THE ROLE OF MERKEL CELLS IN MECHANO-TRANSDUCTION

by

GORDON S. FINDLATER

DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH

1985

The work presented in this thesis is entirely my own, except as indicated in Chapter 2.10.

TABLE OF CONTENTS page no

SUMMARY

In whatever receptor system Merkel cells are found they are always associated with a characteristic, slowly adapting response. The role of Merkel cells in the transduction process of slowly adapting Type I cutaneous mechanoreceptors (SAI receptors or touch domes) of rats and cats was investigated by mechanical and electrical stimulation of SAI receptors and their afferent fibres in various experimental conditions.

In an hypoxic environment, touch domes eventually failed to respond to mechanical stimulation. For receptor failure to occur it was necessary to have an O_2 depleted external environment around the limb as well as an O_2 deficient blood supply. Exposure of the limb surface to O_2 was sufficient to bring about receptor recovery. Correlated with receptor failure was a significant reduction in the number of dense-cored vesicles normally found in the Merkel cell cytoplasm adjacent to the nerve ending innervating the cell. Receptor recovery was associated with a return in the numbers of dense-cored vesicles back to that found in control cells.

When stimulated in the presence of the known calcium channel blockers cobalt and verapamil hydrochloride, SAI receptors again eventually failed to respond to mechanical stimulation. Correlated with receptor failure was a reduction in the number of Merkel cell vesicles as well as an increase in the number of 'synaptic-like' junctions observed between

Merkel cells and their associated nerve endings.

In hypoxic conditions and in the presence of the calcium channel blockers a response could still be produced in the afferent fibre by electrical stimulation of the fibre close to the touch dome.

The results indicate that Merkel cell dense-cored vesicles are necessary for the characteristic slowly adapting response of SAI mechanoreceptors possibly by the secretion of a transmitter substance stored within the vesicles.

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

The Merkel cell in mammals is an integral component of the slowly adapting type I (SAI) cutaneous mechanoreceptors and is found in other slowly adapting receptors of both hairy and glabrous skin. Tastzellen (touch cells) were first introduced to the scientific literature in 1875 by Merkel who described specialized epidermal cells in the skin of several animal species which he investigated. These cells, which have come to be known as Merkel cells, were distinguishable from the surrounding epidermal cells by their large size and pale cytoplasm. When a neurite was found adjacent to the 'Tastzelle' the complex was called a 'Tastkorperchen' - a touch corpuscle. As the neurite frequently appeared to be expanded when adjacent to the cell it was subsequently termed a Merkel's disc by many later authors. The terms Tastzellen and Tastkorperchen used by Merkel reflected his belief that these cells and their accompanying neurites were responsible in an unspecified way for converting physical stimuli applied to the skin to neural activity in the adjacent nerve ending. Merkel's hypothesis however did not receive universal support and as recently as 1955 Weddel,Palmer and Pallie (1955) suggested that Merkel's cells were in fact the same structures described as dendritic cells by Billingham (1949) and that the Merkel's discs were artefacts produced by the histological methods used by earlier workers.

Since then the dispute over the existence of Merkel cell-neurite complexes has been resolved largely through the use of improved histological methods and of the electron microscope. The first EM description in 1962, was by Cauna (1962) and then in 1963 by Iggo and Muir (1963) in Merkel cell-neurite complexes in the touch domes or SAI mechanoreceptors of hairy skin. This was followed by descriptions in the glabrous skin of the opossum snout by Munger (1965) and in the outer root sheath of sinus hair follicles by Andres (1966).

Although prior to this, slowly adapting responses had been obtained from afferent fibres, originally by Adrian and Zotterman (1926), and were later associated with mechanically sensitive spots on the skin, no attempt had been made to identify the underlying structures responsible (Frankenhauser,1949; Maruhashi, Mizuguchi and Tasaki, 1952).

Earlier, in 1949, Frankenhauser (1949), working on the rabbit, showed that steady pressure applied to the area of the leg innervated by the sural nerve gave a slowly adapting response. The frequency of impulses recorded from the afferent unit when pressure was applied to the skin was higher for heavy than light pressure. The receptive field of these afferent units were spot-like, having a skin diameter of about 0.2 mm to which he gave the name 'touch spots'. Maruhashi et al. (1952) reported similar findings in the skin of the toad and cat from what they called 'tonic pressure receptors'.

In 1963 Iggo (1963b), using the method of single unit recording, subdivided slowly adapting mechano-

receptors into two types based on the size of the receptive fields of the receptors and on differences in their electrophysiological responses. Some afferent fibres ended in distinct spots of not more than $250\mu m^2$ in area while other afferent fibres had receptive fields of not less than $1mm^2$ in area. The former were referred to as 'touch spots', the name used by Frankenhauser (1949), and the latter as 'touch fields'. In another publication at the same time, Iggo (1963a) reported on distinct sensory structures in the skin of cats associated with these 'touch spots'. These spots were never innervated by more than one afferent fibre although one afferent fibre could innervate several 'touch spots', a finding earlier reported by Hunt and McIntyre (1960). The diameter of the afferent fibres innervating these structures ranged from 7-16μm. Under binocular microscopic examination these spots were found to be hemispherical domes of 150-250μm diameter. Within each dome was a rich vascular supply in the form of a fine capillary network. The response of these domes to sustained mechanical stimulation was a persistent irregular discharge of impulses which after a period of rapid adaptation showed a slowly falling frequency for as long as the mechanical displacement was maintained. Highest frequencies, in the order of 1100 impulses/sec., were obtained by drawing a glass rod over the surface of the dome or by rapid vertical displacement of the dome. These receptors were also thermally sensitive in that rapid cooling of the skin produced a discharge of impulses in single afferent fibres at frequencies of 50/sec. or less. However Iggo

(1963a) suggested that the thermal response was possibly related to the rate of adaptation of the receptor to mechanical stimulation rather than being directly concerned with thermal sensation. Light microscopic examination of the structures associated with the touch spots (Iggo, 1963a) revealed that the afferent fibre branched repeatedly on entering the touch spot to terminate just beneath the epithelium in the form of circular plates or discs of 10μm diameter and 1μm thickness.

In contrast to the 'touch spots', the 'touch fields' under maintained mechanical stimulation gave a very regular discharge (Iggo, 1963b). Some of these units also had a steady resting discharge in the absence of applied mechanical stimulus and this activity could be altered by moving the skin or altering the skin temperature. In the cat, there was no identifiable surface structure associated with touch fields as there was for touch spots.

The findings of Iggo on touch spots were later confirmed by Tapper (1964, 1965) and Werner and Mountcastle (1965), although some of the receptors examined by Werner and Mountcastle were possibly touch fields and not touch spots.

In 1966, Iggo (1966) described the relationship between the specialized cells in the basal layer of the modified epidermis within a touch spot and the terminal expansions of the afferent fibre axonal twigs. Within a single touch spot there could be between 30-50 of these nerve endings and it was proposed that these terminals corresponded to Merkel's discs which are

commonly found in glabrous skin. Iggo (1966) also suggested that the afferent nerve and its terminals and associated tactile-cells in the epidermis comprised a physiological unit with a characteristic and unique set of functional properties for which the term 'afferent unit' was proposed. The terms 'touch spots' and 'touch fields' were consequently changed to slowly adapting type I and type II cutaneous mechanoreceptors (SAI and SAII) respectively. These terms are now the ones most frequently encountered in the literature particularly when referring to the physiology of these units. Other names for SAI receptors are 'touch domes', 'touch corpuscles', 'tactile pads', 'Iggo domes', ,Iggo-Pinkus domes', 'hair discs' or 'the Haarscheibe'. This last name was the name originally used by Pinkus in 1904 because of the structure's apparent anatomical association with hair follicles. It has also been used more recently by workers who have suggested a functional relationship between tylotrich hair follicles and touch domes, (Mann and Straile, 1965; Smith, 1967, 1977). However as both tylotrich follicles and touch domes are innervated separately (Brown and Iggo, 1967) and in some species show no constant relationship with each other, this term is misleading.

The differences between SAI and SAII units have been reported in various investigations (Chambers and Iggo, 1967; Chambers and Iggo,1968; Burgess,Petit,and Warren,1968; Kenton, 1971) and in 1972 Chambers, Andres, von Duering and Iggo (1972) finally and definitively described the similarities and differences

between these two receptors.

In 1968, Iggo described in some detail the fine structure of the receptor cells in the basal layer of the epidermis and of the adjacent expanded nerve endings. The conclusion reached was that the nerve endings could only be Merkel discs with the specialized receptor cells being Merkel cells.

In the following year Iggo and Muir (1969) published a comprehensive report in which they described the unique structure of the SAI receptor, with its equally distinctive physiological response. In other locations where Merkel cell-neurite complexes were found,the same slowly adapting response was obtained (Munger, Pubols and Pubols, 1971; Gottschaldt, Iggo and Young, 1973) and it is now generally accepted that Merkel cells underlie the characteristic response of SAI mechanoreceptors as a class.

The functional role of the Merkel cell in the Merkel cell-neurite complex has been the focus of attention for some time and several hypotheses, based largely on morphological information, have been proposed. Some investigators have suggested, as did Merkel (1875) that it is a primary receptor cell (Davies, 1961) responding to mechanical deformation by the secretion of a chemical transmitter (Andres, 1966; Iggo and Muir, 1969; Horch, Whitehorn and Burgess, 1974; Hartschuh and Weihe, 1980). An alternative view is that Merkel cell-neurite complexes are abutments making mechanical stimuli efficient in deforming the mechanosensitive nerve ending (Smith, 1977; Gottschaldt

and Vahle-Hinz, 1981).

By looking at investigations into the developmental origin of Merkel cells it was hoped that some insight into their function may be gained. There is however no agreement in the literature on this point. Winkelmann (1977), by drawing comparisons between Merkel cells and cells of the APUD system (Pearse, 1969), believed them to be neural crest migrants to the epithelium. (Pearse in 1969 attached the name APUD to a system of cells which shared certain characteristics, these being amine content, amine precursor uptake, and amino acid decarboxylase activity). Winkelmann's view is supported by Breathnach (1971) and Hashimoto (1972a) who have both found Merkel cells present within the dermis and passing into the epidermis in tissue from human foetuses. At no time did these cells ever resemble keratinocytes (Breathnach, 1971). English (1974, 1977b) on the other hand described cells in the epidermis of cats and rats which were transitional in appearance between Merkel cells and keratinocytes. Lyne and Hollis (1971) found Merkel cells present within sheep epidermis during foetal development but only in the oldest foetuses were these cells associated with neurites. Because the Merkel cells had desmosomal contact with adjacent epidermal cells, they concluded that they were modified epidermal cells. However the opposite order of development to that described by Lyne and Hollis (1971) was found by Kasprzak, Tapper and Craig (1970). They observed in newborn kittens, mechanically sensitive spots which were innervated by type I afferent fibres even though

very few Merkel cells were present. The Merkel cells appeared after innervation had occurred. This supports the findings of Symonowicz (1895) who suggested that Merkel cells differentiated from epithelial cells after the arrival of nerve fibres.

Regardless of the precise function of Merkel cells in Merkel cell-neurite complexes, they are essential for the characteristic slowly adapting response obtained on mechanical stimulation of these complexes (Brown and Iggo, 1963). After nerve crush, the SAI mechanoreceptor with its associated Merkel cell-neurite complexes showed degenerative changes. At various stages of regeneration distal to the crush, mechanical stimulation could still produce nonspecific responses from the tips of the regenerating nerve. It was only when the ingrowing fibres reformed Merkel cell-neurite complexes that the typical slowly adapting response was obtained. This conclusion was supported by the experiments of Kasprzak et al. (1970) who report similar physiological results in developing slowly adapting receptors of new born kittens. It was only when numerous Merkel cells appeared in the epidermis that the receptor developed a capacity for sustained response to a constant mechanical indentation. Denervation experiments in other sensory receptor systems have produced similar effects, both morphologically and physiologically.

The dependence of Merkel cell integrity upon its innervation has been investigated by Burgess, English, Horch and Stensaas (1974) and English (1977a) who found progressive degeneration of Merkel cells and epithelial

cells of SAI receptors in cats following denervation. It was assumed that the type I afferent fibres were 'trophic' neurones as they were juxtaposed to the dermal aspect of the Merkel cell. An effect similar to that of nerve transection can be produced by colchicine or vinblastine - Merkel cells are decreased in number and show degenerative changes interpreted by Chelyshev and Vinter (1983) as evidence suggesting that axonal blockade prevents the secretion of trophic chemical factors from the nerve terminal which are necessary to maintain Merkel cell integrity. Hartschuh and Weihe (1977) however found that nerve transection in the cat had no effect on the number of Merkel cells present in SAI receptors or sinus hair follicles or on the ultrastructure of these cells regardless of the survival time. They concluded that there was no evidence to indicate that Merkel cells in cats were more dependent upon their sensory innervation for their morphological integrity than Merkel cells in rats (Smith, 1967).

In a study on the reinnervation of receptors, Burgess et al.(1974) showed that both crushed and transected type I nerve fibres regenerated preferentially to old receptor sites. In experiments to determine why this should be, Horch (1982) found that the reappearance of type I receptors at old receptor sites following nerve transection appeared to be primarily due to intrinsic properties of the receptor sites rather than to guidance of regenerating axons along Schwann tubes in the distal stump. This result suggests that Merkel cells may in fact be acting as target cells for afferent fibres, an

hypothesis proposed by Scott, Cooper and Diamond (1981).

A feature of the adapted discharge of the SAI mechanoreceptor is an exponential distribution of interspike intervals which would arise if the impulses were generated at sites that were independent of each other (Iggo and Muir, 1969). The structure of the Merkel cell-neurite complex is consistent with the concept of separate generators - each Merkel cell is innervated by the single expanded nerve ending of a branch of the main axon which is myelinated to within a few microns of the Merkel cell. Horch et al.,(1974), on the assumption that SAI receptors did in fact contain multiple impulse generating sites, proposed two models based on the morphological characteristics of the receptor. One model assumed that each Merkel cell-neurite complex acted as an independent oscillator which generated a regular discharge and that the irregular discharge of SAI receptors arose from a mixing of regular discharge patterns. The main assumption in this model was the independence of individual oscillators - impulses produced at one generator site did not influence the generation process of the other oscillator sites. In the alternative model each terminal impulse-generating site was assumed to have an irregular discharge. Reset of one generating site by another may or may not occur. It was suggested in this model that the irregularity of the discharge generated by each terminal could result from variability in transmitter release by Merkel cells. From investigations carried out to test these

models, it was found that the second model - the irregular oscillator model - best described the characteristic discharge pattern of the SAI mechanoreceptors. Horch et al.,(1974) concluded that the most likely cause of such a discharge pattern was transmitter release from the Merkel cell. They also suggested that the unique relationship between Merkel cells and the adjacent expanded nerve terminal was functionally related to the equally distinctive discharge pattern of SAI mechanoreceptors.

Gottschaldt and Vahle-Hinz (1981) disputed this idea of transmitter release from Merkel cells on the grounds that Merkel cell-neurite complexes in the sinus hair follicle of the cat were able to follow, in a 1:1 relationship, vibration frequencies of up to 1500 Hz. This they argued was far too fast for chemosynaptic transmission to occur. They also determined the receptor delay by mechanically stimulating the sinus hair shaft and electrically stimulating the afferent nerve fibre and found this to be about 0.3 ms. Again this was believed to be too fast for chemo-synaptic transmission to occur.

Ultrastructurally the Merkel cell and its adjacent sensory fibre have features suggestive of a neurosecretory function. In particular they contain numerous osmophilic granules which are most abundant between the nucleus of the Merkel cell and the subjacent expanded nerve terminal. The terms 'granules' and 'dense-cored vesicles' are used interchangeably throughout the text. These granules are sometimes found to be concentrated at closely apposed

specialized regions of the Merkel cell and nerve ending membranes (Andres,1966; Iggo and Muir, 1969). Chen, Gerson and Meyer, (1973) described in detail these junctions and the fusion of the Merkel cell granules with these areas of Merkel cell membrane. These results have been confirmed by Smith (1977), Mihara, Hashimoto, Ueda and Kumakiri, (1979) and Hartschuh and Weihe (1980). This contrasts with the observations of other authors who found no evidence suggesting synaptic junctions between the Merkel cell and the adjacent nerve terminal (Munger, 1965; Winkelman, 1977; Hashimoto, 1972b; Smith, 1970). The presence of these structures in several different species of animal led Hartschuh and Weihe (1980) to suggest that the methodology used in the preparation of the tissue for electron microscopy was critical if these junctions were to be visualised. Smith (1970) who reported a lack of membrane specializations later stated on the basis of new evidence that there was no doubting the existence of such specialized junctions and that the appearance of Merkel cell granules fusing with these regions was evidence of synaptic transmission (Smith, 1977). It has been suggested by Hartschuh and Weihe (1980) that the Merkel cell granules function as synaptic vesicles. Munger (1965) found Merkel cells to be PAS (periodic acid-Schiff) positive particularly on the dermal aspect of the nucleus, suggesting the presence of carbohydrates whereas Smith (1977) reported them to be PAS negative, and suggested that the positive reaction found by Munger was to the glycogen stores within the neurite terminal rather than a

reaction to the Merkel cell granules. However the PAS positivity observed by Munger was diastase resistant and therefore unlikely to have been produced by glycogen wherever the reaction occurred.

More recently Hartschuh, Weihe, Buchler, Helmstaedler, Feurle and Forssmann, (1979) demonstrated, at the light microscopic level, a met-enkephalin-like immunoreactivity in the Merkel cells of rats. As the strongest immunoreaction was observed in those parts of the Merkel cell with the highest granule density, they speculated that the granules were the site of the met-enkephalin immunoreaction. This, they suggested, supported the concept that the Merkel cell was a member of the paraneuronal cell system and therefore a potential neuroreceptive cell. In an experiment to test the hypothesis that met-enkephalin acted as a neurotransmitter, Gottschaldt and Vahle-Hinz (1982) made an intravenous injection of naloxone, a known antagonist of met-enkephalin, while mechanically stimulating the sinus hair follicles of the cat. Irrespective of the dose administered, there was no alteration in the response obtained from the type I afferent unit to sustained stimulation from which they concluded that Merkel cells did not function as neuroreceptor cells. In an update of their previous work, Hartschuh, Weihe, Yanaihara and Reinecke (1983) reported that the met-enkephalin immunoreaction was restricted to the Merkel cells of rodents and this reaction could not be demonstrated in Merkel cells of cat, dog, pig or man. They found in all the species investigated except rodents that Merkel cells in

different locations were VIP (vasoactive intestinal polypeptide) -immunoreactive whereas the associated sensory nerve endings gave no such reaction. It was suggested that the 'met-enkephalin-like' material shown to exist in the Merkel cells of rodents is a characteristic of those species only and possibly indicates a separate evolutionary line.

Smith and Creech (1967) found no drugs that could produce spontaneous action potentials in the afferent fibre innervating SAI mechanoreceptors. The response to standard mechanical stimulation was transiently increased by nicotine before the receptor was finally blocked. Lobeline also blocked the response but never caused stimulation. When extracts from SAI mechano-receptors of the cat were injected into other similar receptors, they did not change the response to touch nor did they produce spontaneous action potentials (Smith, 1977). Iggo and Muir (1969) treated some animals for several days, prior to recording electro-physiologically from them, with sufficient reserpine to deplete catecholamine stores. Again they failed to modify the behaviour of the receptors.

In a different approach to determine the involvement of the osmophilic granules in the transduction process, Anand, Iggo and Paintal (1979) tested the effects of extreme hypoxia on granule number and distribution and on the response of receptors to standard mechanical stimuli. This was a brief preliminary study which did not quantify either the changes produced by hypoxia on the electrophysiological response of SAI mechanoreceptors when mechanically

stimulated or on the ultrastructure appearance of Merkel cells in hypoxic conditions.

Cats were ventilated with 99.9% N_2 while at the same time N_2 was passed over the limb containing the SAI mechanoreceptor which was being mechanically stimulated. Immediately upon receptor exhaustion the limb was rapidly perfused with fixative. Subsequent histological examination of the SAI receptor revealed an almost total loss of granules from the Merkel cells. As the author's point out, this result shows a lability of the granular vesicles but does not however, resolve the role of Merkel cells in the transduction process. In an extension of this work Anand et al. (unpublished) found that the effects of hypoxia were reversed by replacing the N_2 around the limb with O_2. When this was done the response returned in less than 30 s. Once the response to mechanical stimulation had returned to 'normal', N_2 was reapplied to the limb and the response quickly faded again. This process could be repeated several times for up to 30 minutes after circulatory arrest.

The basis of the work presented in chapter 3 of this thesis was the need to confirm the preliminary results of Anand et al. (1979) and to quantify the functional and structural changes produced by hypoxia. A detailed introduction to these experiments is presented at the start of chapter 3.

CHAPTER 2

Materials and Methods

MATERIALS AND METHODS

Part 2.1. Choice of experimental animals

In all experiments performed either male or female cats or male albino Wistar rats were used. Rats were used, wherever possible, to confirm data already obtained in cat experiments or for the testing of new experimental procedure. However, cats were used predominantly because of the ease with which SAI mechanoreceptors could be visually located and mechanically stimulated when compared to SAI receptors of other animal species.

In later experiments in which it was necessary to cannulate the femoral blood vessels as well as dissect out the saphenous nerve, cats only could be used. This was because in rats there was an insufficient area of leg available for the location and stimulation of the receptors after the necessary surgical procedures, required in these experiments, had been carried out.

Part 2.2. Anaesthesia

Twenty five cats weighing between 1.8 - 2.8Kg, mean weight 2.3 $\pm$ 0.2Kg (S.E.M., n=25) had anaesthesia induced by a 4% mixture of halothane in O_2 followed by an intravenous injection of chloralose (70mg/Kg). Supplemental doses of chloralose (40mg) were administered intravenously as required.

Four rats weighing between 300-410g, mean weight 334$\pm$43g, (S.E.M., n=4) were injected intraperitoneally

with 25% urethane made up in 0.15M saline (175mg/100g BW). Supplemental doses of anaesthetic (250mg) were adminstered intraperitoneally as required.

Part 2.3. Surgical techniques

The anaesthetised animal was placed on its dorsum on a thermal blanket which was controlled by a rectal probe to maintain a body temperature of 37°C.

A mid-line incision was made in the neck and the trachea cannulated. This cannula maintained a patent airway throughout the experiment and was also used in later experiments to ventilate the cats with N_2. In some experiments the carotid artery was also cannulated and connected to a blood pressure transducer. The blood pressure measured by the transducer was displayed on a chart recorder (Devices MX 212).

If the abominal aorta was to be used for the perfusion of the lower limbs the abdomen was shaved and a 50-75mm mid-line incision made from the umbilicus caudally. The abdominal muscles were parted in the mid-line and loops of intestine removed to gain access to the abdominal aorta and inferior vena-cava which were enclosed in fat and connective tissue deep to the intestine. These blood vessels were dissected free for a short length from the surrounding tissues and threads were passed around them. The ends of the threads were taken out through the incision in the abdominal wall, the intestine was replaced and the abdominal muscles and overlying skin were clipped shut.

The appropriate rear leg was shaved with electric clippers from the area overlying the saphenous nerve to

the foot. An incision was made over the saphenous nerve from the lower edge of the abdominal wall to a point just proximal to the knee. The skin flaps were reflected and threads passed through the cut edges which were then tied to a ring placed over the incision. The tissue within the pool formed by the skin flaps was kept moist by the application of saline from a Pasteur pipette. The limb was then fixed in place by plastering the foot to a wooden block with plaster of Paris bandage.

