are the major prostaglandins synthesized by the endometrium and myometrium, respectively (Poyser, 1983b), although the endometrium synthesizes and secretes 6-keto-$PGF_{1\alpha}$ also (Riley & Poyser, 1987a,b). The effects of caffeine on uterine PGE_2 output were variable. Sometimes caffeine increased PGE_2 output whereas other times it had no effect. In one instance, the stimulatory effect of caffeine on PGE_2 output was delayed. Subsequent analysis of the guinea-pig ages showed that caffeine had no effect on uterine PGE_2 output in younger animals (<4 months) in which basal PGE_2 tended to be higher, but stimulated uterine PGE_2 output in older animals (>4 months) in which basal PGE_2 output tended to be lower. Basal PGE_2 output, but not basal $PGF_{2\alpha}$ output, from the rat uterus also decreases with age and this appears to be due to a reduced response of the ageing uterus to oestradiol and progesterone (Brown *et al.*, 1984). A similar phenomenon in guinea-pigs may explain the reduced basal output of PGE_2 in older animals. However, why the older guinea-pigs should be responsive to caffeine is not clear, except that a lower basal output allows any increase in output to be seen more easily. There is more variability in the output of $PGF_{2\alpha}$ and, to a lesser extent, of the other two prostaglandins on day 15 since output increases progressively at the end of the cycle. Since the cycle may last from 15 to 18 days, day 15 in one animal may not be exactly equivalent to day 15 in another animal which may account for the greater variability in output. This does not apply to day 7 since prostaglandin output from the uterus is consistently low between days 3 and 10 of the cycle. For these reasons, guinea-pig uteri on day 7 of the cycle rather than on day 15 of the cycle were used since the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ are lower and show less variability. The guinea-pigs used on day 7 in any one experiment were approximately of the same age.

Caffeine increased the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus when superfused with calcium-depleted Krebs solution. However, the increase in $PGF_{2\alpha}$ output but not the increases in PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs tended to be slower than when calcium-containing Krebs solution was used. Thus the action of caffeine on $PGF_{2\alpha}$ output, but not on PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs, may depend to a small extent on the presence of extracellular calcium. Therefore, the mechanism by which caffeine stimulates $PGF_{2\alpha}$ synthesis and release may be