Part 2.4. Dissection of fibres from the saphenous nerve

The method employed for the dissection of single identifiable fibres from the nerve was that described by Iggo (1959). An earth electrode was inserted into the gracilis muscle next to the saphenous nerve and stitched in place. The saphenous nerve was then carefully dissected free from the surrounding connective tissue for a distance of 20-30mm and placed on a 10mm wide black perspex dissecting platform. The remaining dissection of the saphenous nerve was performed with the aid of a binocular dissecting microscope (Carl Zeiss, x6 to x40 magnification).

The epineurium around the saphenous nerve was removed for 10-15mm using fragments of a safety razor blade held in pin tongs. Once the sheath was removed nerve fascicles making up the saphenous nerve were seen clearly. The saline within the pool was removed, either by a Pasteur pipette or absorbent cotton wool and replaced by liquid paraffin B.P. warmed to 37°C.

With fine forceps a single fascicle was separated out from the others and the connective tissue around it removed.

The fascicle was divided along its length and the fibres within it laid out carefully on the dissecting platform. It was important not to have the fibres twisted as this made their separation difficult. With fine forceps and pins a small strand of fibres was removed and placed on the recording electrodes. A smooth glass probe was then drawn over the skin to identify the receptive field of the fibres and for the location of SAI receptors in particular. These were recognised by their characteristic high frequency discharge as the probe displaced them laterally. If activity from SAI receptors was present in the strand it was further sub-divided until eventually a fine filament of nerve remained containing a single identifiable unit which innervated one or more SAI receptors. Where necessary, strands of fibres which innervated SAII receptors or hair follicles as well as SAI receptors were located and recorded from.

Part 2.5. Mechanical and electrical stimulation of SAI mechanoreceptors

The basic arrangement for both mechanical and electrical stimulation of SAI receptors and the recording of the responses obtained is illustrated in Figure 2.1. A master oscillator (Digitimer, Devices Ltd.) initiated the sequence of events by triggering the oscilloscope (Tektronix 5103N). Figure 2.2 shows the timing of the output pulses from the Digitimer.

FIGURE 2.1

Illustrated are the general methods used for mechanical and electrical stimulation of a touch dome. Also shown is the basic recording arrangement for the responses produced by this stimulation.

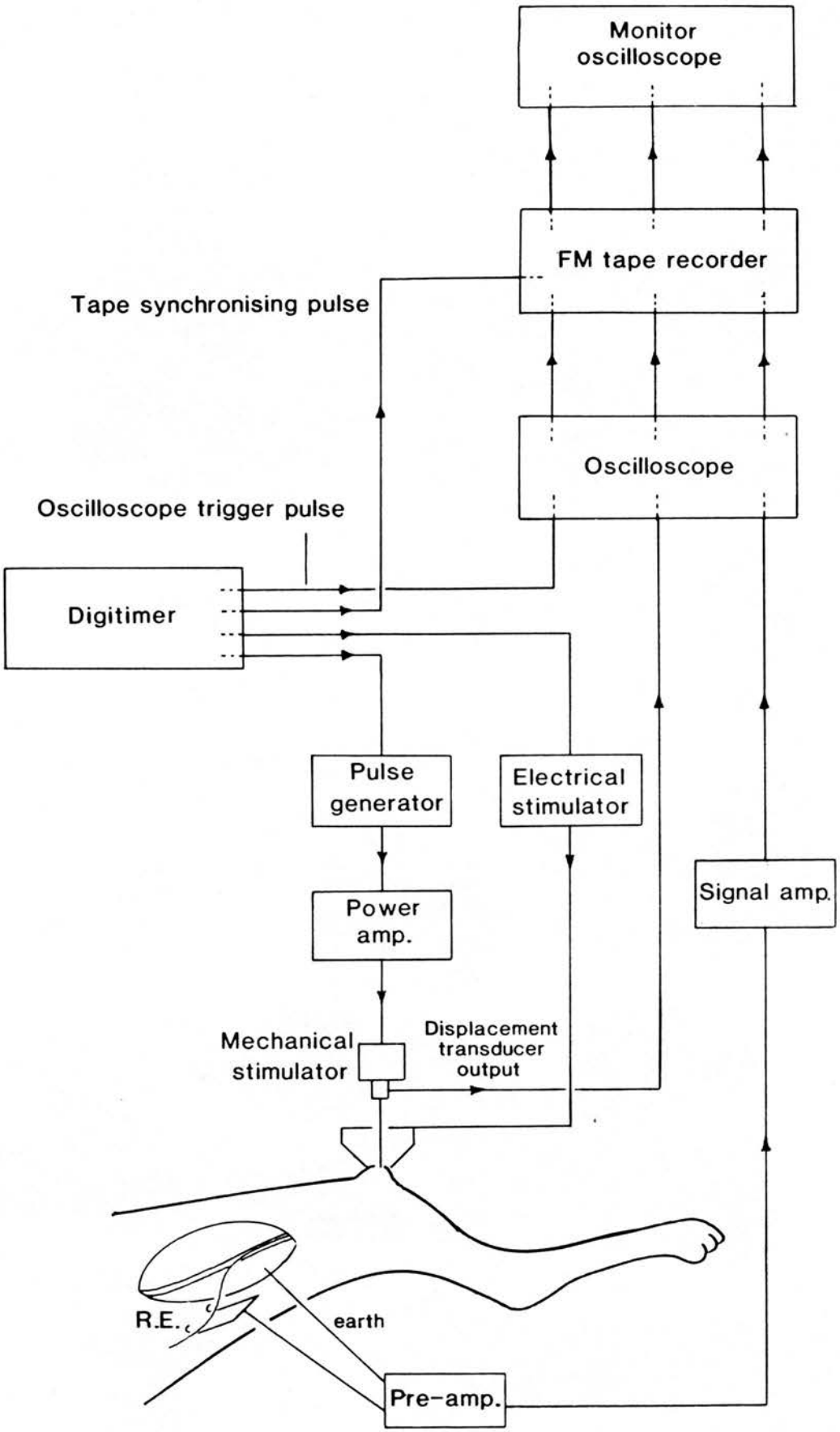
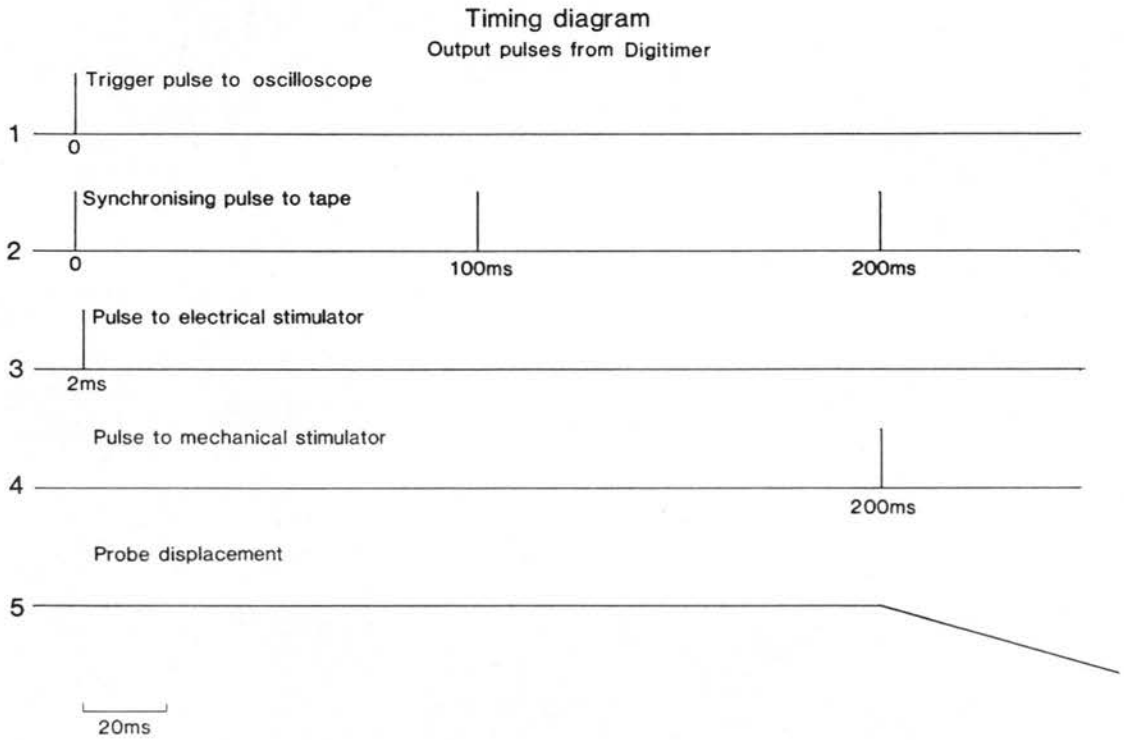

Monitor oscilloscope
FM tape recorder
Tape synchronising pulse
Oscilloscope
Oscilloscope trigger pulse
Digitimer
Pulse generator
Electrical stimulator
Signal amp.
Power amp.
Mechanical stimulator
Displacement transducer output
R.E.
earth
Pre-amp.

FIGURE 2.2

Timing diagram showing the sequence of output pulses from the Digitimer. Each pulse had an amplitude of -12v with a duration of 100μs.

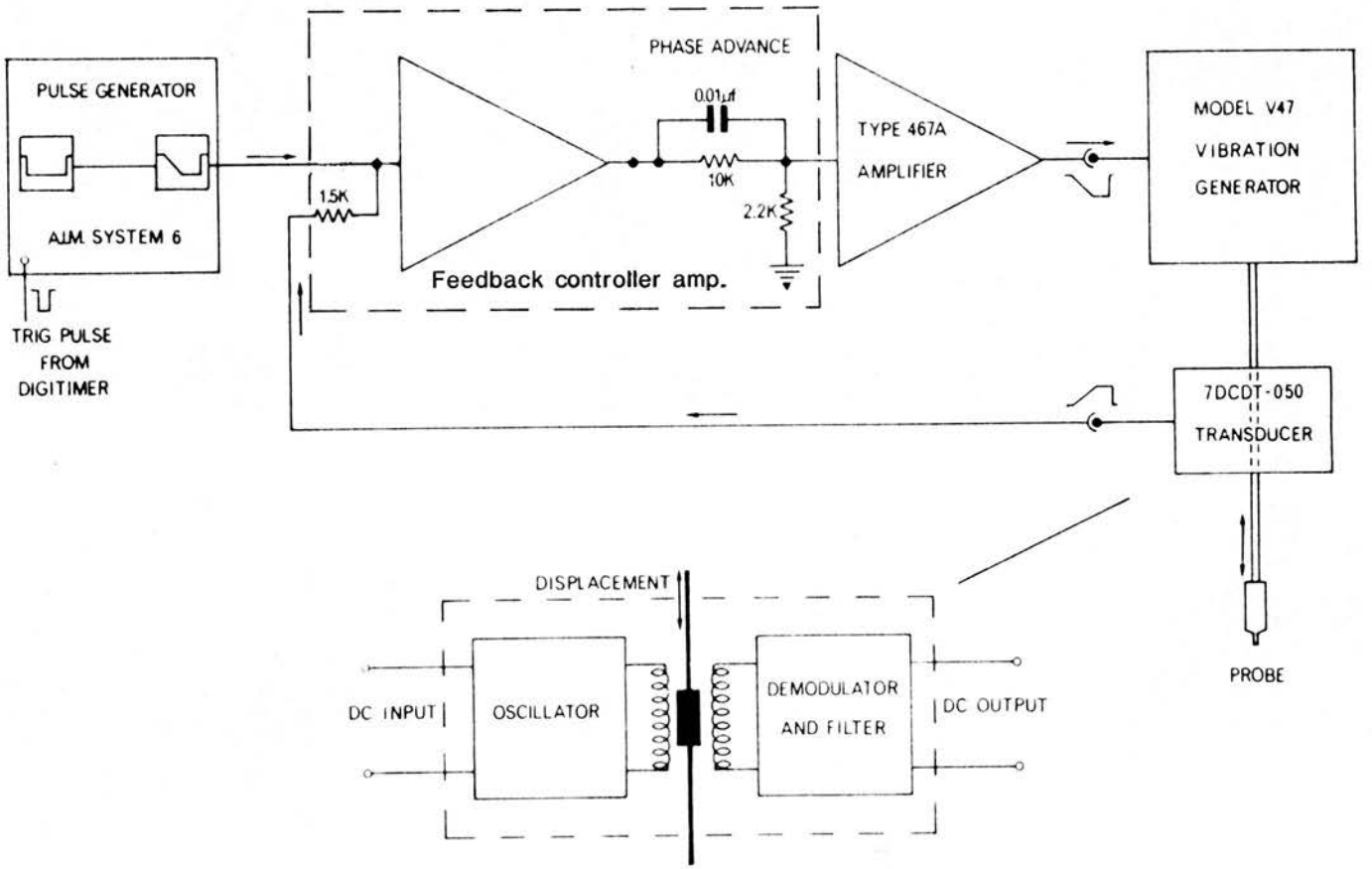

Timing diagram
Output pulses from Digitimer
Trigger pulse to oscilloscope
1
0
Synchronising pulse to tape
2
0
100ms
200ms
Pulse to electrical stimulator
3
2ms
Pulse to mechanical stimulator
4
200ms
Probe displacement
5
20ms

Two hundred milliseconds after triggering the oscilloscope another pulse from the Digitimer was fed into a pulse generator (AIM PWD 103A). This module produced pulses variable in duration between 25ns and 10s which were then fed into a variable rise/fall module (AIM VRF 107). This unit produced pulses of independently variable rise and fall times at any voltage level between $\pm$ 20V. This pulse was passed to a mechanical stimulator system which consisted of a feedback controller amplifier, a power amplifier, a mechanical stimulator and a probe displacement transducer (Figure 2.3). The pulse entered the feedback controller amplifier, the output of which was connected to a power amplifier (Hewlett Packard type 467A). The current produced by the power amplifier was sufficient to drive a mechanical stimulator (Model V47, Goodmans Industries Ltd.,) The stimulator worked on the same principle as a moving coil loudspeaker and followed the shape of the input waveform, turning electrical energy into a mechanical displacement. A thin steel rod was connected to the moving core of the stimulator. The movement of this steel rod was monitored by a displacement transducer (7 DCDT transducer; Hewlett Packard). This gave a D.C. output proportional to the displacement which was fed back into the feedback controller amplifier and which was also displayed on the oscilloscope. The function of the feedback controller amplifier was to improve the tracking of the electrical signal by the mechanical stimulator. Attached to the rod was a 19G needle the point of which had been removed and plugged with solder producing a

FIGURE 2.3

A schematic diagram of the mechanical stimulator system. The pulse from the master oscillator (Digitimer) entered a pulse generator the output of which, after amplification, was passed to the mechanical stimulator. This produced a probe displacement which was monitored by a probe displacement transducer. The output voltage from the transducer was fed back to a feedback controller amplifier which modified the voltage output to the mechanical stimulator. This ensured that the mechanical stimulator followed accurately the electrical signal applied to it.

MECHANICAL STIMULATOR SYSTEM



7DCDT TRANSDUCER SCHEMATIC DIAGRAM

smooth probe tip of diameter 150-200μm.

The stimulator was mounted on a horizontal arm which was attached to an upright pillar. It could be moved along the horizontal arm on a simple sliding carrier which could be angled to bring the stimulator probe normal to the receptor surface. Accuracy of placement of the probe tip was achieved by a fine height adjustment fitted to the carrier. A coarse height adjustment was incorporated in the vertical pillar. At the base of the pillar was a horizontal adjustment giving two fine adjustments at right-angles to each other.

The output from the displacement transducer was displayed on the oscilloscope. The probe displacement, at different transducer output voltages, was measured with a calibrated eye-piece graticule fitted into the dissecting microscope. From this a calibration curve (Figure 2.4) was drawn which was used in all experiments to determine the probe displacement in microns for a given displacement transducer output as displayed on the oscilloscope. The rate of displacement of the probe was also obtained from the oscilloscope by measuring the time taken for the probe to reach its final displacement. Illustrated in Figure 2.5 is a photograph of an oscilloscope trace of the displacement transducer output voltage, and the probe displacement and rate of displacement this represents.

In these experiments the rates of displacement used were approximately 0.75 μm/ms to 1.5μm/ms with a displacement of 150-250μm. The duration of displacement was 1.5s with an interstimulus interval of about

FIGURE 2.4

This calibration curve was used to calculate the probe displacement (μ) from the displacement transducer output (v) as displayed on the oscilloscope.

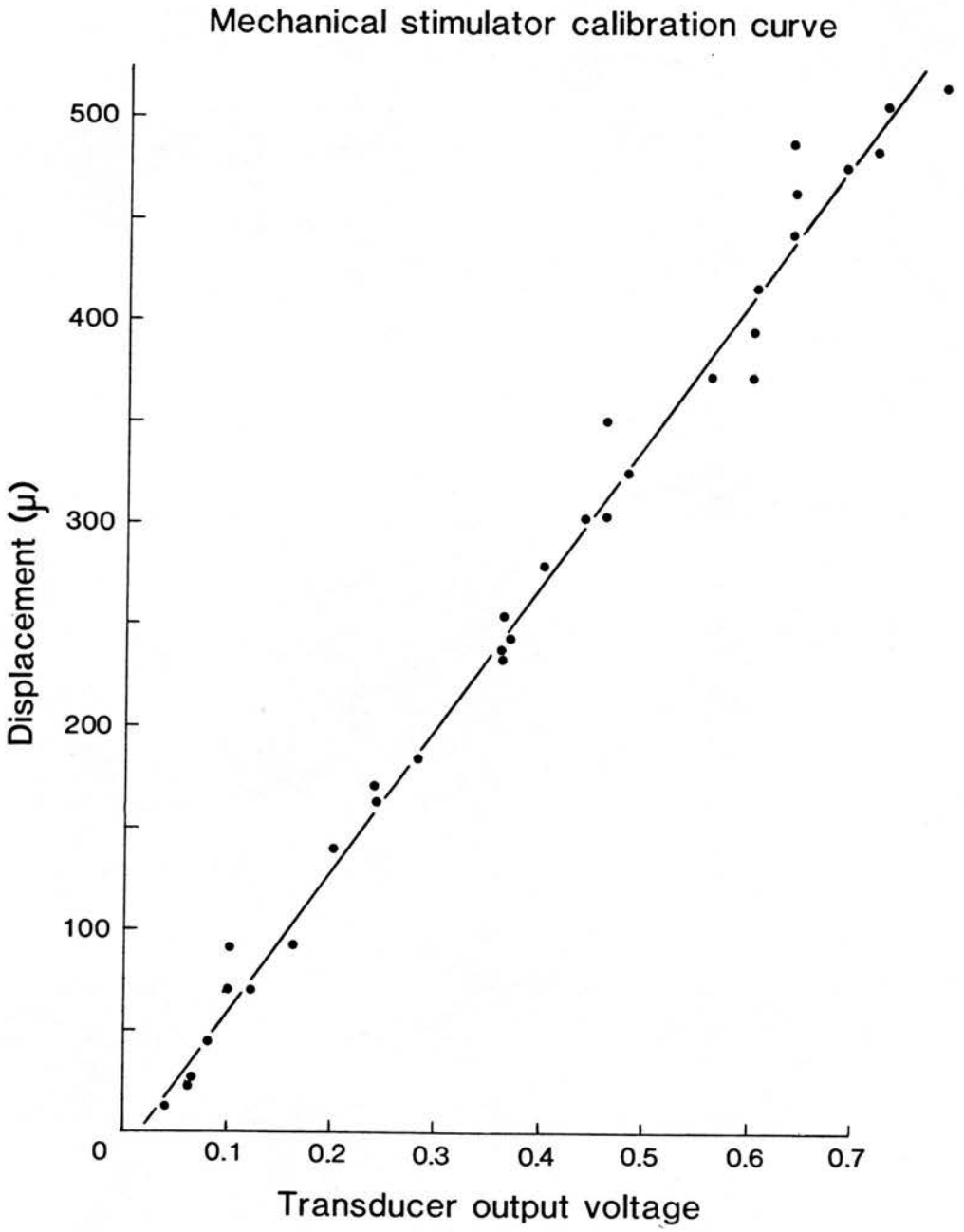
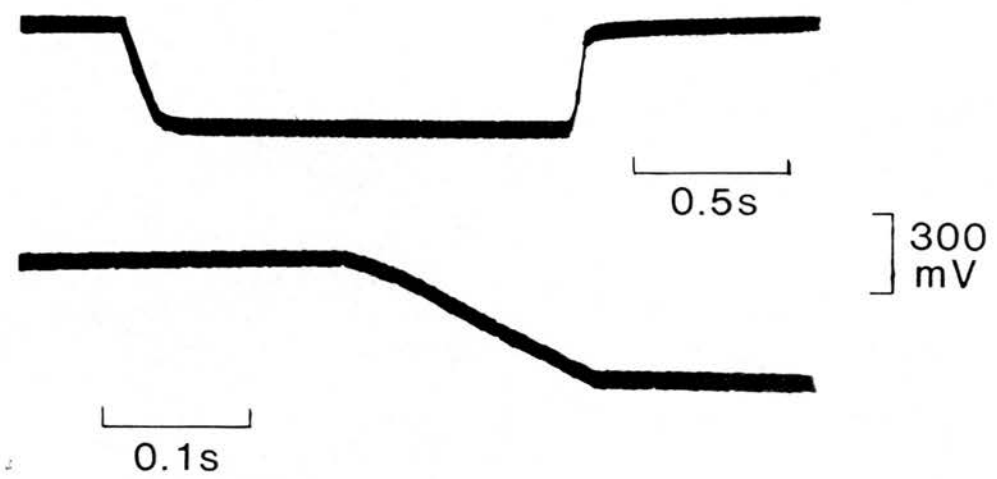

Mechanical stimulator calibration curve
Displacement (μ)
500
400
300
200
100
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Transducer output voltage

FIGURE 2.5

Oscilloscope traces showing the displacement transducer output as it was displayed on the oscilloscope. A downward deflection of the trace reflects a displacement of the probe. The displacement transducer output is approximately 400mv which from the calibration curve in Figure 2.4 represents a displacement of about 250μm. The expanded bottom trace is of that part of the top trace when the probe is moving from rest to its final displacement. It allows the time taken for the probe to reach its final displacement to be accurately measured enabling a calculation of the rate of displacement to be made. In the example shown the 250μm displacement occurs in approximately 150ms giving a rate of displacement of 1.67 μm/ms.

Displacement transducer output



ls. The probe was placed onto the receptor normal to its surface, with the aid of a disecting microscope, until a response was just obtained. This ensured that the receptor was displaced by the same amount at each stimulation. In order to overcome the problem of 'skin creep' away from the probe tip during mechanical stimulation (Pubols, 1982a and b), the probe position was checked and adjusted if necessary so that contact between the probe tip and the receptor was maintained at all times. All adjustments of the stimulator, where carried out, are indicated in the results.

Electrical stimulation of SAI receptor afferent fibres close to the Merkel cells was carried out as a test of nerve conduction in various experimental conditions, particularly when the response to mechanical stimulation had ceased. Two 33G silver-chloride electrodes, which were connected to the output terminals of an isolated constant voltage electrical stimulator (Devices type 2533), were implanted into the skin on each side of the receptor. The voltage necessary to produce an action potential in each afferent fibre for approximately 50% of all stimulations was determined and all subsequent stimulation was carried out at 20% over this threshold voltage. An initial pulse from the Digitimer triggered the oscilloscope followed 2ms later by a pulse which triggered the electrical stimulator. Two hundred milliseconds after this a further pulse from the Digitimer triggered the mechanical stimulator. This timing sequence meant that electrical stimulation of the afferent fibre always preceded mechanical

stimulation of the receptor (Figure 2.2).

Part 2.6 Recording and amplification of afferent fibre activity

In these experiments conventional recording techniques and amplification of electrical signals were used. Figure 2.1 illustrates the arrangement of the recording equipment.

The first pulse from the Digitimer, which triggered the oscilloscope, plus the tape synchronising pulse were recorded on a tape recorder (Ampex PR-500). Another tape recorder channel was used to record the voltage output from the displacement transducer of the mechanical stimulator. This was also displayed visually on the oscilloscope.

The signals produced in the nerve fibres to mechanical and electrical stimulation were initially amplified by an instrumentation amplifier with an input impedance in excess of 10MΩ. This amplifier was situated as close to the preparation as possible to reduce 'noise' problems. The output from this pre-amplifier was fed into a signal processing amplifier with a gain range which was variable between 1 and 1000. There was also a step gain facility which could give gains of X1, X10, X100 and X1000. The amplifier had an A.C. coupled high pass filter and a D.C. coupled low pass filter. The amplified output from this amplifier was fed into a channel of the oscilloscope and on to a channel of the tape recorder.

Part 2.7 Photographic recording of afferent fibre activity

Traces of afferent fibre activity were stored on the oscilloscope screen and photographed with a Tektronix C-5A oscilloscope camera loaded with Polaroid film .

Part 2.8 Blood Gas Analysis

Approximately 1ml of arterial blood was collected in a heparinised glass syringe and, if possible, analysed immediately with a blood gas analyser (IL 213-227 pH-Blood gas analyser). If the sample had to be kept before it could be analysed, the syringe was capped and placed in a vacuum flask packed with ice. Samples were never kept longer than 20 minutes before being analysed.

At the start of every experiment, before samples were analysed, both the balance and slope calibrations for the PCO_2 electrode and the balance and zero calibrations for the PO_2 electrode were carried out. Because the balance calibration for both electrodes drifted over short periods of time, this was checked before each sample was introduced to the machine.

The blood was mixed and warmed by rotation of the syringe between the palms before it was drawn into the auto-sampler. The mode selector was set to PCO_2 and when it was stabilised, the reading on the digitial display was recorded. The same procedure was repeated with the mode selector set to PO_2 for measurement of PO_2 levels in the blood. After analysis of every sample, the sample chamber was flushed out with saline to remove all traces of blood.

Part 2.9. Measure of Co^{2+} and Ca^{2+} levels in the blood

Approximately 1ml samples of arterial blood were removed from the circulation and put into non-heparinised narrow-bore glass tubes. These were placed in a test-tube rack, in a water bath at 37°C, for two hours. The serum subsequently formed was placed in labelled glass vials and deep frozen at -20°C until analysis of samples from several experiments could be made at one time.

Samples were analysed using an atomic absorption spectrophotometer (Unicam SP 90 Series 2; Pye Unicam Ltd.,). This instrument, which measures the total Co^{2+} and Ca^{2+} present in serum, operates on the principle that a hollow cathode lamp, whose cathode incorporates the element to be determined, emits the line spectrum characteristic of that element. This radiation is passed through a flame and the resonance lines are partially absorbed by the atoms of the same element, should these be present in the flame. By comparing the intensities of the light transmitted by the flame before and after the introduction of the sample, the instrument indicates the amount of light absorbed, which is a measure of the concentration of the element in the sample.

Before a series of serum samples were tested , standard solutions containing known concentrations in mM of Co^{2+} and Ca^{2+} were made up in distilled H_2O and introduced into the machine. By plotting the reading on the absorbance readout scale against the concentration of the element being detected in the standard solutions, standard curves were drawn for both

Co^{2+} and Ca^{2+} (Figures 2.6 and 2.7). These curves were drawn, however, after a correction had been introduced for distilled H_2O which appeared to contain trace amounts of Co^{2+}. The absorbance reading for distilled H_2O was subtracted from the absorbance readings of the standard solutions before the graphs were plotted.

Serum samples to be tested were defrosted and diluted 1:5 with distilled H_2O. These were introduced to the spectrophotometer and the absorbance reading noted. From this was subtracted the absorbance readings for H_2O. The concentration of the appropriate element was then obtained from the standard curve for that element and the value obtained was multiplied by x5 to take account of the dilution factor. To check the stability of the instrument a standard solution was chosen at random between every serum sample tested, and analysed in the machine. The readout obtained was checked against the value obtained for that solution when it was measured for the production of the standard curve.

Part 2.10. Histological Procedures

Perfusion fixation Perfusion of the lower limbs was performed through either the abdominal aorta or the femoral artery of the appropriate leg.

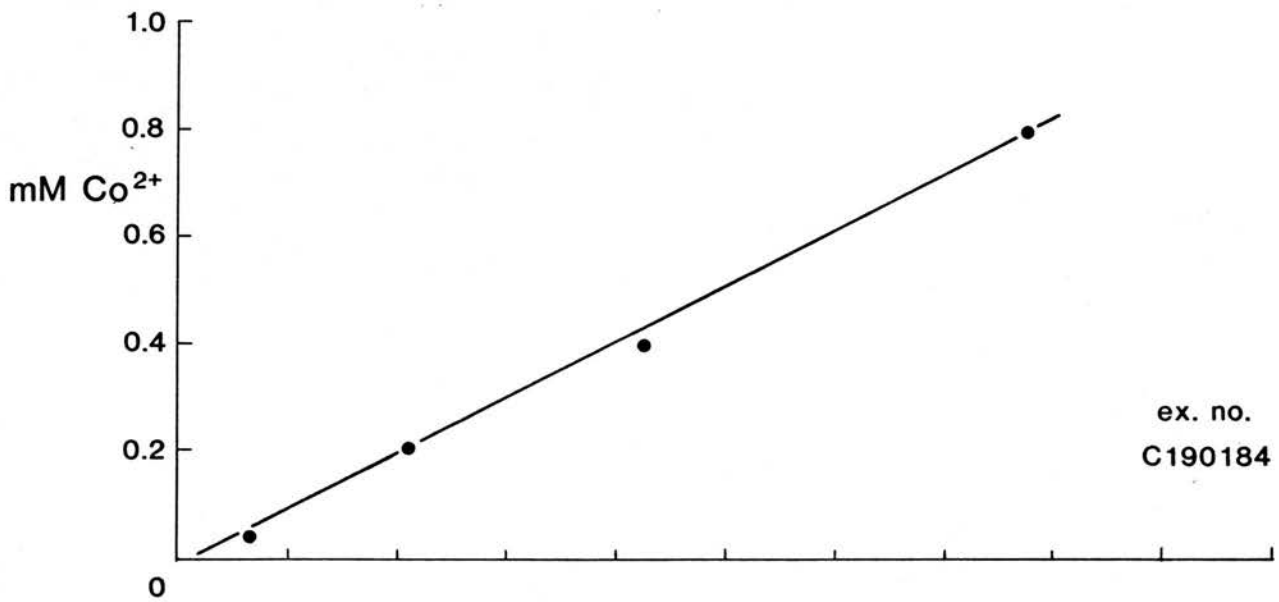
Perfusion via the abdominal aorta was performed in early experiments in which the femoral artery was not cannulated. Part 2.3 describes the method used to locate the abdominal aorta and inferior vena cava. The location of these blood vessels before the start of the experiment was necessary as, in these experiments in

FIGURE 2.6

Cobalt calibration curves for experiments C190184 and C260184. If there was a delay between the testing of samples from different experiments then new calibration curves were drawn for each experiment. These curves were used to calibrate the serum cobalt levels in mM in the limb circulation, from the absorption values obtained when serum was passed into an atomic absorption spectrophotometer.

Standard curve

Cobalt



Cobalt

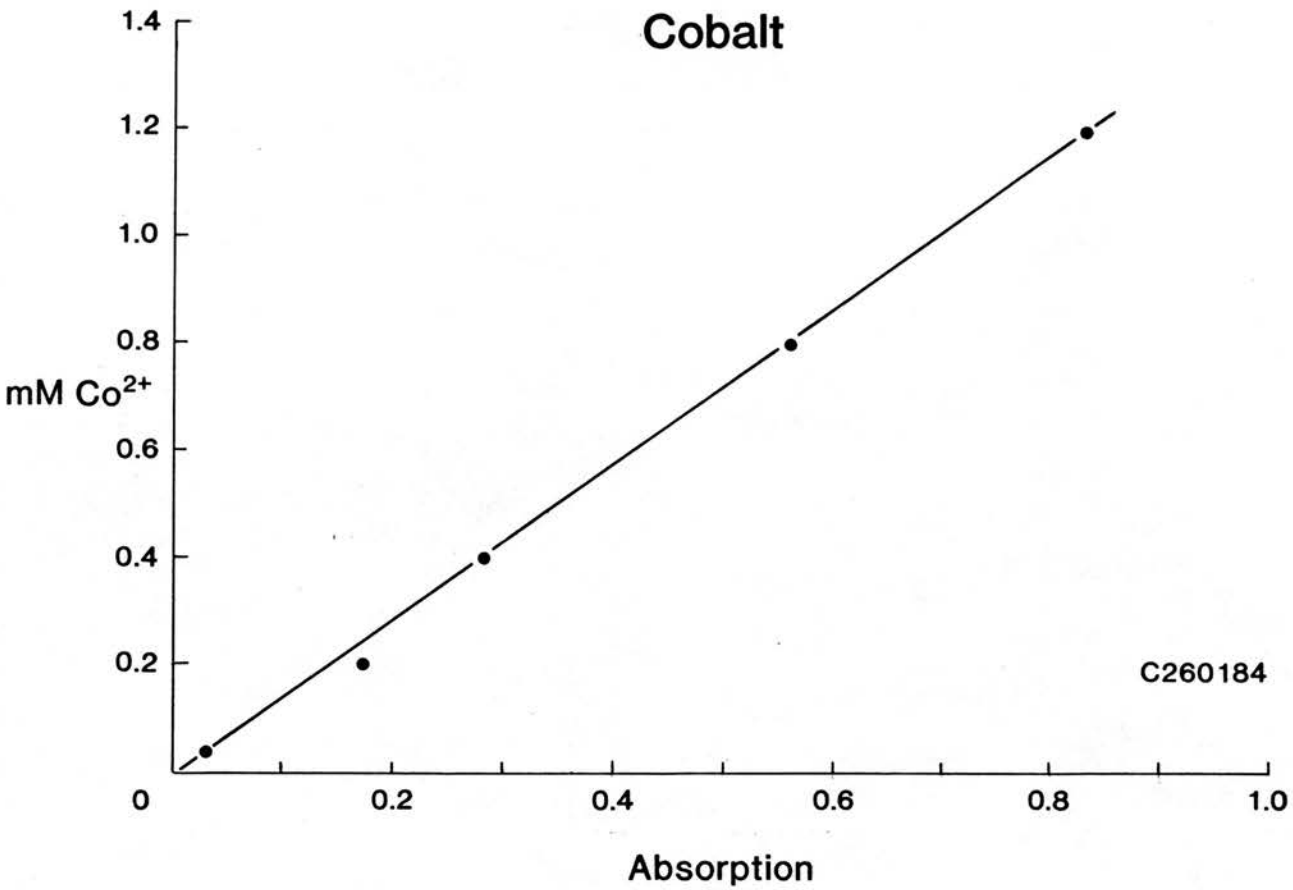
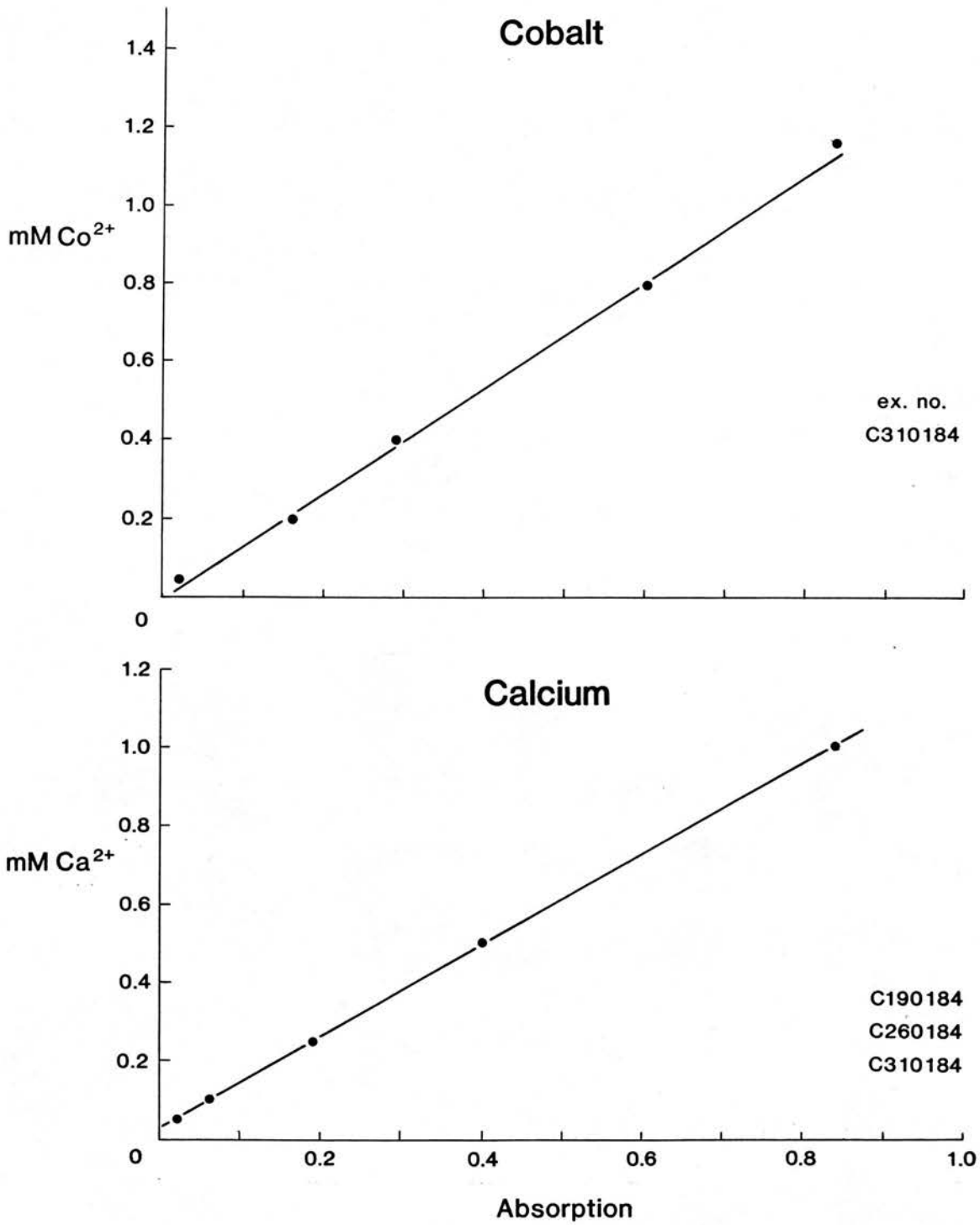


FIGURE 2.7

Cobalt calibration curve for experiment C310184. Also shown is the calcium calibration curve for experiments C190184, C260184 and C310184. Samples from all three experiments were tested at the one time hence the presentation of only one calibration curve.

Standard curve



which this perfusion procedure was used, rapid fixation of the tissues at the end of the experiment was required so that the structure of the Merkel cell at the moment of receptor failure could be visualised.

At the conclusion of the experiment, the clips were removed from the abdominal incision, the intestine was displaced to one side and the abdominal aorta and inferior vena cava located by means of the previously inserted threads. Both blood vessels were occluded and a 13G needle, which was connected to a perfusion pump, was pushed into the peripheral end of the artery. A 50ml prefixative solution of 0.15M NaCl and 0.01% $NaNO_2$ (vasodilator) was pumped into the artery. The inferior vena cava was kept occluded for a short time for pressure to develop in the vascular system of the limbs. The vein was then cut to allow the free flow of the solution around the limbs. Without interruption to the flow, the prefix solution was replaced by 200ml of fixative which consisted of 4% paraformaldehyde/2.5% glutaraldehyde in 0.1M cacodylate buffer at pH7.4. At the same time fixative was applied topically to the surface of the receptors which were removed for histological examination.

Perfusion through the femoral artery was used in later experiments in a preparation which required the cannulation of the femoral blood vessels of the leg being used in the experiment. The surgical procedures involved and the cannulation and inter-connection of the blood vessels is referred to in detail in Chapter 3.

Perfusion of the prefixative solution and the

fixative was carried out by connection of the perfusion apparatus to the cannula inserted into the peripheral end of the femoral artery. Return flow was from the cannula inserted into the peripheral end of the femoral vein.

Immersion fixation Fixative was applied to the receptor surface before it was dissected from the limb.

Tissue processing All tissue processing and the subsequent data analysis of the histological material obtained was carried out by Dr. E.J.Cooksey. Tissue containing the receptors was dissected from the limb with a sharp scalpel blade and placed immediately into vials of fixative. Each piece of tissue was then pinned out under a dissecting microscope for careful removal of individual receptors from the pieces of tissue. These were placed in 4% paraformaldehyde/2.5% glutaradehyde/0.1M cacodylate buffer overnight at $4^{o}C$.

The tissue was then processed as followed :-

1) 0.1M cacodylate buffer wash
   3 x 15 min.
2) post-fixed in 1% OsO_4/0.1M cacodylate
   1 x 60 min.
3) 0.1M cacodylate buffer rinse
   1 x 15 min.
4) dehydration : 30% acetone
   2 x 10 min.
5) dehydration : 60% acetone
   2 x 10 min.
6) " : 90% acetone
   2 x 10 min.

7) " : 100% acetone

2 x 15 min.

8) acetone 1:1 araldite (3)

overnight.

9) araldite (3)

2 x 24 hours.

10) araldite (4)

1 x 24 hours.

The araldite mixture used was :-

DDSA	11 ml	araldite (3)	araldite (4)
CY 212	9 ml		
Dibutyl phthalate	0.75 ml		
DMP 30	0.5 ml		

The receptors were then embedded in araldite (4) in a silicon embedding mould and polymerised at 60°C for 48 hours.

Light and electron microscopic examination Sections were cut on a ultramicrotome (Reichert Ultracut OM U4) using glass and diamond knives. For examination in the light microscope (Nikon), 1µm sections were cut and stained with 1% toluidine blue and 1% borax. Sections of approximately 80Å thickness were collected on Athena 200 thin bar grids, stained with uranyl acetate and lead citrate, and viewed and photographed in a Phillips EM 400 electron microscope.

Part 2.11. Data Analysis

Electrophysiology In order to determine the overall effects of the various experimental conditions on the response of SAI mechanoreceptors to mechanical

stimulation, all data recorded on tape was analysed on a Cromemco System 3 microcomputer using programs RATE 1 and RATSTAT 2 (Short, A.D. unpublished). RATE 1 displayed, stored and plotted the impulse frequency over selected time intervals which were variable from 200 to 4000 ms. RATSTAT 2 gave the average impulses per second over a given time selected from the histogram display produced by RATE 1. Data for graphs and histograms was obtained by totalling the number of spike counts in 10 seconds, in which time there were 4 complete cycles of mechanical stimulation of the receptor, and averaging this to the number of spikes in one second. In the control period of receptor stimulation the average response of the receptor in counts per second ($c.s^{-1}$) over a 2 minute period was taken as the control response of the receptor. All subsequent responses of the receptor were then averaged over 10s periods to the number of counts per second and the values obtained plotted as a percentage of the control response. All responses obtained from different experiments were normalised in this way. This enabled comparison of the responses obtained from different experiments even although the responses of individual receptors in each experiment were different.

The above analysis, as already stated, gave information about the overall effects of hypoxia and Ca^{2+} channel blockers on the response of SAI mechanoreceptors to mechanical stimulation. More precise analysis, similar to that described by Iggo and Muir (1969), was carried out using the data collection program GSPIKE and an analysis program GDISP

(McConnell, G., unpublished). By selecting an appropriate bin-width, in this case 1.0ms, the time between spikes (inter-spike intervals or ISI's) was measured and stored for various periods of mechanical stimulation throughout experiments. The data was represented graphically by a display which plotted successive interval lengths against interval number. For analysis purposes the trigger pulse preceeding each application of the mechanical stimulator to the touch dome was also indicated on the display. This enabled the sampling time for ISI's within each stimulation of the touch dome to be accurately controlled. So that interval collection was carried out when the probability of adaptation of the response was greatest, the sampling period chosen was the last 500 ms of the 1.5s stimulation of the SAI receptor. The ISI's during this time, for up to 20 stimulations of the receptor were collected at different times throughout the various experiments.

Where different afferent fibres innervated different SAI receptors or where both an SAI and an SAII receptor was present, the response of the SAI receptor or the backround activity of the SAII receptor could be differentiated on spike amplitude and duration. By playing the recorded responses of the receptors through a Neurolog spike discriminator (Digitimer Ltd.) it was possible to separate the responses from each receptor for individual analysis.

In all experiments in which the response to mechanical stimulation decreased as a result of some experimental procedure, receptor failure was said to

have occurred when the response had fallen to 10% of the control firing rate. This procedure was adopted as intermittent activity, unrelated to mechanical stimulation, was recorded from several SAI units. This consequently made the time of receptor failure difficult to determine.

Histology Measurement of the numerical density of the vesicles within Merkel cells were made with the aid of a Magiscan (Joyce Loebl). An electron-micrograph of a Merkel cell was viewed on the interactive video display screen. Outer and inner area boundaries were traced manually with a light pen and automatically displayed on the video screen as white lines. It was possible to measure complex or discontinuous areas simply by always keeping the area of interest to the same side of the boundary line. In this way it was possible to determine the area of the cytoplasm alone in the Merkel cell.

The light pen and binary display were also used for the vesicle counting; each vesicle was located with the light pen and recorded as a white point superimposed on the video image of the vesicle, thus avoiding omissions and double counts. On completion of analysis the areas, counts and densities were printed out.

Part 2.12 Statistical Analysis of data

Levels of significance between results were tested by means of the Student's single or 2-tailed 't' test. The 'F test' was used where it was necessary to determine if the difference in variability between

results was significant or not.

In the analysis of the ISI distribution a test for trend in the response during the collecting period (and hence a test for stationarity) was applied. To do this the mean interval length for each 500ms period of stimulation was plotted against the stimulation number and linear regression analysis applied to the data (Cox and Lewis, 1966). The regression coefficient obtained was then tested against a line of zero slope (perfect stationarity) and the difference between the two tested for significance using the Student's 't'-test. Where the response showed stationarity the ISI's during the last 500 mS of each stimulation for the period of the experiment being considered were pooled and a histogram produced of the distribution. This distribution was truncated at the maximum frequency of the histogram and an exponential of the form $f(t) = e^{-\lambda t}$ (where λ is the reciprocal of the mean ISI of the truncated distribution) fitted to it. The 'goodness of fit' of the histogram to the curve was tested using the chi-squared (X^2) test.

The distributions during the sampling periods of an experiment were then compared with the normalized control distribution for that experiment and the difference between the two distributions tested, again using the chi-squared test.

Throughout the thesis, experiment and unit numbers are based on the following naming procedure:

1) the number of the experiment is based on the date on which the experiment was performed.

2) this number is prefixed with a 'C' if cats were

used and 'R' if rats were used.

3) where several units in a single experiment were examined then each experiment number is suffixed with a number which allows identification of the particular unit under investigation.

4) where only one unit in an experiment was examined then the unit number is the same as the experiment number.