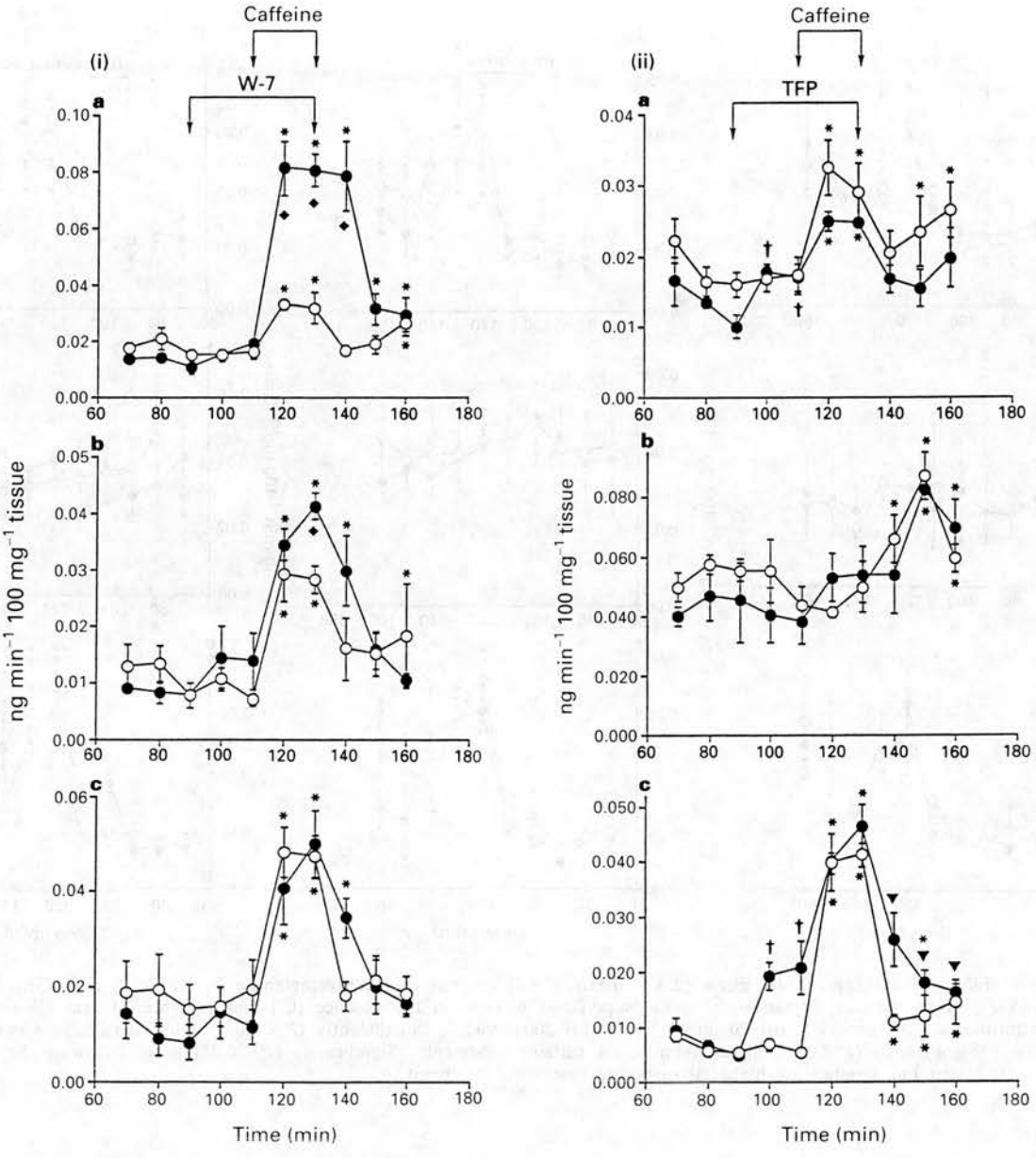

Figure 5 Effect of caffeine on the mean (± s.e. mean, $n = 4$) outputs of (a) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), (b) PGE_2 and (c) 6-keto-$PGF_{1\alpha}$ from the day 7 guinea-pig uterus superfused *in vitro* in the absence (○) and presence (●) of (i) 100 μmol l^{-1} W-7 and (ii) 150 μmol l^{-1} trifluoperazine (TFP). †Significantly ($P < 0.05$) higher than before trifluoperazine treatment alone. *Significantly ($P < 0.05$) higher than before caffeine treatment. ▼Significantly ($P < 0.05$) lower following the end of caffeine treatment but significantly higher than before trifluoperazine treatment. ◆Significantly ($P < 0.05$) higher than corresponding control value (i.e. without W-7 treatment) for the same prostaglandin.

different from the mechanisms by which caffeine stimulates the synthesis and release of PGE_2 and PGI_2 (measured as 6-keto-$PGF_{1\alpha}$). TMB-8 (an intracellular calcium antagonist) completely prevented the stimulatory effect of caffeine on uterine $PGF_{2\alpha}$ output without affecting the stimulatory effect of caffeine on the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$. This finding suggests that the stimulation of uterine $PGF_{2\alpha}$ synthesis and release by caffeine is dependent on intracellular calcium, but the stimulation of uterine PGE_2 and PGI_2 synthesis by caffeine is independent of intracellular calcium or is dependent on a pool of intracellular calcium not affected by TMB-8. Therefore these results provide further evidence that the mechanism by which caffeine stimulates uterine $PGF_{2\alpha}$ synthesis and release is different from the mechanisms by which caffeine stimulates the synthesis and release of PGE_2 and PGI_2 in the uterus. The stimulatory action of caffeine on uterine prostaglandin output was not prevented by the calmodulin antagonists, W-7 and trifluoperazine, indicating that calmodulin does not mediate this action of caffeine. In fact, W-7 greatly potentiated the stimulatory effect of caffeine on $PGF_{2\alpha}$ output. However, W-7 only weakly potentiated the stimulatory effect of caffeine on PGE_2 output and had no effect on the stimulatory effect of caffeine on 6-keto-$PGF_{1\alpha}$ output. Also, trifluoperazine did not potentiate these actions of caffeine. Therefore, W-7 was probably not producing its potentiating effect by inhibiting calmodulin and, since W-7 affected the stimulation of $PGF_{2\alpha}$ output to a much greater extent than the stimulation of PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs, the mechanism by which caffeine stimulates uterine $PGF_{2\alpha}$ synthesis and release again appears to be different from the mechanisms by which caffeine stimulates the synthesis and release of PGE_2 and PGI_2 in the uterus. The reason why W-7 should potentiate the stimulatory action of caffeine on uterine $PGF_{2\alpha}$ synthesis and release is not clear. Oestradiol