CHAPTER 3

Hypoxic Experiments

HYPOXIC EXPERIMENTS

Part 3.1 Introduction

The experiments were based on the preliminary, unconfirmed work of Anand *et al.* (1979) in which they found that mechanical stimulation of SAI mechanoreceptors (also referred to throughout the text as SAI receptors or touch domes) in hypoxic conditions caused the eventual failure of these receptors. Correlated with receptor failure was a depletion in the number of dense-cored vesicles present in the Merkel cell cytoplasm. For receptor failure to occur it was necessary to have an O_2 deficient environment outside the limb as well as an hypoxic blood supply. In later unpublished work these same authors replaced N_2 around the limb with O_2 and found that the response to mechanical stimulation returned even although circulatory arrest had occurred some time before O_2 was applied to the limb. These results indicated a lability of the vesicles which had not previously been demonstrated. It also suggested that the dense-cored vesicles were necessary for the receptor to function normally as vesicle depletion was correlated to a failure of the receptor response to mechanical stimulation.

The aims of the first experiments were to verify the results obtained by Anand *et al.* (1979) and to quantify both the physiological and histological changes associated with mechanical stimulation of touch

domes in hypoxic conditions. Initially the same preparation described by Anand et al. (1979) was used in which hypoxia was induced in the whole animal by ventilating it with N_2. As this procedure produced circulatory arrest within minutes of the application of N_2, only one experiment could be carried out on each animal. In later experiments a new experimental procedure was used in which only the limb containing the receptors to be investigated was made reversibly hypoxic. By using this preparation either several different units could be investigated in one experiment or an experiment could be maintained until a suitable unit was obtained.

In both the preparations used to produce hypoxia, the speed at which the PO_2 levels fell in the circulation could not be controlled. This therefore meant that the time taken to receptor failure, should it be related to the PO_2 levels in the circulation, would be different between one experiment and another. In an attempt to reduce some of the variation in the time to receptor failure, experiments were carried out in which SAI receptors were stimulated in sufficiently hypoxic conditions where it was known that receptor failure would occur. The degree of hypoxia in the circulation was determined by measuring the PO_2 and PCO_2 levels in the circulation particularly at the moment of receptor failure.

Other experiments were carried out to determine the speed at which SAI receptors and the Merkel cells in particular responded to changes in the levels of O_2 present in the external environment. In all

experiments, tissue was removed so that the numerical density of dense-cored vesicles in the Merkel cells at a specific time in an experiment could be related to the electrophysiological response of the receptor at that time.

Afferent fibres innervating SAI receptors were tested for conduction of action potentials by electrical stimulation of the fibre when the receptor response to mechanical stimulation had ceased.

Part 3.2 Experimental Protocol

Hypoxia established in the whole animal ± N_2 applied to the limb.

Preparation of animals. Three cats weighing between 1.8 - 2.3Kg and one rat weighing 300g were used. The animals were anaesthetised, placed on their dorsum on a thermal blanket and the trachea was cannulated. This cannula maintained a patent airway throughout the experiment and was also used later in the experiment to ventilate the cat with N_2. A cannula was also inserted into the carotid artery and connected to a blood pressure transducer for the measurement of blood pressure.

In cat experiments an incision was made in the abdomen and the abdominal aorta and inferior vena cava located (see Chapter 2.2). Threads were passed around these blood vessels so that they could be quickly found at the end of the experiment for perfusion of the lower limbs.

An SAI receptor was located and its response to mechanical stimulation was recorded from fibres

dissected from the saphenous nerve as described in Chapter 2.4. This period of mechanical stimulation of the touch dome before the animal was made hypoxic was taken as the control response of the receptor with which all other responses of that receptor were compared.

Induction of Hypoxia. A polythene sock, with entry points for the mechanical stimulator probe and a gas tube, was placed around the limb and made as gas tight as possible. A Douglas bag which had been previously filled with N_2, was emptied and refilled with N_2. This procedure was adopted to reduce the possibility of there still being traces of O_2 left in the Douglas bag.

The animal was paralysed with an intravenous injection of gallamine triethiodide (flaxedil), 1ml in cats, 0.1ml in rats, and immediately connected via the tracheal cannula to a respirator, the stroke volume of which was adjusted to maintain the blood pressure at its previously recorded level. The Douglas bag was connected to the respirator input and the animal ventilated with N_2. At the same time N_2 from a cylinder was blown at the rate of 4-5 l/min, into the polythene sock around the limb. In subsequent analysis of the receptor response to mechanical stimulation, the timing of events was from the onset of hypoxia. In this preparation the onset of hypoxia was taken as the time when N_2 was applied to both the limb and the animal. The response of the receptor to mechanical stimulation was recorded throughout the experiment.

Hypoxia established in the limb only + N_2 applied to the limb

Preparation of animals. A total of 11 units, in 5 cats weighing between 1.9 - 2.4Kg, were used in 7 experiments. Each animal was anaesthetised, placed on its dorsum on a thermal blanket and its trachea cannulated.

In this preparation (Figure 3.1) the incision over the saphenous nerve was extended on to the lower abdominal wall for about 10 mm to allow access to the femoral blood vessels and their branches as they passed from under the abdominal muscles into the leg. The femoral artery and veins were dissected free from the surrounding connective tissue for a length of 10-15 mm. All large branches of the artery and vein were located and tied to restrict the circulation in the limb to that area supplied by the femoral blood vessels. Heparinised cannulae were then inserted into the peripheral and central ends of the vein and artery and interconnected by a system of taps and tubing. All the necessary preparatory work for this procedure was carried out in advance of the cannulations to minimize the time the limb was without an arterial supply during cannulation.

Venous blood returned passively from the limb to the general circulation of the animal whereas arterial blood was actively pumped down the leg by a roller pump (MHRE Mk III Flow Inducer; Watson-Marlowe Ltd.,). The animal's arterial blood pressure was measured by a pressure transducer connected to the cannula in the central end of the femoral artery. The pressure of the blood at the pump output was also measured by a blood pressure transducer and the pump speed was adjusted so

FIGURE 3.1

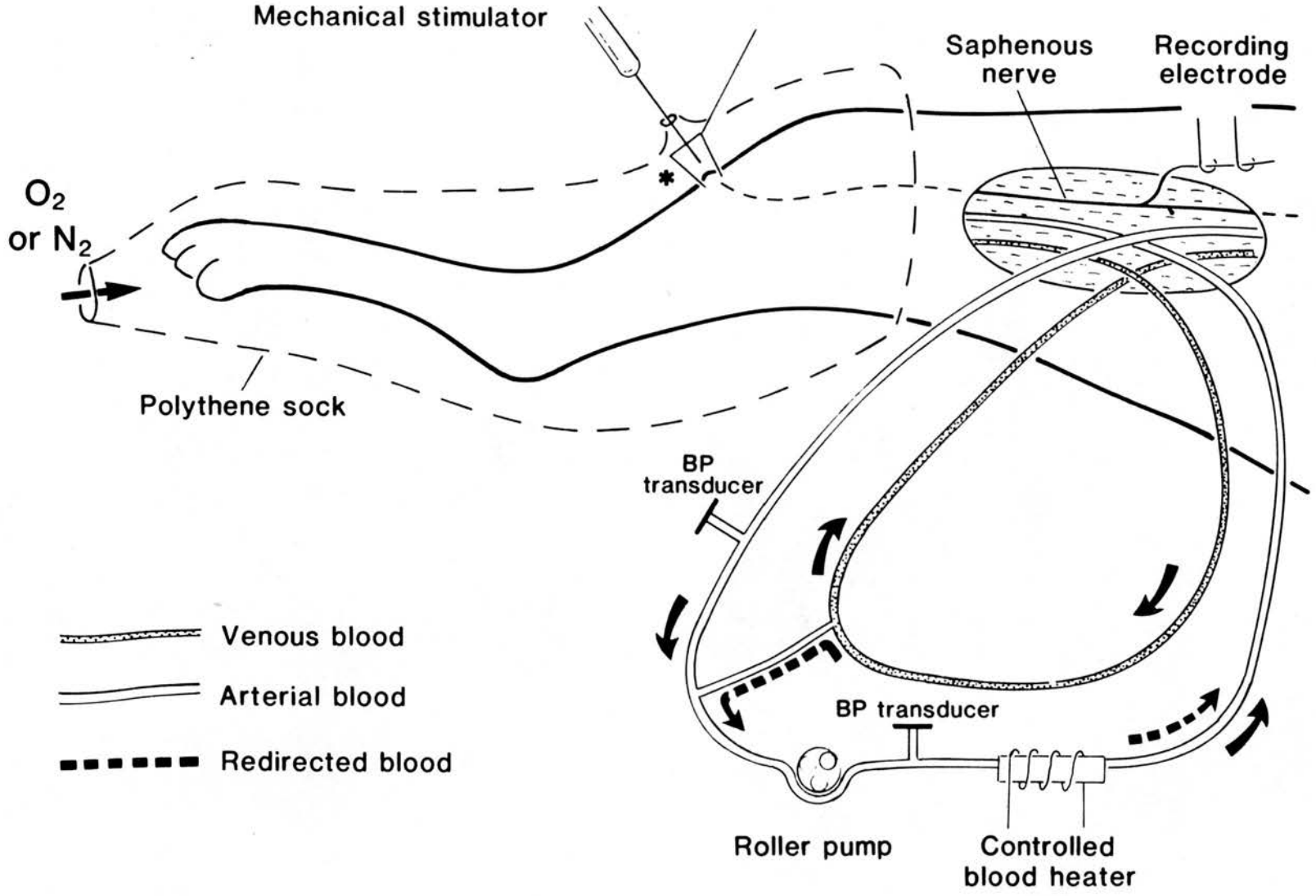
Illustrated is a schematic diagram of the preparation used to make the limb only hypoxic.

The polythene sock attached around the limb enabled manipulation of the gas content of the environment surrounding the limb. In 'normal' conditions a control response was recorded from the touch dome when arterial blood was pumped from the central to peripheral ends of the femoral artery. The roller pump speed was adjusted so that the pressure of the blood passing into the peripheral end of the artery equalled the blood pressure in the central end of the artery. The blood temperature as it circulated outwith the limb was maintained by passing it through a blood heater (see Figure 3.2 for circuit details). The venous blood passed passively back from the limb to the general circulation of the animal.

To make the limb hypoxic the arterial circulation was occluded and the venous blood was redirected via the roller pump back into the limb. N_2 was also blown over the limb. In N_2/O_2 experiments the hypoxic circulation was maintained whilst the N_2 around the limb was replaced by O_2.

Also shown is the method used to electrically stimulate touch dome afferent fibres (*). Two electrodes, which were connected to an electrical stimulator, were impaled into the skin on each side of the touch dome. Electrical stimulation of the afferent fibre always preceded mechanical stimulation of the touch dome (see timing diagram, Figure 2.2).

Using this preparation, several experiments could be carried out by restoring the arterial circulation to the limb and allowing the venous drainage of the limb to pass back to the general circulation.


Mechanical stimulator
Saphenous nerve
Recording electrode
O_2 or N_2
Polythene sock
BP transducer
Venous blood
Arterial blood
Redirected blood
BP transducer
Roller pump
Controlled blood heater

that the blood pressure of the arterial circulation perfusing the limb was the same as that measured in the central end of the artery.

Blood temperature control. As the blood circulated outwith the animal, its temperature was maintained close to 37°C by passing it through a blood heater. This consisted of a 60mm length of heat resistant tubing of 1mm internal diameter around which was wrapped several turns of nichrome resistance wire. The temperature of the blood as it passed out of the heater was detected by a calibrated thermistor which controlled the power supplied to the resistance wire. The thermistor output could also be displayed on a channel of the oscilloscope so that the blood temperature could be monitored from time to time throughout the experiment. A schematic diagram and description of the electronic circuitry of the blood heater control unit is presented in figure 3.2. The heater and thermistor were fitted on the output side of the roller pump as near to the peripheral end of the femoral artery as possible. This reduced the heat loss from the blood between leaving the blood heater and entering the limb. In the course of an experiment the blood temperature fluctuated slowly between 36°C and 38°C.

Induction of Hypoxia To make the limb hypoxic, the central end of the femoral artery was occluded and the venous return from the limb was redirected into the peripheral end of the artery. In this way the circulation formed a closed loop within the limb. N_2 was then applied to the polythene sock which had been

FIGURE 3.2

Illustrated is a circuit diagram of the blood heater control unit. The current from a negative temperature coefficient thermistor is fed into an integrated circuit amplifier (1) the output voltage of which is proportional to the temperature change detected by the thermistor. This amplifier has 2 variable controls, variable resistor A which sets the low temperature reference point and variable resistor B which sets the gain of the amplifier. The voltage measured at the temperature monitoring point is proportional to the thermistor temperature.

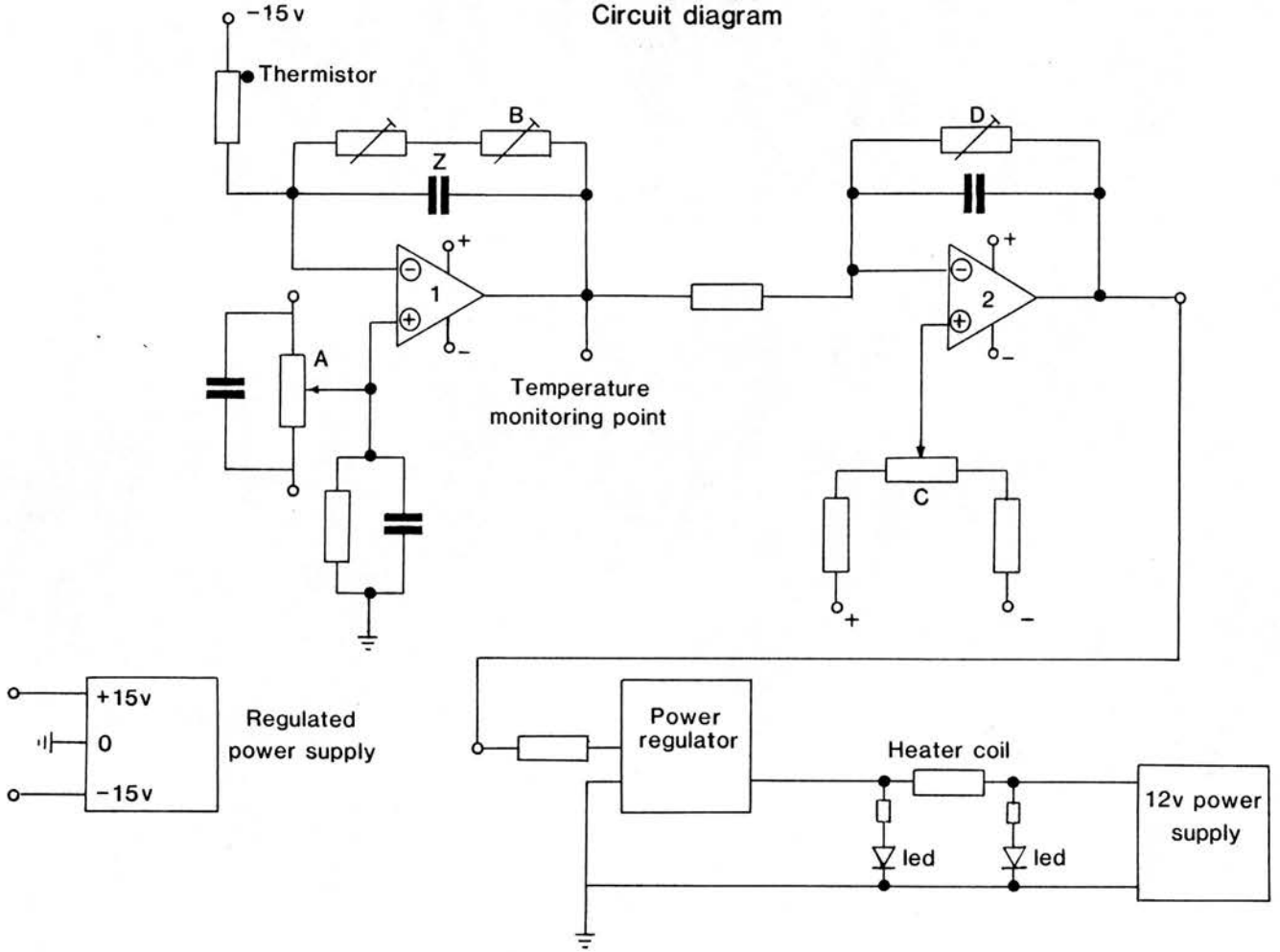
The output from amplifier 1 is fed directly into a voltage amplifier (2) which is used to condition the output of amplifier 1 to operate the power regulator. The offset of amplifier 2 is adjusted by resistor C and its gain by resistor D.

The power output from the power regulator, which is proportional to its voltage input, supplies the heater coil. The light emitting diode (led) connected to the output of the power regulator indicates when there is input to the heater coil; another led indicates when the 12v power supply is on.

The output from the regulated power supply is connected to amplifiers 1 and 2.

Blood heater control unit

Circuit diagram



previously placed around the limb. The onset of hypoxia for the subsequent timing of events was taken as the time when venous blood first entered the femoral artery and when N_2 was applied to the limb. As hypoxia became established in the limb, a small drop in blood pressure occurred as a result of vasodilation of the limb blood vessels. This was compensated for by increasing the pump speed until the blood pressure was back to its pre-hypoxic level.

The leakage of blood between the general circulation and the isolated circulation of the limb was tested with a 1% solution of Evans blue made up in 0.15M NaCl. When injected into the general circulation, the time taken for the dye to first appear in the limb circulation was approximately 15-20 minutes indicating that only a small amount of blood entered the isolated limb circulation over a period of time.

Electrical stimulation. In total, 7 units were investigated in 5 experiments in which the afferent fibres innervating touch domes were both electrically and mechanically stimulated. The method used was that described in detail in chapter 2.5.

Mechanical stimulation of touch domes in established hypoxic conditions. These experiments were an attempt at reducing some of the variation in the time to receptor failure by eliminating as far as possible the variation in the time to the production of an hypoxic environment sufficient to cause receptor failure. In one cat, 3 different units were mechanically stimulated for the first time in conditions in which it was known that receptor failure would occur. In order to do

this, it was necessary to have at least two functional touch domes in the strand of nerve fibres being recorded from. One touch dome was then stimulated to exhaustion and then the mechanical stimulator was immediately transferred to one of the adjacent, previously unstimulated, touch domes. It was the time to failure of this receptor, in an environment in which it was known that receptor failure would occur, that was measured.

Histological procedures. In all hypoxic experiments as soon as receptor failure occurred the limb was fixed by perfusion. Prefixative and fixative solutions, which had been deoxygenated by passing N_2 through them, were perfused into the circulation as detailed in Chapter 2.10. Fixative was also applied topically to the surface of both stimulated and non-stimulated receptors which were subsequently removed from the limb for histological preparation and examination. Control Merkel cells were cells taken from SAI receptors which were in an hypoxic environment but which had not been stimulated.

Hypoxic experiments ± N_2 and O_2 alternately applied to the limb. Both experimental procedures described above for the establishment of hypoxia in the circulation were used in experiments in which N_2 and O_2 were alternately applied to the limb. Two cats weighing 2.1 and 2.4Kg were made hypoxic by ventilating them with N_2. Four other cats weighing between 2.0 - 2.4Kg had hypoxia produced in the limb by the recirculation of venous blood around the limb.

A single tube connected via a 'T' piece to the

flow-meters of both N_2 and O_2 cylinders, was pushed through an entry point of the polythene sock which was around the limb. N_2 was applied to the limb and a touch dome stimulated until it failed. When this occurred the N_2 blowing on to the limb was replaced by O_2. This process of alternately applying N_2 and O_2 to the limb was repeated several times in each experiment.

In an attempt to investigate more precisely the way in which O_2 and N_2 affected SAI receptors, the mean responses to mechanical stimulation in successive 5s periods after the application of O_2 or N_2 to the limb were plotted as a percentage of the control response for each receptor. For one receptor (C090583/2) which was stimulated for the first time when the circulation was already hypoxic, the control response was taken as the response in the 30s immediately preceding the first application of N_2 to the limb.

In all hypoxic experiments tissue was fixed and removed for histological examination either when the receptor had failed with N_2 around the limb or when the response had returned when O_2 was applied to the limb.

Blood gas analysis

In experiments in which the whole animal was to be made hypoxic, control blood samples were removed from the arterial circulation after the animal had been paralysed with gallamine triethiodide and was being ventilated artificially with room air. Other blood samples were removed after the onset of hypoxia and particularly at the time that receptor failure occured. In the local limb preparation, arterial blood samples were removed from the limb circulation both before and

after the establishment of hypoxia in the limb. All blood samples were analysed as detailed in Chapter 2.8.

Data analysis

Data analysis of the histological and electrophysiolgical results was carried out as described in Chapter 2.11. Analysis of the ISI distribution was carried out on 2 units, 1 each from experiments C151282 and C251083.

Part 3.3 Results

Electrophysiology- all experiments

Hypoxic experiments with N_2 applied to the limb.

Mechanical stimulation of SAI mechanoreceptors in hypoxic conditions caused the eventual failure of these receptors. In all eleven units investigated, receptor failure occurred within 11 minutes of the onset of hypoxia. This is in contrast to normal animals in which a response can still be obtained 40 minutes after the start of mechanical stimulation.

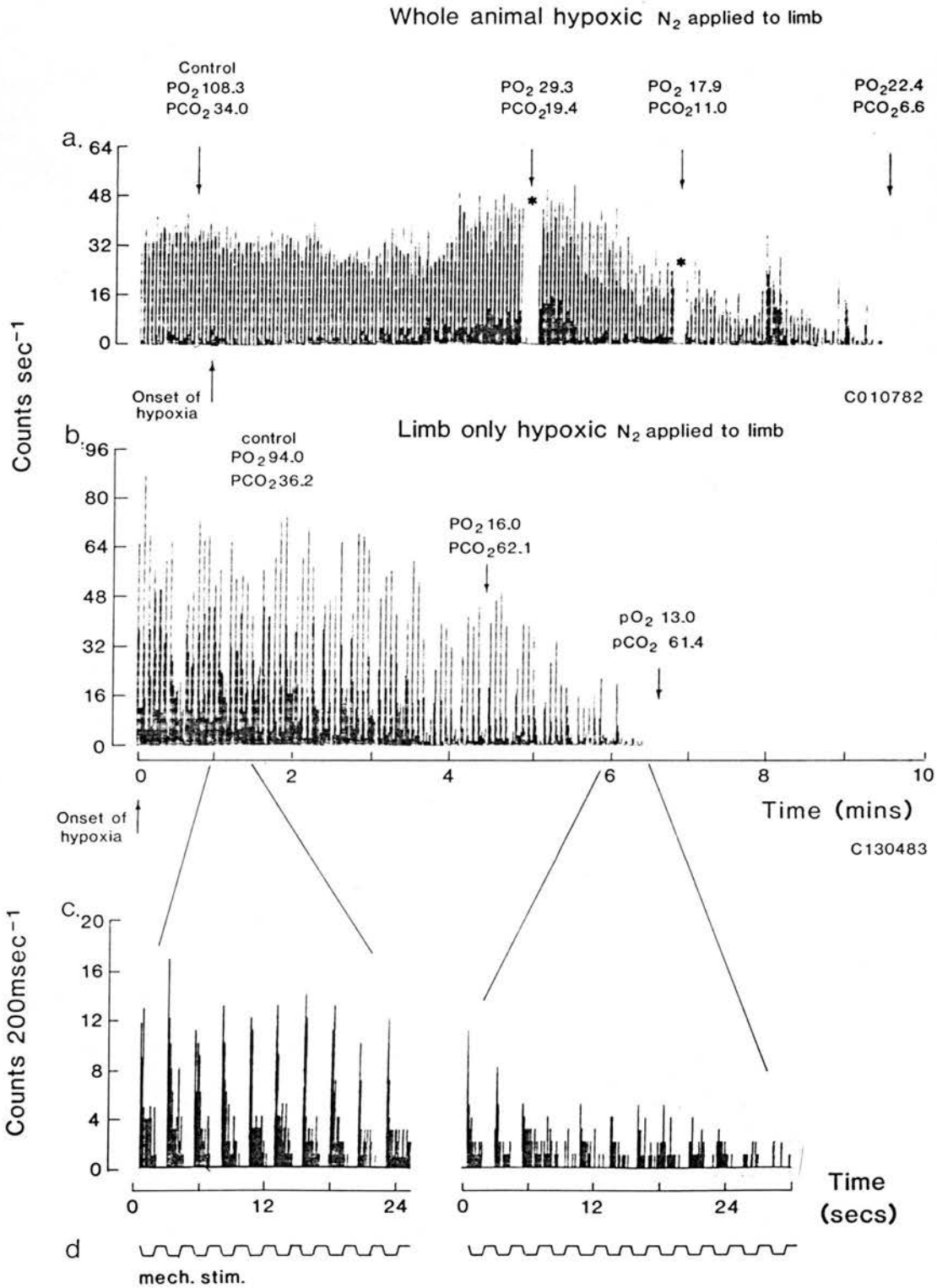
Although two quite different preparations were used to produce hypoxia in the circulation the end result was the same. Figures 3.3a and b show two examples of the results obtained when SAI receptors were stimulated in hypoxic conditions. One example is from an experiment in which the whole animal was made hypoxic and the other from an experiment in which the limb only was made hypoxic. Figure 3.3c also illustrates the form in which, in all units examined, data was collected from the tape recorder. This data was analysed using the computer program described in

FIGURE 3.3

Illustrated are the results of 2 units, one from each experimental preparation, when SAI receptors were stimulated to exhaustion in hypoxic conditions. Presented is raw data where each bar represents the total number of spike counts/s. Graphs were produced from this data by totalling the number of spikes in a 10s period and averaging this to the mean spike count/s. Trace (c) shows an expansion of selected periods of the data presented in (b) and enables the response of each stimulation of the receptor to be seen. Each bar in (c) represents the total number of spike counts/200ms. Trace (d) shows the mechanical stimulator displacement which can be directly related to the response in (c).

In both experiments shown failure of the touch dome to respond to mechanical stimulation occurs. These results are typical of all experiments in which touch domes were stimulated in hypoxic conditions. Also indicated are the PO_2 and PCO_2 levels in the limb circulation at different times throughout the experiments.

(* - blood sample taken)


Whole animal hypoxic N_2 applied to limb
Control
PO_2 108.3
PCO_2 34.0
PO_2 29.3
PCO_2 19.4
PO_2 17.9
PCO_2 11.0
PO_2 22.4
PCO_2 6.6
a.
64
48
32
16
0
Onset of hypoxia
C010782
Counts sec^{-1}
Limb only hypoxic N_2 applied to limb
b.
96
80
64
48
32
16
0
control
PO_2 94.0
PCO_2 36.2
PO_2 16.0
PCO_2 62.1
pO_2 13.0
pCO_2 61.4
0
2
4
6
8
10
Time (mins)
Onset of hypoxia
C130483
c.
20
16
12
8
4
0
Counts 200msec^{-1}
0
12
24
0
12
24
Time (secs)
d
mech. stim.

Chapter 2.11. The results presented in Figure 3.3 are reproduced in Figure 3.4 along with the results of other hypoxic experiments from both experimental preparations. Table 3.1 summarises the results obtained in all hypoxic experiments carried out. The data presented in the table are shown separately for each preparation to illustrate the variations in the results that occurred between them. However, the only result that is significantly different between the two preparations ($p<0.005$ Student's 2-tailed 't' test) is the level of PCO_2 in the circulation at the moment of receptor failure. This difference results from the two different ways in which hypoxia was produced. When animals were ventilated with N_2, CO_2 was removed from the circulation by normal respiration, but was not produced by cell metabolism in the tissues due to the reduced PO_2 level. When the venous blood was re-directed around the limb the CO_2 produced by metabolism remained in the limb circulation causing the observed rise in PCO_2 levels with time. The large variation in the PCO_2 levels between both preparations indicates that receptor failure is unrelated to the levels of PCO_2 in the circulation.

For individual animals there were considerable variations in the electrophysiological responses obtained by mechanical stimulation of different touch domes a point illustrated clearly in Table 3.1b by experiment C130483. In this one animal three different touch domes were stimulated with three different control responses and three different times to receptor failure. Between the mechanical

FIGURE 3.4

a. Illustrated are normalized graphs of results from 3 experiments in which touch domes were stimulated to exhaustion when the general circulation of the animal was hypoxic. Adjacent to each experiment number is the response of the touch dome to mechanical stimulation in counts per second ($c.s^{-1}$), before hypoxia was induced. This was regarded as the control response of the touch dome. The * indicates the time of circulatory arrest in each experiment. It can be seen from this that the time to receptor failure from circulatory arrest is variable.

b. The graphs presented are of the results from 3 experiments in which the limb only was made hypoxic.

Included in a. is experiment C010782 and in b. experiment C130483. The raw data for both these experiments is presented in Figure 3.3. From these graphs it is evident that the time to receptor failure in hypoxic conditions varies from one experiment to another. In some experiments the response falls steadily from the onset of hypoxia (C130483) whereas in others the response continues almost at control levels for some time before eventually starting to fail (C160683, C010782).

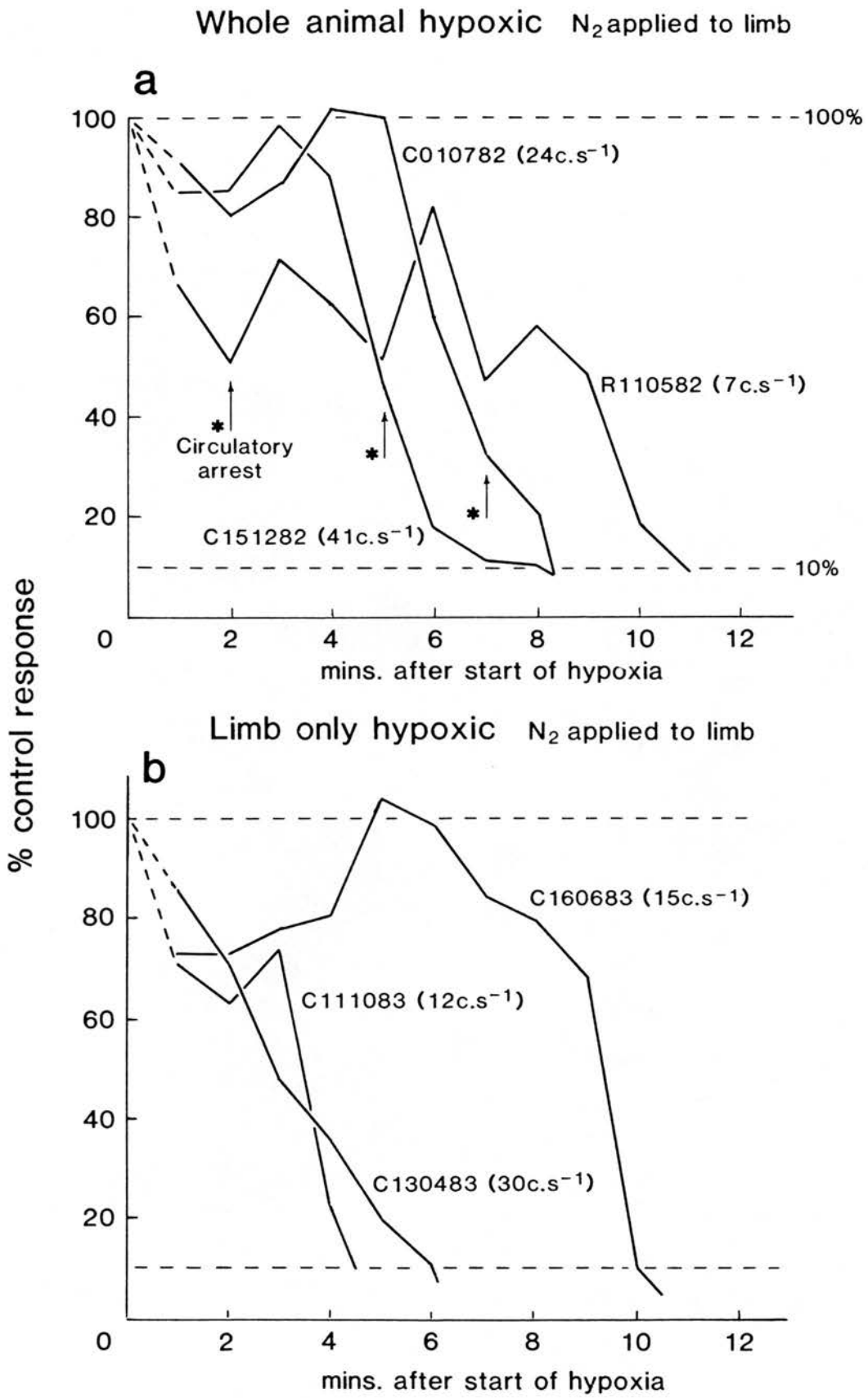

Whole animal hypoxic N2 applied to limb
a
100
80
60
40
20
0
100%
10%
C010782 (24c.s-1)
R110582 (7c.s-1)
C151282 (41c.s-1)
Circulatory arrest
2
4
6
8
10
12
mins. after start of hypoxia
% control response
Limb only hypoxic N2 applied to limb
b
C160683 (15c.s-1)
C111083 (12c.s-1)
C130483 (30c.s-1)
mins. after start of hypoxia

TABLE 3.1

Summary table showing the results obtained in all hypoxic experiments. Times are in minutes from the onset of hypoxia.

TABLE 3.1

	UNIT NO.	TIME TO CIRCULATION ARREST (min)	CONTROL RESPONSE Count/s	TIME TO RECEPTOR FAILURE (min)	CONTROL PO_2 (mm Hg)	CONTROL PCO_2	FAILURE PO_2 (mm Hg)	FAILURE PCO_2
A. WHOLE ANIMAL HYPOXIC	R110582	< 2	7 ± 1.9	10.8	-	-	-	-
	C010782	< 7	23.8 ± 0.9	8.3	108.3	34.0	22.4	6.6
	C151282	< 5	40.7 ± 4.9	8.3	115.8	30.7	11.7	9.5
	C020383	< 4	15.8 ± 2.3	5.5	111.4	28.4	9.0	7.4
	MEAN			8.25 ± 1.1	111.0 ± 3.8	31.0 ± 1.6	14.4 ± 4.1	7.8 ± 0.9
B. LIMB ONLY HYPOXIC	C13048-11	-	30.1 ± 5.4	6.17	94.0	36.2	13.0	61.6
	" -12	-	13.4 ± 3.5	5.33	94.0	36.2	11.2	51.1
	" -13	-	8.8 ± 3.2	8.17	-	-	-	-
	C090583	-	19.9 ± 4.8	8.0	99.2	29.2	20.1	47.3
	C160683	-	14.6 ± 2.3	10.5	-	-	25.6	23.5
	C111083	-	12.4 ± 1.9	4.5	-	-	-	-
	C151283	-	14.2 ± 2.6	4.67	101.4	30.4	22.3	44.1
	MEAN			6.76 ± 0.8	97.15 ± 1.7	*33.0 ± 1.9	18.4 ± 0.36	*45.5 ± 6.2

(mean ± S.E)

* difference between levels of PCO_2 significant $p < 0.005$ (Student's 2-tailed 't' test.)

stimulation of each touch dome, hypoxic conditions were removed by the restoration of the arterial circulation to the limb and by exposing the limb to room atmosphere.

When hypoxia was produced in the whole animal, the time to circulatory arrest was less than 7 minutes in all experiments. This time was measured from when the animal started to breath N_2 to when the B.P. of the animal fell to zero. Because this was a gradual event only an estimate of the time that this took was made (see Table 3.1). The time taken for circulatory arrest to occur was unrelated to the time taken for the receptor to fail. In the rat experiment, R110482, the circulation had ceased within two minutes whilst the response to mechanical stimulation continued for a further 8-9 minutes. In contrast to this was experiment C010782 in which it took almost seven minutes for the circulation to cease and only a further 1-2 minutes after that for the receptor to fail. There was also no significant correlation found in the time taken for receptor failure to occur and the PO_2 levels in the circulation at the time of receptor failure.

In the experiments carried out in which touch domes were stimulated in known hypoxic conditions (at times ranging from 13 to 23 minutes after the onset of hypoxia) receptor failure occurred in 2.1 $\pm$ 0.26 minutes (S.E.M., n=3). This was significantly less time ($p < 0.01$ Student's 2-tailed 't' test) than the mean time taken for receptor failure to occur from the onset of hypoxia when the limb only was hypoxic. This comparison was made because all 3 receptors stimulated

in established hypoxic conditions were in the preparation in which the limb only was made hypoxic. Illustrated in Figure 3.5 is the response of one touch dome of an SAI unit as it fails in hypoxic conditions. At the moment of receptor failure the stimulator was moved to an adjacent previously unstimulated touch dome and the time taken for this receptor to fail was recorded. The values obtained for the three receptors in which this procedure was used are presented in Table 3.2 and illustrated graphically in Figure 3.5b. As these touch domes were stimulated for the first time in hypoxic conditions there was no control response with which the failing receptor response could be compared. For this reason, the response in each 10 second period of mechanical stimulation was plotted as a percentage of the response present in the first 10s period after the start of mechanical stimulation of the touch dome. The time of receptor failure was taken as the mid-point of the 10s period during which the response fell to <10% of the response in the first 10s period. With the exception of the first 20s of receptor C090583-05, which showed a transient rise in the response to mechanical stimulation, all receptor responses decreased steadily with time. Figure 3.5c which is of the pooled data from all three receptors shows that this decrease in receptor response with time is linear, with a regression coefficient of -0.77. This is not the case where touch domes were stimulated to failure from the onset of hypoxia (see Figure 3.4).

Electrical stimulation of touch dome afferent fibres in control and hypoxic conditions. Electrical

FIGURE 3.5

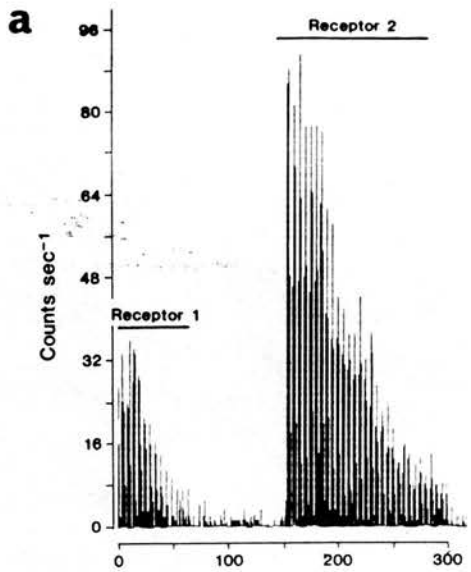
Receptor failure in known hypoxic conditions.

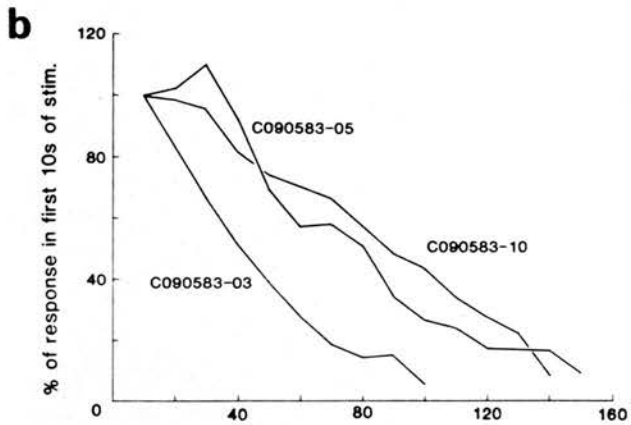
a. A touch dome (receptor 1) was stimulated to exhaustion in hypoxic conditions. At the moment of receptor failure the stimulator probe was then moved to an adjacent touch dome (receptor 2) which was innervated by the same afferent fibre. It was the time taken for this touch dome to fail, in established hypoxic conditions, that was recorded.

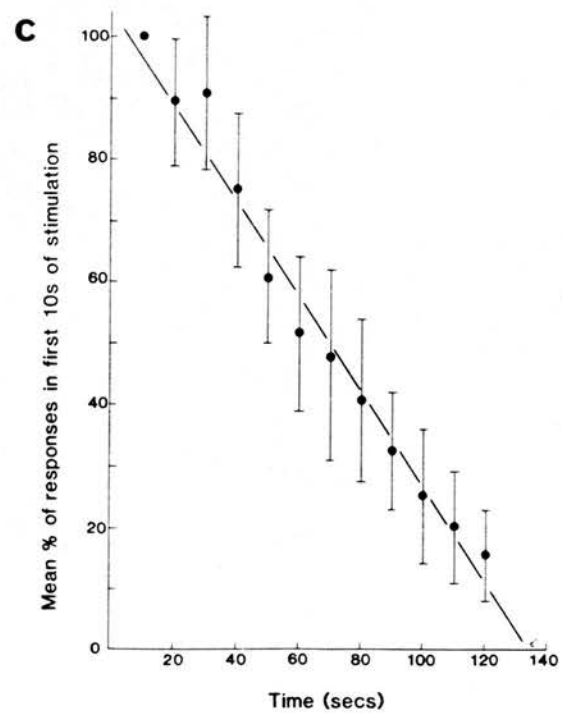
b. Data presented refers to the 2nd receptor of a pair.
The results of the 3 experiments in which touch domes were stimulated to exhaustion in conditions in which it was known that receptor failure would occur are shown. The response in each 10s period is plotted as a % of the response obtained from each touch dome in the first 10s of mechanical stimulation.

c. Pooled data from the 3 experiments presented in b. There is a linear decay in the mean % response with time. Regression coefficient (b) = -0.77.

(error bars indicate standard errors of the mean).


a
Receptor 2
Receptor 1
Counts sec⁻¹
96
80
64
48
32
16
0
0
100
200
300


b
% of response in first 10s of stim.
120
80
40
0
C090583-05
C090583-10
C090583-03
40
80
120
160


c
Mean % of responses in first 10s of stimulation
100
80
60
40
20
0
20
40
60
80
100
120
140
Time (secs)

-D·77

TABLE 3.2

Results of the experiment in which 3 touch domes were stimulated for the first time in established hypoxic conditions. Shown are the responses in each 10s period given as a % of the response in the first 10s of stimulation.

TABLE 3.2

Time (S)	C090583 - 03 % of response in 0-10s.	C090583 - 05 % response in 0-10s.	C090583 - 10 % response in 0-10s.	Mean % response (n=3)
0 - 10	100	100	100	100
10 - 20	68.9	102	98.7	89.4 ± 10.5
20 - 30	66.7	110	95.9	90.9 ± 12.7
30 - 40	51.2	91.9	82.0	75.0 ± 12.5
40 - 50	38.7	68.8	74.2	60.6 ± 11.0
50 - 60	27.8	57.1	70.4	51.8 ± 12.6
60 - 70	18.5	57.9	66.3	47.6 ± 14.7
70 - 80	14.2	50.6	57.5	40.8 ± 13.4
80 - 90	15.2	34.2	48.1	32.5 ± 9.5
90 - 100	5.7	26.2	43.1	25.2 ± 11.0
100 - 110	2.9	24.1	34.0	20.3 ± 9.2
110 - 120	1.9	17.4	27.6	15.6 ± 7.4
120 - 130		16.6	22.7	
130 - 140		16.6	8.9	
140 - 150		8.8	4.3	
Time to receptor failure	95s (1.58 m)	145s (2.42 m)	135s (2.25 m)	

Mean time to receptor failure = 2.1 ± 0.26 (m) (Mean ± SE)

stimulation of the afferent fibre close to the receptor produced an action potential in both control and hypoxic conditions. An example of this is illustrated in Figure 3.6. It shows that in hypoxic conditions when the response to mechanical stimulation had practically ceased a response could still be produced consistently in the afferent fibre by electrical stimulation. The threshold voltage to stimulate each afferent fibre, using 0.1ms square pulses, varied between 1.6V and 3.4V with a mean of 2.3 $\pm$ 0.7V (S.E.M., n=7). In all experiments the stimulating voltage for each receptor (threshold + 20% threshold - see Materials and Methods Part 2.5) both in control and hypoxic conditions remained unchanged.

Hypoxic experiments with N_2 and O_2 alternately applied to the limb. When N_2 around the limb was replaced by O_2 after receptor failure, the response to mechanical stimulation quickly recovered. When N_2 was reapplied to the limb thus removing O_2, the response of the receptor fell rapidly to the level present before O_2 had been applied. This process of alternately applying N_2 and O_2 to the limb was carried out in both experimental procedures, and was repeated several times in each experiment.

Figure 3.7 shows the results of two experiments, one from each preparation, when O_2 and N_2 were alternately applied to the limb. The results illustrated in Figure 3.7a are from an experiment in which hypoxia was established in the general circulation, and commence at approximately 5m 30s after the onset of hypoxia and 30s after the occurrence

FIGURE 3.6

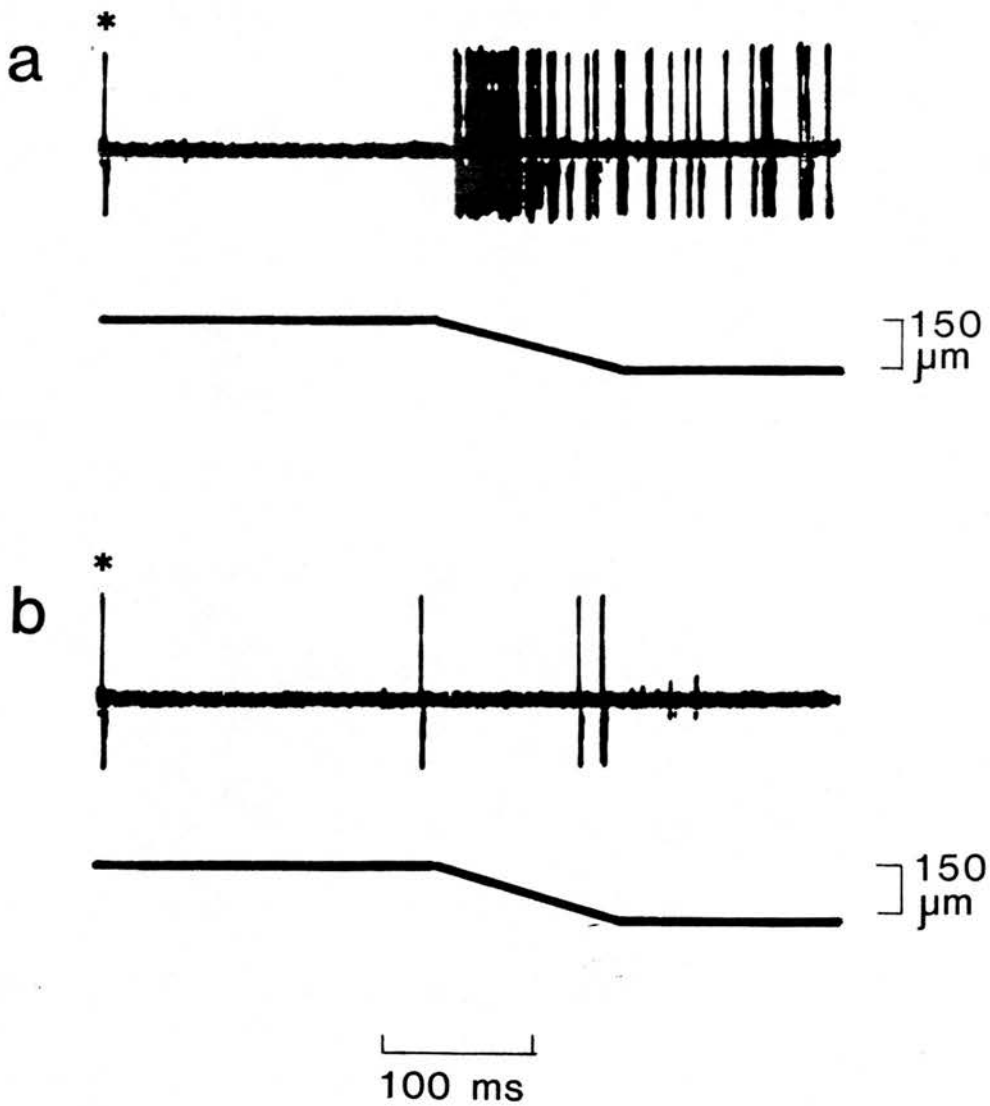
a. 1. Response produced by electrical (*) and mechanical stimulation before the onset of hypoxia.