acting on a progesterone-primed uterus can selectively 'switch on' $PGF_{2\alpha}$ synthesis by the guinea-pig uterus (Poyser, 1983a), so perhaps W-7 is promoting the 'switching on' by caffeine of this steroid-dependent pathway of $PGF_{2\alpha}$ synthesis.

Caffeine acts on ryanodine receptors Types 1 and 2 to release intracellular calcium, an effect blocked by ruthenium red and high concentrations of ryanodine (see Sorrentino & Volpe, 1993). Ruthenium red and ryanodine had no inhibitory effect on the increase in output of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ induced by caffeine from the day 7 guinea-pig uterus. Thus caffeine appears to stimulate uterine prostaglandin synthesis by mechanisms which are not dependent upon the activation of Type 1 or Type 2 ryanodine receptors. However, the fact that ruthenium red and ryanodine caused the uterus to relax and abolished spontaneous contractile activity suggests that these two compounds are possibly preventing the release of calcium from an intracellular calcium pool. Ryanodine at the two higher concentrations (20 and 200 $\mu mol\,l^{-1}$) altered the time course of the increase in $PGF_{2\alpha}$ output produced by caffeine. The 'peak of increased release' was flatter and broader in the presence of ryanodine. In addition, ryanodine alone at the two higher concentrations increased the output of 6-keto-$PGF_{1\alpha}$. Why ryanodine should have these two actions is not apparent.

A third type of ryanodine receptor has been described in mink lung epithelial cells (see Sorrentino & Volpe, 1993). This receptor is also present in rat brain and smooth muscle (McPherson & Campbell, 1993). However, this receptor, although binding ryanodine, is not activated by caffeine. Thus it is unlikely that caffeine is stimulating uterine prostaglandin synthesis by acting on a ryanodine Type 3 receptor. Caffeine-sensitive calcium stores have been reported as being present in adrenal chromaffin cells (Burgoyne *et al.*, 1989), liver (Shoshan-Barmatz, 1990) and pancreas (Schmid *et al.*, 1990). The receptor type involved in the chromaffin cells and liver may be one of the three main ryanodine types. However, the receptor type involved in the pancreas is not one of these three ryanodine types since it is not sensitive to ryanodine, but it is blocked by ruthenium red (Dehlinger-Kremer *et al.*, 1991). Consequently, since in the present study the effects of caffeine are not prevented by ryanodine or ruthenium red, caffeine does not appear to be stimulating $PGF_{2\alpha}$ synthesis in the guinea-pig uterus by acting on any of the caffeine and/or ryanodine intracellular calcium pools described so far. Recently, an intracellular calcium pool has been described in hepatocytes which is caffeine-sensitive and ryanodine-insensitive. Additionally, the effect of caffeine in hepatocytes is not blocked by ruthenium red (McNulty & Taylor, 1993). This stimulation by caffeine and lack of inhibition by ryanodine and ruthenium red in hepatocytes is exactly the same profile as that seen in the stimulation of $PGF_{2\alpha}$ output by caffeine in the guinea-pig uterus. Thus, it is possible that caffeine-sensitive calcium pool present in hepatocytes is also present in the uterus and is involved in the stimulation of uterine $PGF_{2\alpha}$ synthesis by caffeine. However, theophylline also stimulated uterine prostaglandin synthesis yet theophylline does not release calcium in hepatocytes (McNulty & Taylor, 1993). Theophylline may be acting to stimulate uterine prostaglandin synthesis by a mechanism different from that of caffeine or, if caffeine and theophylline are acting through the same mechanism, then caffeine may be stimulating calcium release in hepatocytes and $PGF_{2\alpha}$ synthesis in the uterus by different mechanisms.

Since caffeine and theophylline are phosphodiesterase inhibitors, they may be acting to increase adenosine 3′:5′-cyclic monophosphate (cyclic AMP) concentrations in the uterus. This may be the mechanism by which caffeine and theophylline relaxed the uterus and abolished spontaneous uterine contractions. However, a previous study has shown that increasing cyclic AMP concentrations in the guinea-pig uterus (by using forskolin) does not lead to an increase in the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ from the uterus (Poyser, 1987). Thus, it is unlikely that caffeine and theophylline increased uterine prostaglandin output by elevating the cyclic AMP concentration. The effects of caffeine and theophylline on the intracellular calcium concentration of uterine cells in the presence and absence of extracellular calcium merit further study to investigate whether the actions of caffeine and theophylline on uterine prostaglandin synthesis involve the release of intracellular calcium and/or the influx of extracellular calcium.

In conclusion, caffeine stimulated the synthesis and release of $PGF_{2\alpha}$ from the guinea-pig uterus by an action which may be dependent on the release of intracellular calcium since TMB-8 (an intracellular calcium antagonist) prevented the action of caffeine. However, the action of caffeine does not appear to be mediated by any of the three ryanodine receptors identified so far. Caffeine also stimulated the synthesis and release of PGE_2 and 6-keto-$PGF_{1\alpha}$ from the uterus, apparently by mechanisms different from the one by which it stimulated $PGF_{2\alpha}$ production. The mechanism controlling PGE_2 production by the uterus is age-dependent since it was not present in young guinea-pigs. The results of this study showing that caffeine stimulates uterine prostaglandin production raise the possibility that caffeine, which is consumed in beverages by many women each day, may be one of the extrinsic factors responsible for or contributing to menstrual disorders in some women. A high concentration of caffeine was used in the present studies since this is the optimum concentration found in other tissues to release intracellular calcium. However, other studies have shown that concentrations of caffeine as low as $1\,\mu g\,ml^{-1}$ stimulate prostaglandin output from rat blood vessels (Naderali & Poyser, unpublished observations). The average cup of coffee contains about 100 mg caffeine and, after drinking 300 ml of a beverage containing 250 mg caffeine, the plasma concentration obtained 1 h later is between 4.2 and $26\,\mu g\,ml^{-1}$ (Robertson *et al.*, 1978). Therefore, there is some evidence that caffeine can release prostaglandin from tissues in concentrations that are obtained after drinking 1 or 2 cups of coffee. Consequently the present findings in guinea-pigs suggest that similar studies should now be performed on human uterine tissues. In one study, ryanodine increased the free intracellular calcium concentration in human cultured myometrial smooth muscle cells in the presence and absence of extracellular calcium, which indicates that a ryanodine receptor is present in human myometrium. However, caffeine had no such stimulatory effect on calcium release (Lynn *et al.*, 1993), which suggests that caffeine may not stimulate prostaglandin production by human myometrial smooth muscle cells. The endometrium is the main source of prostaglandin during the cycle (see Poyser, 1981), so an investigation of the effect of caffeine on human endometrium is merited.

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PROSTAGLANDINS LEUKOTRIENES AND ESSENTIAL FATTY ACIDS

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The Effect of Caffeine on Prostaglandin Output from the Perfused Mesenteric Vascular Bed of the Rat

E. K. Naderali and N. L. Poyser

Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ UK (Reprint requests to NLP)

ABSTRACT. **Caffeine significantly ($p < 0.05$) increased the output of prostacyclin (PGI_2) from the perfused rat mesenteric vascular bed. The outputs of PGE_2 and $PGF_{2\alpha}$ were also increased by caffeine. This stimulatory response to caffeine did not show rapid desensitization. Ryanodine also increased PG output, suggesting that caffeine may be acting via the stimulation of a ryanodine receptor. The increased production of a vasodilator such as PGI_2 from blood vessels following exposure to caffeine may explain why caffeine has a beneficial effect in angina.**