2. Displacement transducer output.

b. 1. Response to electrical (*) and mechanical stimulation in established hypoxic conditions when the response to mechanical stimulation has almost ceased. The response to electrical stimulation was consistently present compared to the intermittent response produced by mechanical stimulation of the touch dome.

2. Displacement transducer output.

Elect. & mech. stim. of SAI receptor
before and during hypoxia

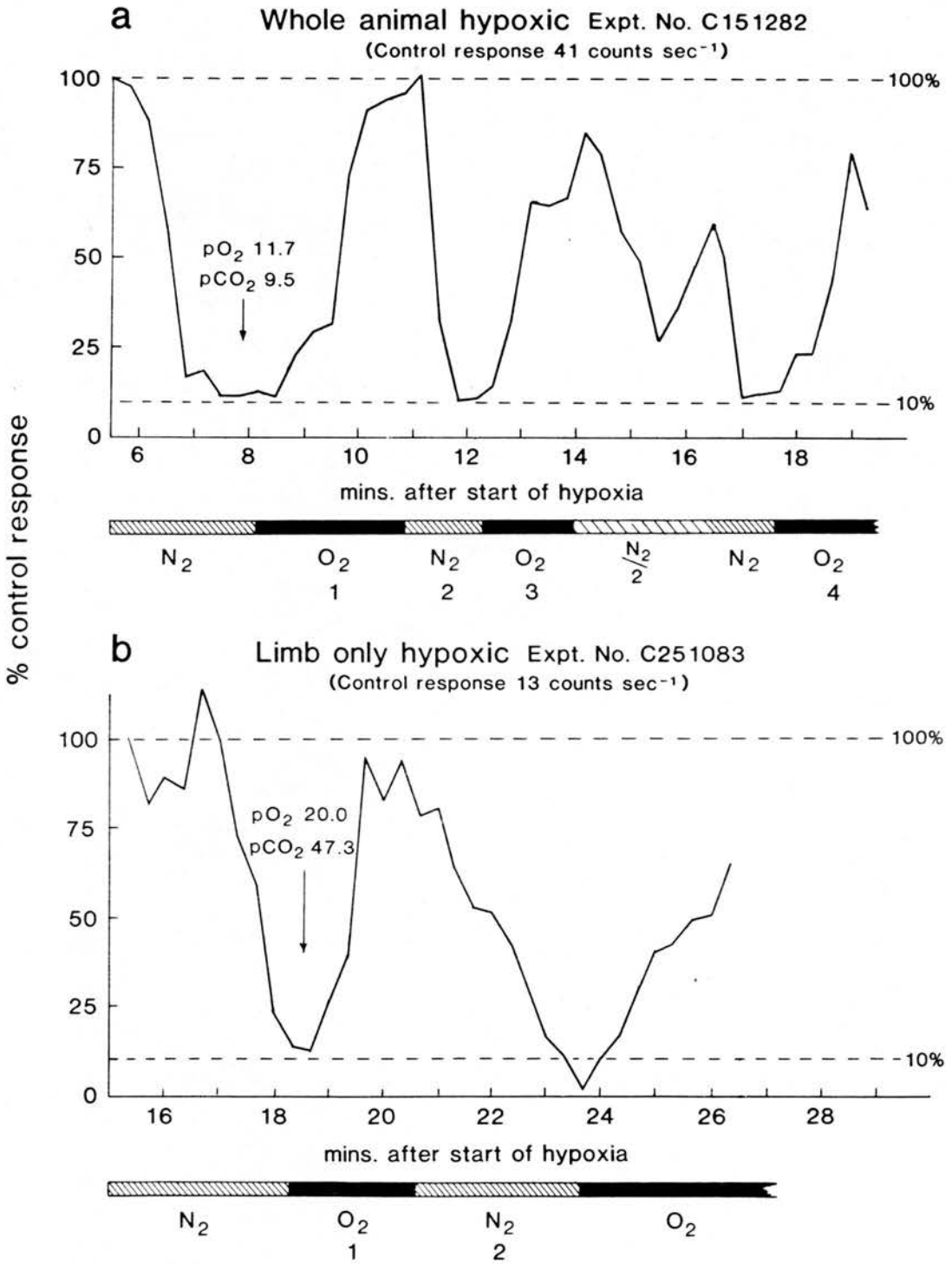


C220683

FIGURE 3.7

a. Illustrated are the effects of mechanical stimulation of a touch dome when the general circulation was hypoxic and when N_2 and O_2 were alternately blown into the polythene sock around the limb. The timing of the application of N_2 and O_2 to the limb is indicated by the bar below the abscissa. The results shown start approximately 30s after circulatory arrest, therefore receptor recovery on the third application of O_2 to the limb is occurring 13 minutes after circulatory arrest. Reduction by half of the flow rate of N over the limb, 14 minutes after the onset of hypoxia, causes a reduction only in the response to mechanical stimulation before recovery starts to occur. Restoration to the normal flow rate of N_2 causes immediate receptor failure.

b. A similar experiment to that described in a. with the exception that hypoxia was produced in the limb only. In this experiment the receptor was stimulated for the first time 15 minutes after the onset of hypoxia.
In both experiments the PO_2 and PCO_2 levels at receptor failure are indicated.
In both graphs a) and b) the numbers below the abscissa refer to the appropriate columns in Tables 3.3 and 3.4.


a
Whole animal hypoxic Expt. No. C151282
(Control response 41 counts sec⁻¹)
100%
10%
pO2 11.7
pCO2 9.5
% control response
mins. after start of hypoxia
N2
O2
1
N2
2
O2
3
N2/2
N2
O2
4
b
Limb only hypoxic Expt. No. C251083
(Control response 13 counts sec⁻¹)
100%
10%
pO2 20.0
pCO2 47.3
mins. after start of hypoxia
N2
O2
1
N2
2
O2

of circulatory arrest. It is, therefore, evident from Figure 3.7a that when O_2 was applied to the limb 13 minutes after circulatory arrest, the response returned within seconds to almost 80% of that receptor's control response. When hypoxia was produced in the limb circulation only (Figure 3.7b), receptor recovery still occurred 24 minutes after the onset of hypoxia. An unusual feature of the data presented in Figure 3.7b is the time taken (170s) for receptor failure to occur. This is almost three times longer than the mean time to failure for all other receptors in which this was measured (see Table 3.4).

The sensitivity of the receptor to the presence or absence of O_2 in the external environment is evident in Figure 3.7a. At approximately 14 minutes after the onset of hypoxia, N_2 was blown over the limb at 50% of its normal flow rate. The receptor, which initially showed signs of failing, continued to respond erratically until N_2 flow was restored to its normal rate after which failure occured within 30s.

The results of application of O_2 and N_2 to the limb are presented in Tables 3.3 and 3.4 and show respectively the recovery and failure of the resonse. These tables should be considered together. The numbers at the tops of columns indicate the order in which O_2 and N_2 were applied in each experiment. The appropriate column numbers are also shown for units C151282 and C251083 in Figures 3.7a and b. In all experiments the failure of the receptor on the first application of N_2 to the limb was not included because the time to receptor failure on the initial application

TABLE 3.3

Receptor recovery when O_2 applied to the limb.
This table should be considered in conjunction with table 3.4. The mean response over a 5s period is presented as a percentage of the control response (C.R.) for each unit. The numbers at the top of each column indicate the order in which, for each experiment, events occurred. For units C151282 and C251083 the data in the appropriate columns can be related to the graphs presented in Figures 3.7a and b.

TABLE 3.3

TIME TO RECEPTOR RECOVERY WITH O_2 ON LIMB

TIME(s)	C151282 CR=41c/s			C090583 RECEPTOR 1 CR=20c/s		C090583 RECEPTOR 2 CR=15c/s		C251083 CR=13c/s	MEAN % RESPONSE
	1	3	4	1	3	1	3	1	(± S.E.M.)
0-5	9.8	9.8	8.0	11.0	9.5	11.3	6.7	17.4	10.4 ± 1.1
5-10	14.0	23.4	9.4	6.3	9.5	7.3	5.3	16.4	11.5 ± 2.1
10-15	12.1	27.3	12.2	7.0	14.5	4.0	12.7	23.0	14.1 ± 2.7
15-20	11.7	37.5	17.0	7.1	9.5	14.0	7.3	23.2	15.9 ± 3.6
20-25	47.3	56.2	11.0	6.4	8.5	20.7	10.0	25.5	23.2 ± 6.7
25-30	55.1	75.4	13.4	5.0	5.7	33.3	20.7	29.9	29.8 ± 8.7
30-35	22.6	60.4	13.6	6.5	6.5	34.7	29.3	30.4	25.5 ± 6.2
35-40	14.0	61.0	14.6	10.0	6.5	34.7	46.0	32.6	27.4 ± 6.8
40-45	18.2	61.7	17.3	16.2	6.5	56.7	54.0	37.3	33.5 ± 7.7
45-50	22.8	65.7	20.7	19.1	8.5	55.3	54.0	51.9	37.2 ± 7.6
50-55	27.6	68.2	24.7	18.2	7.7	52.0	69.3	66.8	41.8 ± 8.9
55-60	41.6	79.6	30.9	32.0	8.6	65.3	73.3	88.6	52.5 ± 10.0
60-65	58.1	65.3	20.1	29.5	48.0	78.7	76.7	98.2	59.3 ± 9.2
65-70	69.1	58.1	20.3	54.2	91.5	88.3	88.7	118.8	73.6 ± 10.6
70-75	72.4	60.1	21.6	67.9					
75-80	75.8	69.1	25.6	89.3					
80-85	86.7	74.0	33.4						
85-90	88.5	81.2	36.8						
90-95	87.6	82.6	51.2						
95-100	87.6	88.2	66.2						
100-105	91.0		72.6						
105-110			88.0						

(MEAN ± S.E.)

TABLE 3.4

Receptor failure when N_2 applied to the limb. Data is presented in a similar way to that described in Table 3.3.

TABLE 3.4

TIME TO RECEPTOR FAILURE WITH N_2 ON LIMB

TIME(s)	C151282 CR=41c/s	C090583 RECEPTOR 1 CR=20c/s		C090583 RECEPTOR 2 CR=15c/s		C251083 CR=13c/s	MEAN% RESPONSE
	2	2	4	2	4	2	(± S.E.M.)
0-5	111.2	83.0	98.0	87.3	117.3	89.3	97.7 ± 5.7
5-10	107.4	49.0	61.5	48.7	132.0	75.3	80.0 ± 13.8
10-15	97.6	25.0	50.0	34.7	111.3	72.1	65.1 ± 14.1
15-20	90.2	31.0	85.5	18.0	82.0	67.9	62.4 ± 12.5
20-25	80.7	31.5	53.0	18.0	62.7	78.6	54.1 ± 10.3
25-30	60.3	26.0	14.5	18.0	56.7	84.4	43.3 ± 11.4
30-35	29.8	15.5	6.5	15.3	40.7	75.1	30.5 ± 10.2
35-40	14.0	9.5	11.5	14.0	24.0	71.1	24.0 ± 9.6
40-45	12.6	9.5	8.5	9.3	20.7	69.4	21.7 ± 9.7
45-50	12.2				19.3	66.0	
50-55	11.7				18.0	57.1	
55-60	11.2				11.3	53.7	
60-65	11.1				2.7	50.4	
65-70	11.2					48.0	
70-75	11.1					53.6	
75-80	11.0					52.6	
80-85	10.8					51.7	
85-90	10.5					49.9	
90-95	9.2					50.4	
95-100						49.6	
100-110						35.4	
110-120						46.2	
120-130						33.8	
130-140						23.2	
140-150						16.9	
150-160						13.8	
160-170						7.9	

(MEAN ± S.E.)

of N_2 also included the time taken for hypoxia to become established in the circulation. Also, as mentioned above, for unit C151282 (Figure 3.7a) the flow rate of N_2 over the limb was reduced approximately 14 minutes after the onset of hypoxia and this data is therefore not included in Table 3.4. This explains why in Table 3.3 unit C151282 has three recovery periods with O_2 as opposed to only one period of failure with N_2 (Table 3.4). Receptor 2 of C090583 was stimulated for the first time after the onset of hypoxia. The control response for this receptor was taken as the response of the same receptor 30s prior to the first application of N_2 to the the limb. When the mean percentage response in each successive 5s period is plotted against time, the response recovery with O_2 fits an exponential function (Figure 3.8) with a correlation coefficient of +0.98 ($p < 0.002$, Student,s 2-tailed 't' test). This is in contrast to the first 45s after the application of N_2 to the limb which shows a linear decay of the response with a correlation coefficent of -0.98 ($p < 0.002$, Student,s 2-tailed 't' test) and a regression coefficient of -1.867.

There was an exponential distribution of ISI's in the adapted response during the control period of mechanical stimulation in experiments C151282 and C251083. However the distribution of ISI's during the application of N_2 and O_2 to the limb in these experiments could not be tested due to non-stationarity of the response during the sampling period. This is evident from Tables 3.3 and 3.4 where the trend in the response with time is clearly seen. Table 3.5

FIGURE 3.8

a. Graph showing recovery of the receptor response with time in N_2/O_2 experiments when O_2 was applied to the limb. There is an exponential relationship between the recovery response and time.

b. Graph showing the linear decay in the response with time when N_2 applied to the limb in N_2/O_2 experiments.

Regression coefficient (b) = -1.87.

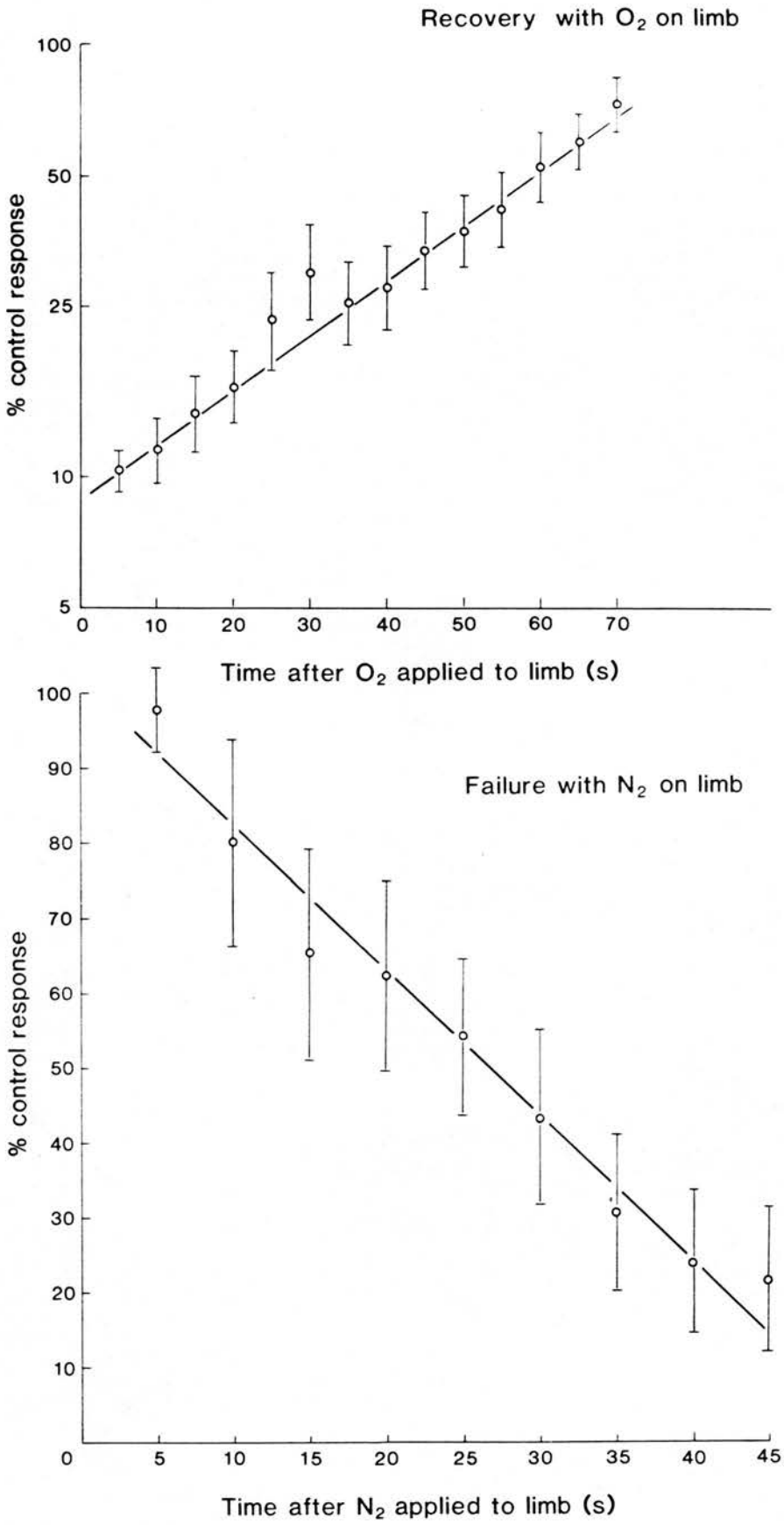

Recovery with O_2 on limb
% control response
100
50
25
10
5
0
10
20
30
40
50
60
70
Time after O_2 applied to limb (s)
Failure with N_2 on limb
% control response
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
45
Time after N_2 applied to limb (s)

TABLE 3.5

Shown are the results of the analysis of the distribution of ISI's for the units examined in experiments C151282 and C251083. Chi-squared values (χ^2), degrees of freedom (d.f.), and the probabilty levels (p) are shown both for the 'goodness-of-fit' of the distribution to an exponential and for the comparison of the distribution at various times throughout the experiment with the normalised control distribution. The point at which the histogram was truncated (maximum frequency of the histogram) is also shown and it was against the truncated histogram that the 'goodness-of-fit' of the distribution to an exponential was tested.

The trend in the response with time during the application of N_2 and O_2 to the limb accounts for the lack of stationarity and hence the inability to compare the response at these times with an exponential distribution.

TABLE 3.5

	COMPARISON WITH EXPONENTIAL			COMPARISON WITH NORMALISED CONTROL			HISTOGRAM TRUNCATED AT
C151282	X^2	d.f.	p.	X^2	d.f.	p.	(ms)
Control	20.2	17	>0.05	-------			44.0
N_2	NO STATIONARITY (N.S.)			N.A.			
O_2	"			"			
N_2	"			"			
O_2	"			"			
N_2	"			"			
O_2	"			"			
C251083							
Control	13.5	17	>0.005	N.A.			27.0
N_2	NO STATIONARITY			"			
O_2	"			"			
N_2	"			"			
O_2	"			"			

N.S. - NO STATIONARITY
N.A. - NOT APPLICABLE

summarises the results of this analysis. This table also shows the point at which the histogram was truncated (see Materials and Methods Part 2.11).

Histology.

Merkel cells from normal unstimulated touch domes

Figure 3.9 shows a Merkel cell of an unstimulated touch dome taken from a limb which had a normal arterial blood supply.

In the plane of sectioning illustrated Merkel cells have a diameter parallel to the skin surface of approximately 12μm. They are situated in the basal layer of the epidermis and are attached to the overlying epidermal cells by desmosomal contacts. These points of contact are found on the cell membrane between cytoplasmic processes which push up into the adjacent epidermal cells. There are no desmosomes between the processes and epidermal cells.

Closely apposed to the dermal aspect of the Merkel cell is an expanded nerve ending of the afferent fibre which innervates the Merkel cell. Both the nerve ending and the Merkel cell are situated within the basement membrane of the epidermis. The main structural features of the nerve ending are numerous mitochondria around which are scattered a number of small (<50 nm) clear vesicles.

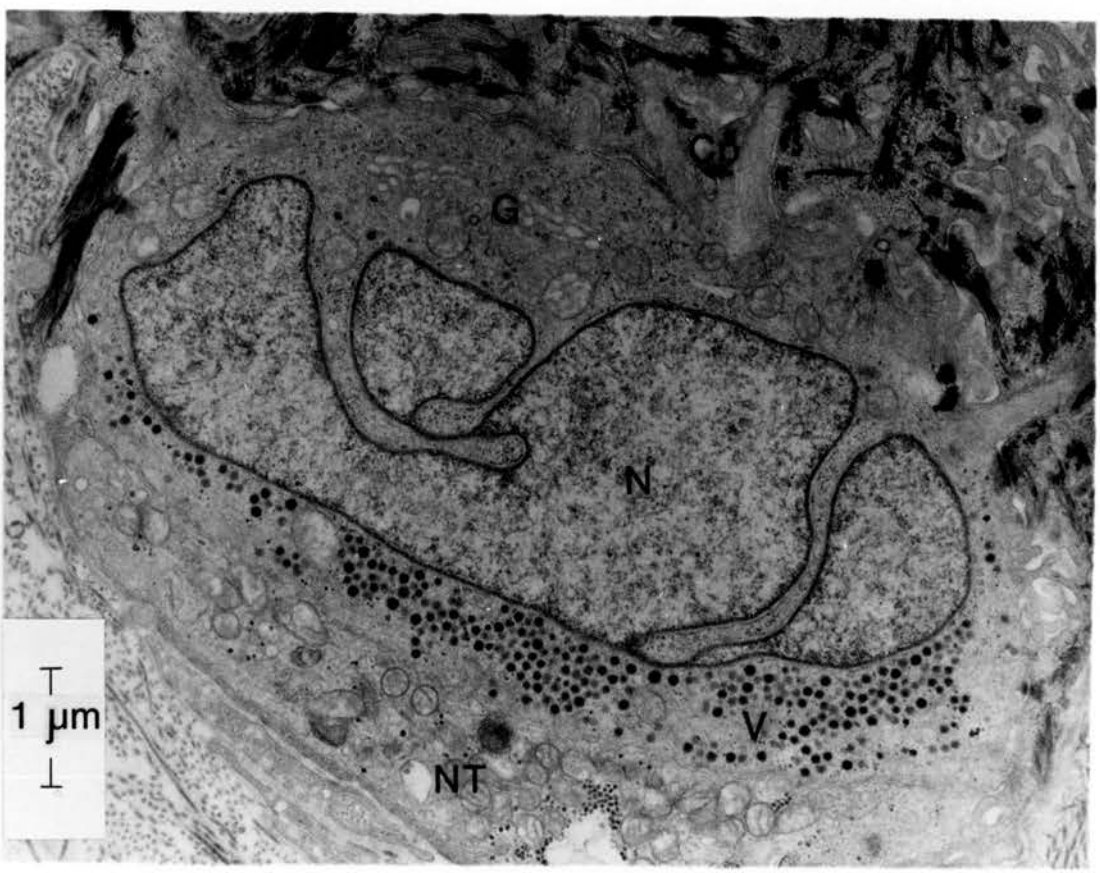
The Merkel cell has a lobulated nucleus and is most frequently observed with its long axis parallel to the skin surface. The convolutions of the nuclear membrane are on the epidermal side of the nucleus whereas the basal side of the nucleus is more regular with few, if any, indentations. There is a distinctive

FIGURE 3.9

Illustrated is a Merkel cell and its adjacent nerve terminal from an unstimulated touch dome in 'normal' conditions. In this and in all subsequent micrographs the overlying epidermal cells and the touch dome surface are to the top of the picture.