INTRODUCTION

It is generally believed that high caffeine intake (in the form of coffee and other beverages) may cause coronary heart disease and acute myocardial infarction. However, only 50% of the recent case-control and perspective studies have shown any statistically significant correlation between coffee intake and the risk of developing coronary heart disease or acute myocardial infarction (1). Consequently, it is not proven that caffeine has detrimental effects on health. In fact, as stated in a recent review, 'positive effects of coffee are being increasingly reported. Coffee has been shown to be an effective bronchodilator in young patients with asthma, and as a booster of pain-free walking time for patients with chronic stable angina. Ingestion of one and two cups of coffee increased the exercise duration until onset of angina by 8% and 12%, respectively, whereas decaffeinated coffee had no effect' (2). Why caffeine should have a beneficial effect in angina is not clear. Drugs used to treat angina are mostly vasodilators, so caffeine may stimulate the production by blood vessels of a vasodilator such as prostacyclin (PGI_2), especially as PGI_2 plays an important role in the regulation of vascular tone (3). Consequently, the effect of caffeine on prostaglandin output from blood vessels, using the perfused mesenteric vascular bed of the rat, has been studied.

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METHODS

The mesenteric vascular bed of male rats (250–300 g) was perfused in vitro with McEwen's solution (4 ml/min), which was heated to 37°C and aerated with 5% carbon dioxide and 95% oxygen, as described by Lennon and Poyser (4). After an initial settling period of 30 min, samples of perfusate were collected for periods of 1 min. In experiment 1 ($n = 6$), caffeine in increasing doses (1 µg to 1 mg) was injected into the perfusing fluid at intervals of 15 min. In experiment 2 ($n = 4$), three successive injections of 1 mg caffeine at 15 min intervals were made into the perfusing fluid. In experiment 3 ($n = 7$), 10 µg and 50 µg ryanodine were injected into the perfusing fluid with an interval of 3 min between doses. In experiments 1 and 2, samples of perfusate were collected from 2 min before to 6 min after, and at 9 and 13 min after caffeine administration. In experiment 3, samples of perfusate were collected from 1 min before the first dose to 2 min after the second dose of ryanodine (i.e. for 6 min altogether). The amounts of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ in the samples of perfusate were measured by radioimmunoassays previously described (4). The intra-assay coefficients of variation were < 12% for all three assays. The inter-assay coefficients of variation were 9.5%, 9.8% and 9.1% for the 6-keto-$PFG_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ assays, respectively.

Sources of material

Caffeine and ryanodine were purchased from Sigma

Chemical Co., Poole, Dorset, UK, and from Calbiochem-Novabiochem, Nottingham, UK, respectively.

Statistical tests

The results were analysed by one-way analysis of variance (ANOVA) and by the paired t-test.

RESULTS

6-Keto-$PGF_{1\alpha}$ was the major prostaglandin released from the rat mesenteric vascular bed; the basal outputs of PGE_2 and $PGF_{2\alpha}$ were 50–70% lower. Caffeine significantly ($p < 0.05$) increased the output of 6-keto-$PGF_{1\alpha}$ at doses between 1 µg and 1 mg by 1.7- to 3.3-fold. The maximum effect was obtained with 5 µg caffeine. Caffeine also increased the outputs of PGE_2 and $PGF_{2\alpha}$ between 1.3- and 2.4-fold. These increases were significant ($p < 0.05$) at the higher doses (Fig. 1). The maximum increase in PG output usually occurred within the first minute following caffeine administration, and an increase in PG output lasted for at least 2 min. Caffeine (1 mg) administered three times at 15 min intervals significantly ($p < 0.05$) increased the ouput of 6-keto-$PGF_{1\alpha}$ between 2.4- and 3.6-fold following each dose of caffeine. The outputs of PGE_2 and $PGF_{2\alpha}$ increased between 1.4- and 2.3-fold, although these increases were not statistically significant (Fig. 2).

Ryanodine (10 µg) increased the outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ by 1.3- to 1.6-fold, but these increases were statistically significant ($p < 0.05$) only for PGE_2 and $PGF_{2\alpha}$. Ryanodine (50 µg) significantly ($p < 0.05$) increased the outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ by 2.8-, 2.7- and 2.0-fold, respectively (Fig. 3).