The characteristic features of the Merkel cell are its multilobulated nucleus (N) and membrane bound dense-cored vesicles (V) which are found mainly between the Merkel cell nucleus and the nerve terminal (NT). Also present are the cytoplasmic processes Cp) which extend up into the adjacent epidermal cells and a distinct Golgi region (G).

The main structural features of the nerve terminal are numerous mitochondria and small clear vesicles. At the magnification of this micrograph these clear vesicles are not seen.


G
N
V
NT
1 µm

Golgi area, which is clearly seen in Figure 3.9, situated in the cytoplasm on the epidermal side of the nucleus.

The most distinctive feature of Merkel cells is the presence of numerous, membrane bound dense-cored vesicles which are found concentrated within the cytoplasm on the side of the nucleus adjacent to the nerve ending. The vesicles have a diameter of approximately 120 nm and are usually separated from the Merkel cell membrane by a 200 nm gap.

Previous, briefly reported studies (Anand et al., 1979) indicated that vesicle number was reduced in stimulated, hypoxic Merkel cells. For this reason detailed counts were made in the present investigation. Table 3.6 summarises the data on vesicle numerical density for each experimental condition. The numerical density of the vesicles, expressed as vesicles per square micron, in normal cells was 14.6 $\pm$ 1.1 vesicles/μm^2 (S.E.M., n=17). A second feature of the Merkel cell-neurite complex is the occasional presence of 'synaptic-like' junctions between the Merkel cell and the nerve terminal (see figure 4.5). These consisted of thickened regions on both the Merkel cell membrane and the nerve ending membrane which were approximately 400 nm long. The vesicles were mainly concentrated between the Merkel cell nucleus and the nerve ending in normal unstimulated Merkel cells, but were never found accumulated adjacent to these thickened areas of cell membrane.

The results of experiments in which hypoxia was induced in the whole animal and those in which the limb

TABLE 3.6

Table of vesicle numerical-density in Merkel cells in the different experimental conditions in which tissue was removed. Also shown are the levels of signifance when the numerical density in these different conditions are compared with each other.

TABLE 3.6

EXPERIMENTAL CONDITION	NUMERICAL DENSITY (vesicles/Um^2)
NORMAL	14.6 ± 0.97 (n = 17)
CONTROL Hypoxic unstimulated	8.3 ± 0.7 (n = 16)
Hypoxic stim, N_2 atmosphere	4.3 ± 0.48 (n = 20)
Hypoxic stim, O_2 atmosphere	7.9 ± 1.1 (n = 7)

(mean ± 1SD.)

LEVELS OF SIGNIFICANCE BETWEEN CONDITIONS

NORMAL			
p 0.001	CONTROL HYPOXIC UNSTIMULATED		
p 0.001	p 0.001	HYPOXIC STIM. N_2 ATMOSPHERE	
p 0.001	NOT SIGNIFICANT	p 0.001	HYPOXIC STIM. O_2 ATMOSPHERE

Student's 2-tailed 't' test.

only was made hypoxic yielded identical histological results and will therefore be treated together.

Merkel cells from control touch domes In hypoxic experiments control Merkel cells were cells taken from touch domes which had an hypoxic circulation and N_2 around the limb, but which were not mechanically stimulated. It was with these cells that other Merkel cells from hypoxic experiments were compared. Such a cell and its adjacent nerve terminal are illustrated in Figure 3.10. The most obvious effect is a disruption of the mitochondria within the Merkel cell and, to a lesser extent, within the nerve terminal. In these hypoxic but unstimulated Merkel cells the numerical density of the vesicles was reduced to 8.3 ± 0.7 vesicles/μm^2 (S.E.M., n=16), which is significantly less ($p < 0.01$ Student's 2-tailed 't' test) than the granule density in normal Merkel cells.

Merkel cells from hypoxic stimulated touch domes and with N_2 around the limb. Touch domes were removed from the limb when the response to mechanical stimulation had ceased. Figure 3.11 shows that Merkel cells taken from these receptors also have distorted mitochondria similar to those in control Merkel cells. They are also deficient in dense-cored vesicles, the density being reduced to 4.3 ± 0.8 vesicles/μm^2 (S.E.M., n=20) This is a significant reduction ($p < 0.01$. Student's 2-tailed 't' test) when compared to the vesicle density in 'control' Merkel cells.

Merkel cells from hypoxic touch domes and with O_2 around the limb. When the N_2 around the limb was replaced by O_2 and the response had returned, tissue

FIGURE 3.10

Micrograph of a 'control' Merkel cell. This cell was from a touch dome exposed to hypoxic conditions but which was not stimulated. The most obvious effect of hypoxia is disruption of the mitochondria both in the Merkel cell and the nerve terminal (NT). Still present, however, between the Merkel cell nucleus (N) and the nerve terminal, are the dense-cored vesicles (V).

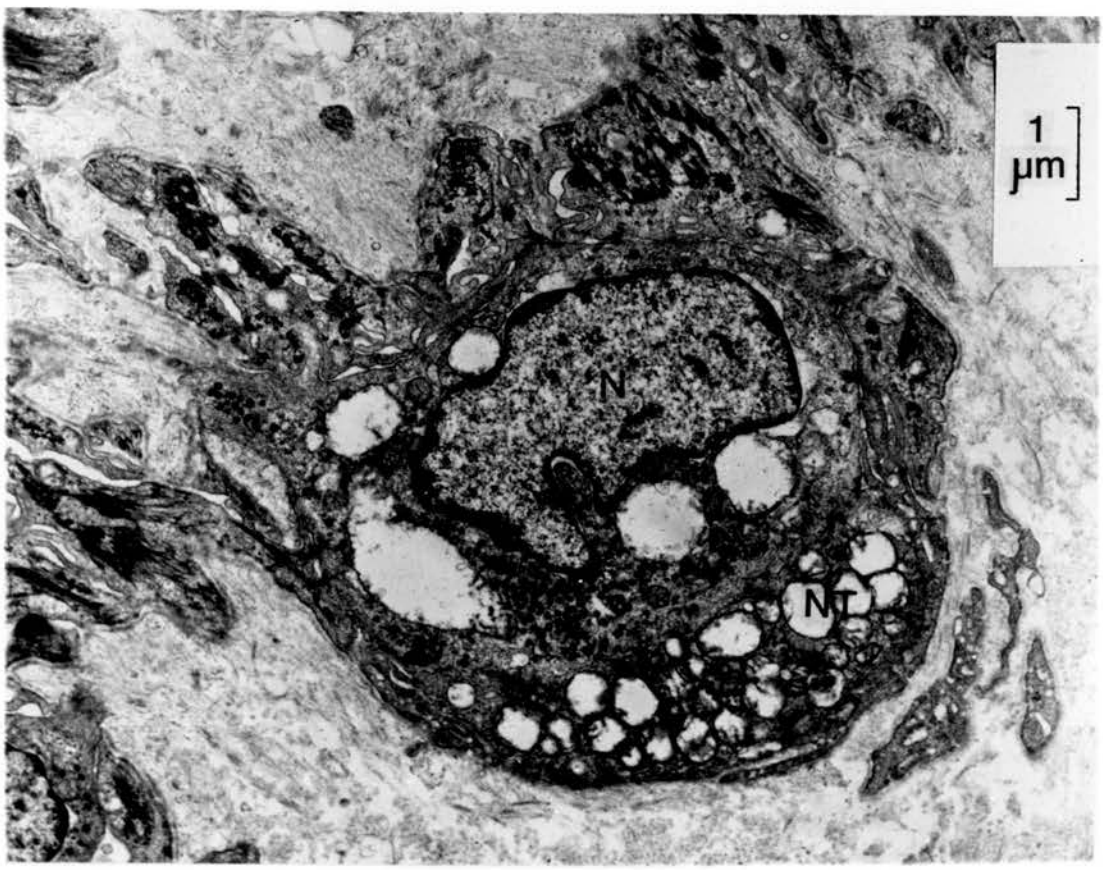
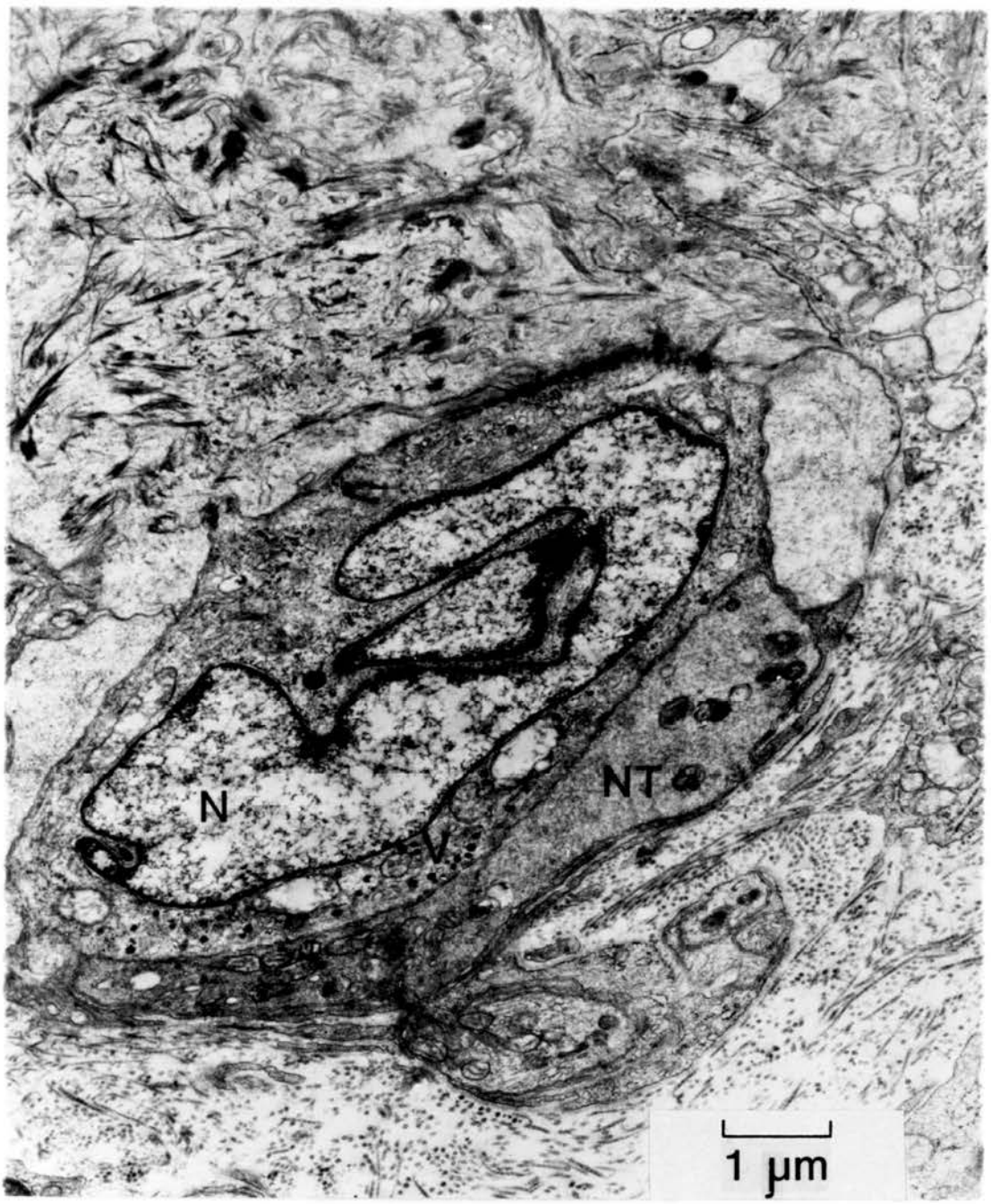

1
µm
N
NT

FIGURE 3.11

Merkel cell and nerve terminal from an hypoxic stimulated touch dome with N_2 around the limb. The effects of hypoxia, particularly on the mitochondria, are still evident. However the difference between this Merkel cell, which was from a touch dome which had ceased to respond to mechanical stimulation in hypoxic conditions, and the control Merkel cell presented in Figure 3.9 is a significant reduction in the number of dense-cored vesicles (V).

(N - Merkel cell nucleus;

NT - nerve terminal).


N
NT
1 µm

was taken for examination. Figure 3.12 is of a Merkel cell from such a receptor. The effects of hypoxia are evident in the disrupted mitochondria both in the Merkel cell and in the nerve ending. However, the vesicle density in these cells, at 7.9 $\pm$ 1.1 vesicles/μm^2 (S.E.M., n=7) is significantly greater ($p < 0.01$, Student's 2-tailed 't' test) than the vesicle density of Merkel cells taken from failed receptors. There was no significant difference between the vesicle numerical density of 'control' Merkel cells and the vesicle density of Merkel cells taken from SAI receptors when the response had returned after the application of O_2 to the limb.

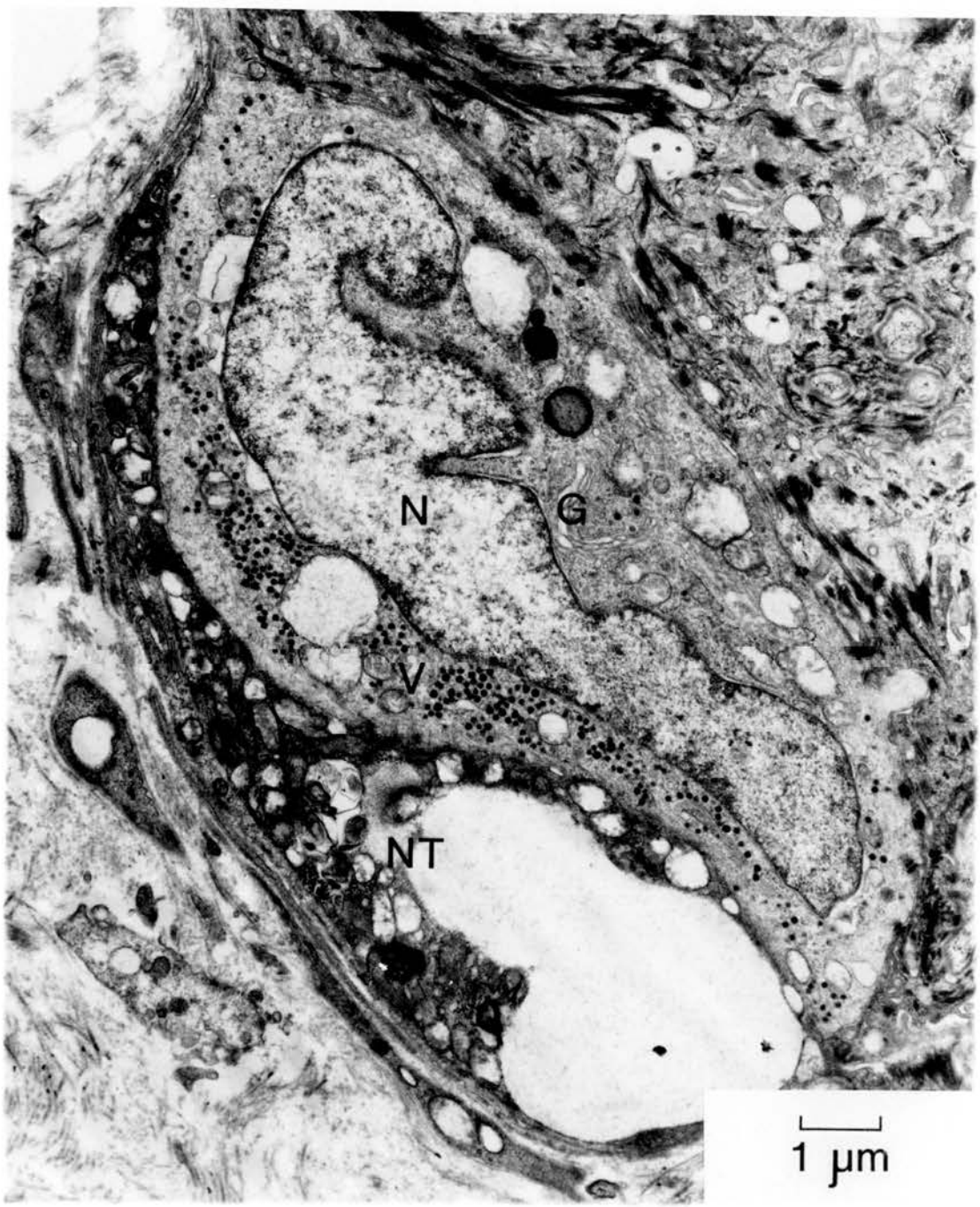
Part 3.4 Summary of Results

SAI receptors when mechanically stimulated in hypoxic conditions eventually fail to respond. In experiments in which animals were ventilated with N_2 the time taken for circulatory arrest to occur was unrelated to the time taken for the receptor to fail. There was also no relationship between the PO_2 and PCO_2 levels in the circulation at the time of receptor failure and the time taken for this to occur.

In experiments designed to eliminate the variation between animals in the time taken for hypoxia to become established, there was a significant decrease in the time to receptor failure when compared with the time taken from the onset of hypoxia. The failure of the receptor when first stimulated in established hypoxic conditions shows a linear decrease in the response with

FIGURE 3.12

Micrograph of a Merkel cell and nerve terminal from an hypoxic stimulated touch dome with O_2 around the limb. The touch dome was removed from the limb when the response to mechanical stimulation had returned. Correlated to a return of receptor response is the reappearance of the dense-cored vesicles (V) in the Merkel cell cytoplasm (c.f. Figure 3.10).
(N - Merkel cell nucleus;
G - Golgi region;
NT - nerve terminal).


N
G
V
NT
1 µm

time.

There was an exponential relationship between the returning response and time when N_2 around the limb was replaced by O_2. When N_2 was reapplied there was a linear decline in the response with time.

Electrical stimulation of the afferent fibre when the response of the receptor to mechanical stimulation had ceased still initiated an action potential in the afferent fibre.

The ISI distribution of the adapted response was exponential during the control period. The distribution of intervals during failure with N_2 and recovery with O_2 could not be determined due to non-stationarity of the response.

Correlated with receptor failure was a significant decrease in the number of dense-cored vesicles found in the Merkel cell cytoplasm when compared with Merkel cells from control receptors. When O_2 was applied to the limb, recovery of the receptor response was correlated to reappearance of the vesicles. The granule density in these cells was not significantly different from that of control Merkel cells.

Part 3.5 Discussion

The results of these experiments show that hypoxic conditions cause the eventual failure of slowly adapting type I (SAI) cutaneous mechanoreceptors to respond to mechanical stimulation. Correlated with receptor failure is a significant depletion in the number of dense-cored vesicles present in the Merkel cells of these receptors when compared to the density

of vesicles in Merkel cells of adjacent unstimulated receptors. The prerequisite for vesicle depletion in hypoxic conditions would therefore appear to be mechanical stimulation of the SAI receptors. Mechanical stimulation of these receptors in normal conditions causes a slight but insignificant reduction in the Merkel cell granule population (Iggo, personal communication). When the response to mechanical stimulation had ceased, electrical stimulation of the afferent fibre innervating the receptor still initiated a response in the fibre. This evidence suggests that Merkel cell vesicles are an essential requirement for the normal function of SAI receptors, possibly by the secretion of a neurotransmitter. This possibility has received support recently from the work of Hartschuh and Weihe (1983) who have demonstrated, at the light microscopic level, an immunohistochemical reaction in Merkel cells to the neurotransmitter peptides met-enkephalin and VIP. These reactions were localised in the Merkel cell to the area of cytoplasm with the greatest vesicle density suggesting that the vesicles are the site of this reaction and that they contain these neurotransmitter substances. However this has not been verified at the electron microscopic level. Should the vesicles contain peptide neurotransmitters then their production, storage, transport and release from the Merkel cell would be a metabolic or O_2 dependent process. If this is the case then mechanical stimulation of the receptor in hypoxic conditions may be expected to cause vesicle depletion if vesicle transport and release were less O_2 dependent or had

preferential use of the available O_2 than the production and storage of the vesicles. If this were not the case then the Merkel cells at receptor failure would still contain numerous vesicles.

Close analogues exist in both structure and function between the Merkel cell-axon complex of SAI receptors and glomus cells of the carotid body and its neural innervation, particularly after exposure to hypoxia (Hoffman & Birrell, 1958; Blümcke, Rode & Niedorf, 1967). Glomus cells normally contain dense-cored granules which in extreme hypoxic conditions, move to the cell periphery where they fuse with the cytoplasmic membrane of the glomus cell. They then discharge their entire contents into the intracellular space. Hypoxia also causes in the carotid body an initial increase in the discharge of the afferent fibre innervating the glomus cells followed by a depression of the response (Eyzaguire & Koyano, 1965). There is also an accompanying increase in the release of catecholamines from the carotid body with hypoxia (González & Fidone, 1977; Hanbauer & Hellstrom, 1978). This has led to the conclusion that the glomus cells respond to hypoxia by the secretion of a transmitter substance stored in the glomus cell granules. In Merkel cells hypoxia does not produce an increase in activity in the afferent fibre and on its own is insufficient to cause vesicle depletion.

In the work described in this paper the degree of hypoxia present in each experiment was determined by measuring the PO_2 and PCO_2 levels of the circulating arterial blood. From the results of the experiments in

which whole animals were suubsequently made hypoxic, the mean control PO_2 level of 111.0 ± 3.8mm Hg (Table 3.1a) was considerably higher than the 'normal' mammalian arterial PO_2 level of approximately 94.0mm Hg (Albritton 1952). The mean control PCO_2 level of 31.0 ± 1.6mm Hg was also considerably lower than the 'normal' PCO_2 level of approximately 41.0mm Hg (Albritton, 1952). As the control blood samples were taken whilst the animals were being artificially ventilated with room air, these results suggest that the cats were being hyperventilated prior to the induction of hypoxia. The consequence of this may have been to delay the effects of N_2 breathing and hence to prolong the time to receptor failure. However from the results available (table 3.1a) there is no obvious relationship between the PO_2 levels prior to the onset of initial hypoxia and the time to receptor failure for individual experiments. When the mean times to receptor failure for both preparations are compared there is a slight but insignificant reduction in the 'limb only hypoxic' preparation where the mean PO_2 level prior to the onset of hypoxia (97.1 ± 1.7mm Hg) was near the normal value quoted above.

Ventilation of the cats with 100% N_2 would have caused a rapid fall in both PO_2 and PCO_2 until a point was reached when circulatory arrest occurred. Thereafter it was unlikely that the blood PO_2 would have changed significantly. Therefore the PO_2 measured at the time of receptor failure would probably be unaltered from that present at circulatory arrest. In the 3 experiments for which results are available the

arterial PO_2 levels at receptor failure (hence the PO_2 levels at circulatory arrest) would appear to be directly related to the time to circulatory arrest but not to the time to receptor failure. This last observation is also true for experiments in which the limb only was made hypoxic. The variation in the blood PO_2 levels at receptor failure for both preparations (9.0 - 25.6mm Hg. see Table 3.1) suggests that receptor failure was more dependent on external sources of O_2 rather than on O_2 from the circulation. Receptor recovery after the replacement of N_2 around the limb with O_2 is consistent with this explanation and further illustrates the dependence of the Merkel cell-neurite complex on the O_2 levels in the external environment. The return of the response coincided with the return of the vesicles in the Merkel cell cytoplasm.