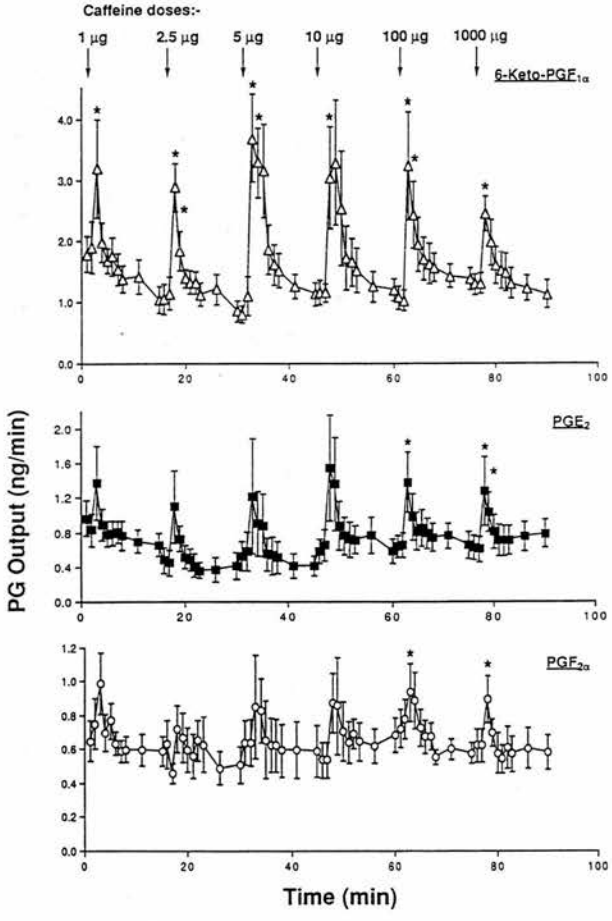

Fig. 1 Effects of caffeine on mean (± SEM, $n = 6$) outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ from the perfused mesenteric vascular bed of the rat. *Significantly ($p < 0.05$) increased by caffeine.

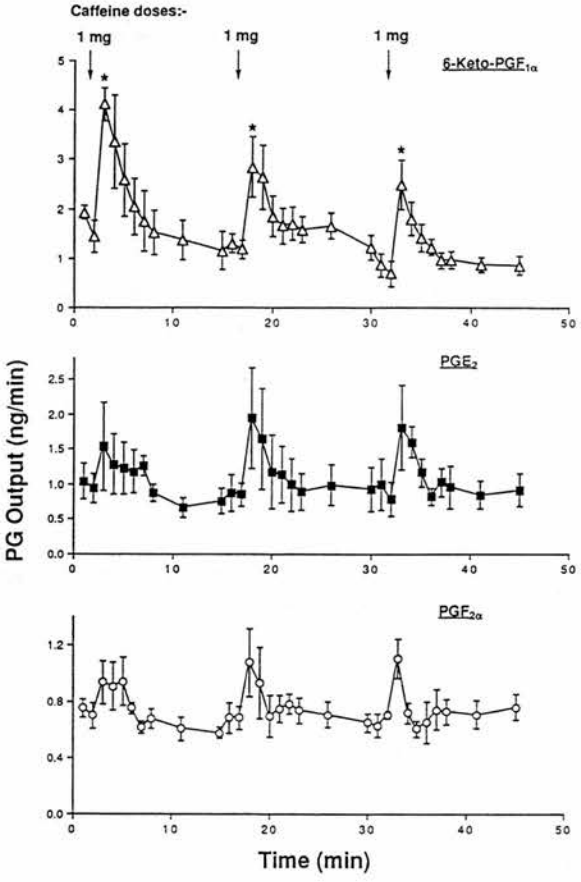

Fig. 2 Effects of a high dose of caffeine (1 mg) administered three times on mean (± SEM, $n = 4$) outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ from the perfused mesenteric vascular bed of the rat. *Significantly ($p < 0.05$) increased by caffeine.

DISCUSSION

Caffeine stimulated the output of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$), together with smaller quantities of PGE_2 and $PGF_{2\alpha}$, from the perfused mesenteric vascular bed of the rat. Both the smooth muscle and endothelial cells of rat blood vessels synthesize PGI_2 (as well as PGE_2 and $PGF_{2\alpha}$), with the smooth muscle having the higher synthesizing capacity per unit length of vessel (4). The average cup of coffee contains about 100 mg caffeine and, after drinking 300 ml of a beverage containing

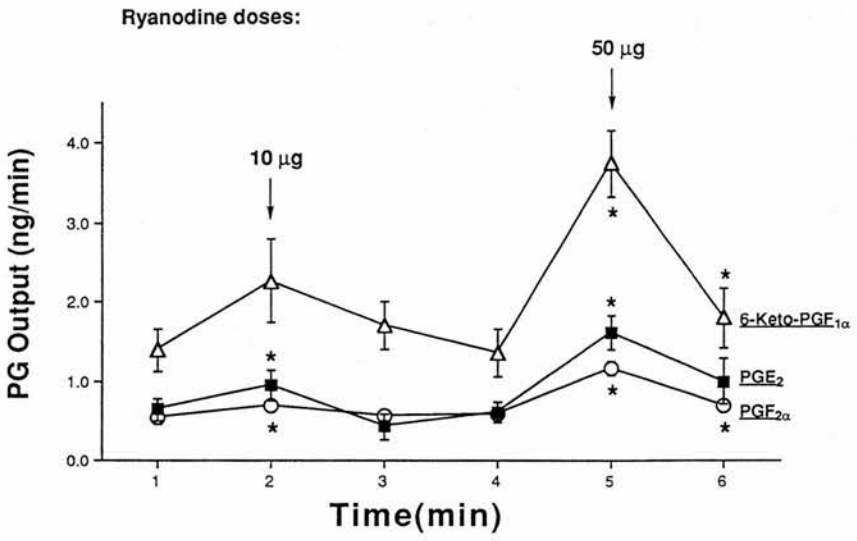

Fig. 3 Effects of ryanodine on mean (± SEM, $n = 7$) outputs of 6-keto-$PGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$ from the perfused mesenteric vascular bed of the rat. *Significantly ($p < 0.05$) increased by ryanodine treatment.

250 mg caffeine, the peak plasma concentration of caffeine obtained 1 h later is between 4.2 and 26 µg/ml (5). Caffeine (5 µg) administered into the perfused rat mesenteric vascular bed produced a maximum increase in PGI_2 output, and even 1 µg caffeine produced an increase in PGI_2 output which was 50% of the maximum increase. Due to dilution effects, the concentration of caffeine reaching the mesenteric blood vessels would be considerably less than 5 and 1 µg/ml, respectively. If human blood vessels are as responsive to caffeine as rat blood vessels, then the concentration of caffeine in the blood after swallowing one or two cups of coffee would appear sufficient to stimulate PGI_2 synthesis by blood vessels. Furthermore, the administration of a high dose (1 mg) of caffeine at 15 min intervals over a period of 30 min to the perfused rat mesenteric vascular bed did not result in any reduction in the response. Consequently, tolerance does not occur rapidly, so the repeated intake of caffeine over a short period should not reduce its stimulatory effect on PGI_2 output.

Since PGs are not stored in tissues, their release is immediately preceded by their synthesis. The rate-limiting step in PG synthesis is the release of arachidonic acid from phospholipids, usually by the action of phospholipase (PL) A_2. This enzyme requires calcium to be active, so substances which stimulate PG synthesis usually have to increase the intracellular free calcium concentration. Caffeine can release intracellular calcium by acting on ryanodine receptors (6), and caffeine releases intracellular calcium in both vascular smooth muscle and endothelial cells (7–9). Ryanodine, like caffeine, rapidly increased the output of PGI_2 from the perfused rat mesenteric vascular bed. After allowances are made for ryanodine's higher molecular weight, caffeine and ryanodine were of similar potency. Consequently, caffeine and ryanodine may be initiating vascular PGI_2 synthesis and release by the same mechanism, i.e. through releasing intracellular calcium by combining with a ryanodine receptor.

Overall, caffeine stimulated the output of PGI_2 (a vasodilator) from rat blood vessels by a mechanism which does not rapidly become desensitized. Experiments in dogs have shown that PGI_2 in low doses induces hypotension accompanied with increases in cardiac ouput, stroke volume and increased cardiac contractility (10). Similar actions of PGI_2 in humans may explain, at least in part, the beneficial effect of caffeine in patients suffering from angina, assuming that in humans (as in the rat) caffeine stimulates the production of PGI_2 by blood vessels.

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E. K. Naderali is a MRC research student.

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