Although there was considerable overlap in the arterial PO_2 levels at receptor failure between all experiments for both preparations, there was possibly an even greater variation in the tissue PO_2 levels due to the Bohr effect (Haldane and Priestly, 1935). A reduction in blood PCO_2 levels, as occurred in the 'whole-animal hypoxic' preparation, causes the O_2 present in the blood to be more firmly bound to the haemoglobin. The consequence of this is that the tissue PO_2 adjacent to the capillaries is less than that measured in the arterial blood. The opposite effect would be the case in the 'limb-only hypoxic' preparation since the PCO_2 level in the blood was considerably increased above normal. This would cause the haemoglobin to give up its O_2 more readily and

hence make available to the tissues, adjacent to the capillaries, more of the O_2 present in the blood.

Normal tissue PO_2 outside capillaries, although very variable, averages 40mm Hg (Guyton, 1976). In isolated mitochondria the first perceptible drop in the rate of O_2 consumption occurs at an estimated PO_2 of 1.0mm Hg. A PO_2 of 0.1mm Hg will still maintain half maximal rate of cellular respiration (Jöbsis, 1974). However the metabolic pathways involved in enzymatic reactions within cells require much higher levels of PO_2. Jöbsis (1974) also concluded that the normal oxidative metabolism served by enzymes (such as may occur in Merkel cells) other than the respiratory chain appears susceptible to relatively minor fluctuations in normal PO_2. Davis, Carlsson, Macmillan and Siesjo (1973) demonstrated that during periods of lowered PO_2 (20.0 - 50.0 mmHg) acheived by changes in ventilatory gas mixtures, the rate of 5-hydroxytryptophan (5-HTP) production decreased. The relationship between 5-HTP production and PO_2 level was linear. This is similar to the result found when touch domes were stimulated to exhaustion in hypoxic conditions - there was a linear decay in the response with time both when receptors were stimulated for the first time in hypoxic conditions (Figure 3.5) and when N_2 was applied to the limb in N_2/O_2 experiments (Figure 3.8b). This assumes that afferent fibre activity reflects metabolic activity within the Merkel cells in which case failure of the receptor system may be due to a decreased rate of transmitter production within the Merkel cell.

The greater the distance cells are from the

capillaries the more readily they are affected by a reduction in tissue PO_2 (Jöbsis, 1964). Because Merkel cells, situated within the basement membrane of the skin, are some distance from the nearest capillaries it is likely that they are some of the first cells affected by a reduction in blood PO_2 levels. However this may not affect the normal function of the respiratory chain or normal enzymatic function if sufficient O_2 diffuses through the skin. When N_2 is blown around the limb it is possible that enough O_2 remains for cellular respiration to occur but that normal enzymatic function eventually fails. The time to Merkel cell failure would be dependent upon the metabolic rate of all the cells in the surrounding tissue. The higher the metabolic rate the more rapidly the remaining O_2 would be depleted and the quicker receptor failure would occur. The opposite would also be true - a reduced metabolic rate would increase the time to receptor failure. This may explain the variation in the time to receptor failure from the onset of hypoxia and the independence of this time on the arterial PO_2 at receptor failure. The decrease in the time taken for another touch dome, in the same hypoxic environment, to cease to function possibly indicates that the majority of the time taken to receptor failure is for the establishment of a sufficiently hypoxic environment around the outside of the limb and, once this is achieved, receptor failure occurs relatively quickly. It may also indicate that hypoxia had already affected the Merkel cell's within these touch domes and that the significantly shorter

time to receptor failure resulted from these changes. The histological effects of hypoxia on unstimulated receptors remains to be investigated.

The apparent dependency shown by Merkel cells on the O_2 levels around the limb as blood PO_2 levels fall is supported by the findings of Shaw and Messer (1931). They found that even at very low partial pressures of O_2 the skin absorbed from the atmosphere around it what O_2 was available to it. Fitzgerald (1957) has also shown that the outward loss of O_2 from the skin even to an atmosphere of pure N_2 was negligible despite its partial pressure in the tissues. It was suggested by Shaw and Messer (1931) that all the O_2 passing into the skin was utilized in tissue oxidation and that the higher the O_2 tensions outside the skin the greater the utilization of O_2 from external sources rather than from the blood. If epidermal cells appear to use O_2 from the atmosphere for oxidative metabolism then Merkel cells are equally likely to do so particularly as PO_2 levels in the blood and surrounding tissues decrease. As mentioned earlier if both the internal and external environments of the limb are depleted of O_2 then all metabolic activities in the cell will cease. This would be especially true if the transduction process in SAI mechanoreceptors was by chemosynaptic transmission from the Merkel cell to the adjacent nerve ending as mechanical stimulation of the receptor would increase the energy requirements of the cell. Lack of O_2 in these circumstances would inhibit the production of further transmitter substance, eventually causing receptor failure.

There was a linear relationship between the receptor response and time when touch domes were first stimulated in established hypoxic conditions. This was also true in N_2/O_2 experiments when O_2 around the limb was replaced with N_2. However the rate of failure was different in the different experimental situations. This is evident from the slope of the lines in figures 3.5 and 3.8b, the regression coefficient in the former case being -0.77 and in the latter -1.87. The more rapid failure with N_2 in N_2/O_2 experiments may be an indication that O_2 was applied to the limb for an insufficient length of time for complete recovery to occur. This is borne out by the reduced number of dense-cored vesicles present in Merkel cells after recovery of the receptor response with O_2 around the limb (see Table 3.5). There was, in contrast to failure with N_2, an exponential increase in the response to mechanical stimulation when O_2 was blown over the limb (see Figure 3.8a).

Hypoxia had no effect on the level of electrical stimulation used (threshold voltage + 20%) to initiate an action potential in its afferent fibre even though the response of SAI receptors to mechanical stimulation had ceased. This may confirm that the failure of SAI receptors is due to vesicle depletion in the Merkel cells of the receptor and that the afferent fibre is unaffected by hypoxia. However we do not know at what position the afferent fibre is stimulated and it may be that hypoxia does in some way affect the nerve ending adjacent to the Merkel cell causing failure of the receptor. The resolution of this problem awaits

experimental procedures which will, in some way, allow electrophysiological separation of the Merkel cell and nerve ending.

The ISI distribution of the adapted response during the control period of stimulation did not vary significantly from an exponential ($p > 0.05$, X^2-test; see Table 4.9). This is similar to the findings of Iggo and Muir (1969) who showed that the response of a touch dome to sustained mechanical stimulation fitted an exponential pattern for long intervals (>45ms) but failed to do so for shorter intervals. In the 2 units examined, C151282 and C251083, the distribution was exponential for intervals greater than 44.0ms and 29.0ms respectively, these values being the maximum frequencies of the histograms.

An exponential distribution corresponds to the time intervals between successive occurrences of random impulses, the distribution of the impulses being a Poisson. This would occur if the impulses were generated at sites that were independent of each other. As Iggo and Muir (1969) point out the anatomical organisation of the Merkel cell-neurite complex within a touch dome is consistent with the hypothesis that individual Merkel cell's and their corresponding nerve terminals function as independent generators. If this is the case, then as more and more Merkel cells become exhausted in hypoxic conditions, the response of the receptor to mechanical stimulation should have became more regular. Horch et al. (1974), by using small diameter probes, stimulated small areas of touch domes and found, in fact, that the smaller the portion of the

dome stimulated the more regular was the reponse. However because of the rapidity of receptor failure and the resultant non-stationarity of the response, the effects of hypoxia on the ISI distribution of the adapted response of the SAI receptor could not be tested.

Part 3.6 Conclusions

The results of the work presented in this chapter verify and extend the findings of Anand *et al.*(1979) and indicate that transduction in SAI mechanoreceptors is an O_2 dependent process.

As electrical stimulation of the afferent fibre close to the Merkel cell produces an action potential when there is no response to mechanical stimulation, this suggests that it is the Merkel cells that have failed and not the afferent fibre. This conclusion, though based on indirect evidence, is reinforced by the significant depletion in the numbers of dense-cored vesicles in the Merkel cell at the time of receptor failure and their reappearance with receptor recovery.

However no firm conclusions can be made about the vesicle involvement in the transduction process. The possibility that they contain a neurotransmitter substance has neither been proved nor disproved and requires further investigation.

CHAPTER 4

Pharmacological Experiments

PHARMACOLOGICAL EXPERIMENTS

Part 4.1. Introduction

The main conclusion reached from the results of the hypoxic experiments presented in Chapter 3 was that transduction in SAI mechanoreceptors was an O_2 dependent process. Correlated with receptor failure was a depletion in the numbers of dense-cored vesicles present in the Merkel cell cytoplasm. This could be expected if the vesicles contained a neurotransmitter particularly in slowly adapting receptors where the maintenance of the response to static mechanical displacement would require a high turnover of transmitter substances.

The Merkel cell-nerve terminal arrangement has features suggestive of a neurotransmitter function. The rod-like processes of the Merkel cell which extend up into the adjacent epidermal cells are analagous to the sensory hairs or stereocilia of the mammalian auditory receptor cells. These sensory hairs are tubular projections of the cell membrane arising from the cell surface. Bending of the sensory hairs is brought about by displacement of auxiliary sensory structures to which the tips of the hairs are attached. The displacement of the sensory hairs is the first step in the excitation process in the receptor cell. It is now known that the fine filaments running down the length of each stereocilium are composed of actin (Flock and Cheung, 1977). Similar filaments are found within Merkel cell processes. Deformation applied to

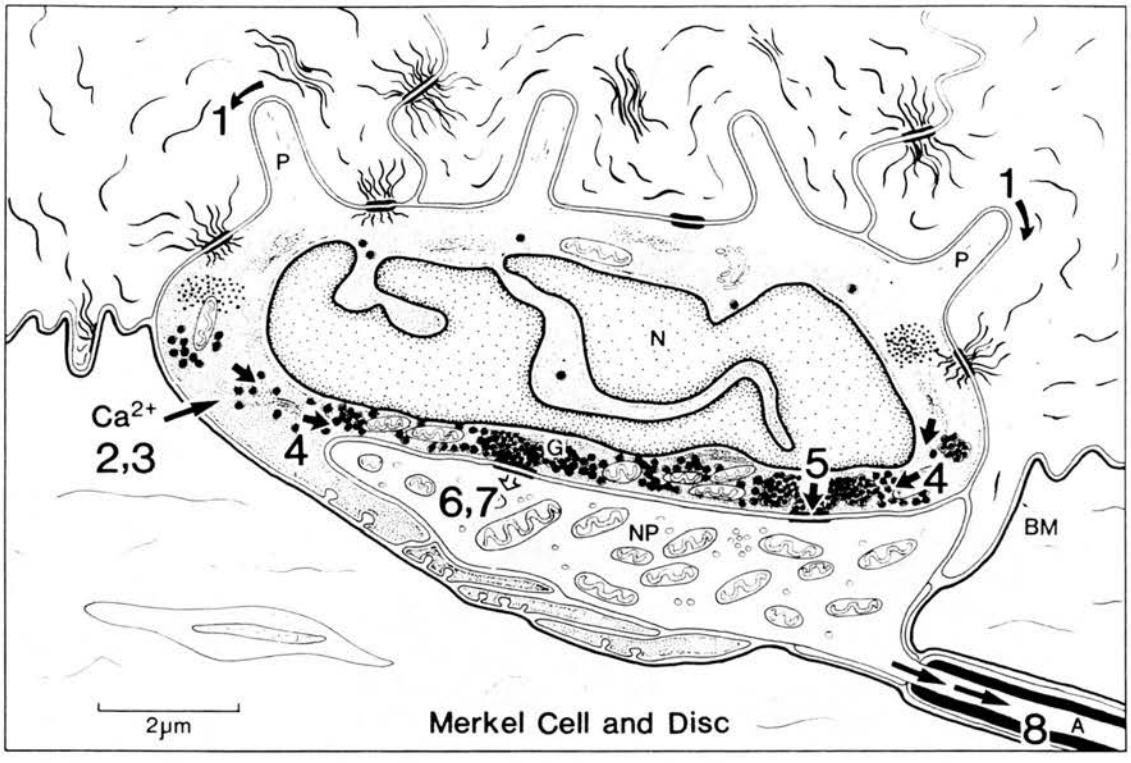
the skin overlying the Merkel cell-neurite complex could be expected to cause these processes to bend in a similar manner to that of the stereocilia of the auditory receptor cells. The simplest transduction process to visualise is presented schematically in Figure 4.1.

It is based on evidence that Ca^{2+} is required for the secretion of substances, including neurotransmitters, from secretory cells. Katz and Miledi (1965, 1967a and b) were first to show that Ca^{2+} was directly concerned with the release of aceylcholine from motor nerve terminals. Since then voltage dependent Ca^{2+} channels have been demonstrated in photoreceptors (Kaneko and Shimazaki, 1975; Ross and Stuart, 1978) and it has recently been shown the release of catecholamines from the adrenal medulla is dependent on the presence of Ca^{2+} (Baker and Knight, 1981). In the carotid body, a receptor system with both structural and functional similarities to that of the Merkel cell-neurite complex, Ca^{2+} binding sites have been identified in the membrane of the dense-cored vesicles within the glomus cells. (Hess, 1977; Hansen and Smith, 1979). Calcium binding sites have also been demonstrated on the cell membrane of vertebrate sensory hair cells (Moran, Rowley and Asher, 1981). On that basis the following hypothesis was proposed. Bending of the Merkel cell processes causes stretching or compression of the cell membrane. Assuming Ca^{2+} channels to be present in the cell membrane, alterations in the tension across the cell membrane alters the structural configuration of the channels

FIGURE 4.1

An illustration of a Merkel cell and the adjacent nerve ending. Shown are the main structural features and the suggested steps in the proposed transduction process.

Deformation of the overlying skin causes
(1) bending of the cytoplasmic processes
(P) which produces
(2) a change in membrane permeability
allowing
(3) the entry of Ca^{2+} into the Merkel cell.
Ca^{2+} entry mobilizes
(4) the Merkel cell granules (G) towards the regions of membrane specialization between the Merkel cell and the nerve terminal where
(5) they release a neurotransmitter. This binds with receptor sites
(6) on the nerve ending (NP) causing
(7) a graded depolarization. If sufficient transmitter is released then
(8) an action potential is initiated in the afferent fibre (A).
(N - Merkel cell nucleus; BM - basement membrane of epidermis).


1
P
1
P
N
Ca2+
2,3
4
G
5
4
6,7
NP
BM
2µm
Merkel Cell and Disc
8
A

allowing the entry of Ca^{2+} into the cell. This in turn causes the release of neurotransmitter from the vesicles of the Merkel cells producing a graded depolarization of the adjacent nerve terminal. With sufficient stimulation, transmitter release would be such that an action potential would be initiated in the afferent fibre.

This hypothesis was tested in the following series of experiments in which two known Ca^{2+} channel blockers, cobalt chloride ($CoCl_2$) and verapamil hydrochloride, were either slowly injected into the localised limb circulation of the cat or intradermally injected under SAI mechanoreceptors in both cats and rats. If Ca^{2+} channels were involved in the transduction process then the prevention of Ca^{2+} entry into the Merkel cell would be expected to cause receptor failure. Cobalt, which is an inorganic Ca^{2+} channel blocker, was chosen as its effects on the neuromuscular junction are well documented (Weakly, 1973; Kita and van der Kloot, 1973). It is also an effective blocker of transmission between photo-receptors and horizontal cells in the turtle eye (Cervetto and Picolino, 1974).

In order to verify that the effects of $CoCl_2$ were not the result of a direct effect of Co^{2+} on the Merkel cell or its adjacent nerve terminal the organic Ca^{2+} channel blocker, verapamil hydrochloride, was used. Verapamil is known to reduce significantly the Ca^{2+} current in cardiac muscle (Kohlhardt, Bauer, Krause and Fleckenstein, 1972) and to reduce the Ca^{2+} current in the snail neuron (Kostyuk, Krishtal, Shakhovalov,

1977). The responses of SAI receptors after the injection of both $CoCl_2$ and verapamil were compared with the responses after the injection of physiological saline - the control solution.

Where possible the effects of $CoCl_2$ and verapamil were tested on SAII receptors, which are known not to have an accessory receptor cell, (Chambers et al. 1972), and on hair follicles. Electrical stimulation of the afferent fibre close to the Merkel cell was carried out as a test of nerve conduction particularly when the response of the SAI receptor to mechanical stimulation had ceased.

In experiments in which $CoCl_2$ was injected into the limb circulation, serum samples were removed so that the Co^{2+} concentration in the serum could be measured. This was done to give an indication of the Co^{2+} levels present in the circulation at the time when the response to mechanical stimulation had ceased.

Part 4.2. Experimental Protocol

Slow injection of physiological saline, cobalt chloride and verapamil hydrochloride into the limb circulation of cats.

Animal Preparation A total of nine units were investigated in six cats which weighed between 2.1 - 2.6 Kg. After induction of anaesthesia each animal was placed on its dorsum on a thermal blanket and its trachea cannulated.

An incision was made over the saphenous nerve and the femoral blood vessels were cannulated and interconnected as described in Chapter 3.2. In these

experiments however, the polythene sock was not placed around the limb.

Electrical and mechanical stimulation An SAI mechanoreceptor was stimulated both electrically and mechanically and control responses recorded (see Chapter 2.5 and 2.6). Electrical stimulation was carried out by implanting into the skin on each side of the receptor, two fine electrodes which were connected to the output of an electrical stimulator. The timing of events was arranged so that electrical stimulation always preceded each mechanical stimulation of the receptor (see Figure 2.2).

Preparation of solutions Before each experiment the following solutions were prepared as required.

Physiological saline A solution of 0.15M NaCl was prepared and kept in a water bath at 37°C. This was used as the control solution - the receptor response to mechanical stimulation after the injection of either $CoCl_2$ or verapamil hydrochloride was compared with the receptor response after injection of 0.15M NaCl.

The pH and osmolality of the saline solution was measured on a pH meter (EIL model 38A) and a depression of freezing point osmometer (Vogel) respectively. Both instruments were calibrated using standard solutions before the start of each experiment.

Cobalt chloride Solutions of 5, 10 and 15mM $CoCl_2$ were made up in 0.15 NaCl and kept in a water bath at 37°C until required. The pH and osmolality of these solutions were measured and compared with the values for 0.15M NaCl. With the exception of 15mM $CoCl_2$ which showed a slight drop in pH, all other values were

practically unaltered from the values for 0.15M NaCl and therefore did not require adjusting.

Verapamil Hydrochloride A 100μM solution of verapamil hydrochloride was made up in 0.15M NaCl and kept at 37°C until required. The pH and osmolality of this solution were also unaltered from the values for physiological saline and therefore were not adjusted.

Injection of solutions Before the injection of a solution into the circulation, the central end of the femoral artery was occluded and the venous return from the limb redirected into the peripheral end of the artery. This procedure is fully described in Chapter 3.2. Ten ml of the appropriate solution was then injected, over 1 to 2 minutes, into the localised limb circulation. Three experiments were carried out in which 0.15M NaCl was injected, three experiments in which 5mM $CoCl_2$ was injected, one experiment each in which 10 and 15mM $CoCl_2$ was injected and one experiment in which 100μM verapamil was injected.

The effects of each solution on the SAI receptor response to electrical and mechanical stimulation was recorded and compared to the evoked receptor response after the injection of 0.15M NaCl. The effects of each solution on the background activity of an SAII receptor was also investigated in three experiments.

Blood sampling Blood samples were taken before the injection of a solution and at the end of each experiment for blood gas analysis. In the five experiments in which $CoCl_2$ was injected, blood samples were also taken throughout the experiment for the measurement of Co^{2+} and Ca^{2+} levels in the limb

circulation, as described in Chapter 2.9.

Recovery of receptor response To test if the effects of the substances injected were reversible, the general circulation was restored to the limb and the response of the receptor to both electrical and mechanical stimulation recorded.

Histological procedures. At the end of each experiment the limb was perfused via the peripheral end of the femoral artery, with prefixative and fixative solutions. Outflow of fixative was from the peripheral end of the femoral vein. Fixative was also applied topically to the surface of both stimulated and non-stimulated touch domes which were then removed from the limb and processed for light and electron microscopic examination (see Chapter 2.10).

Data Analysis Analysis of both histological and electrophysiological data was carried out as described in Chapter 2.11.

Intradermal injection of physiological saline, cobalt chloride and verapamil hydrochloride under SAI receptors Because solutions were being injected locally under individual SAI receptors, several of them could be tested in each animal. Physiological saline (0.15M NaCl) was injected under three touch domes in one cat; cobalt chloride was injected under three touch domes in one cat and one touch dome in another cat and verapamil hydrochloride was injected under three touch domes, two of which were in a cat and one in a rat. With the exception of one touch dome which had both physiological saline and $CoCl_2$ injected under it, all other touch domes were innervated by separate afferent

fibres. In all, 3 cats weighing between 2.0 - 2.5Kg and two rats weighing 340 and 410g were used.

Animal preparation In cat experiments the same procedures as those described for the slow injection of solutions were carried out. In rat experiments the femoral blood vessels were not cannulated. However the carotid artery was cannulated and connected to a blood pressure transducer for the measurement of the animal's blood pressure.

Preparation of solutions Solutions of 0.15M NaCl, 5mM $CoCl_2$ and 100μM verapamil hydrochloride in 0.15M NaCl were prepared as described above and stored in a water bath at 37°C, until required.

Injection of solutions The solution to be injected was drawn up into a glass syringe which was graduated in microlitres. Attached to the syringe was a 27G needle which, by being connected to a terminal of the electrical stimulator, acted as one of the electrical stimulating electrodes when inserted into the skin close to the touch dome. The other electrode was implanted into the skin on the other side of the receptor. The syringe was mounted on a micro-manipulator which enabled the needle to be positioned accurately, close to the touch dome. It also held the syringe stable throughout the experiment, particularly when a solution was being injected. In all but two experiments, 100μl of each solution was injected. In the other two experiments 25μl and 50μl of 5mM $CoCl_2$ were injected.

Electrical and mechanical stimulation Electrical and mechanical stimulation was applied before, during

and for some time after the injection of a substance and the response of the SAI receptor recorded continuously. In one experiment 100µl of 100µM verapamil was injected under hair follicles which were stimulated by a motorised brush stimulator. Supplementary doses of verapamil were injected and again the hair follicles were stimulated. Because of the nature of the mechanical stimulation the response of the hair follicles when stimulated could not be quantified.

Histology At the end of the cat experiments, receptors were prepared for histological examination by either perfusion or immersion fixation, the methods for which are described in Chapter 2.10. No tissue was removed in rat experiments.

Data analysis Data analysis was carried out in accordance with the methods described in Chapter 2.11. Analysis of ISI distributions was carried out on the adapted responses obtained when receptors were stimulated in each experimental situation.

Part 4.3 Results

Slow injection of 0.15M NaCl (physiological saline) Electrophysiology Slowly adapting type I (SAI) cutaneous mechanoreceptors continued to respond to mechanical stimulation for up to 40 minutes after the slow injection of saline into the localised limb circulation of cats. Figure 4.2 shows the responses in an experiment in which the afferent fibres being recorded from contained fibres from both an SAI and an

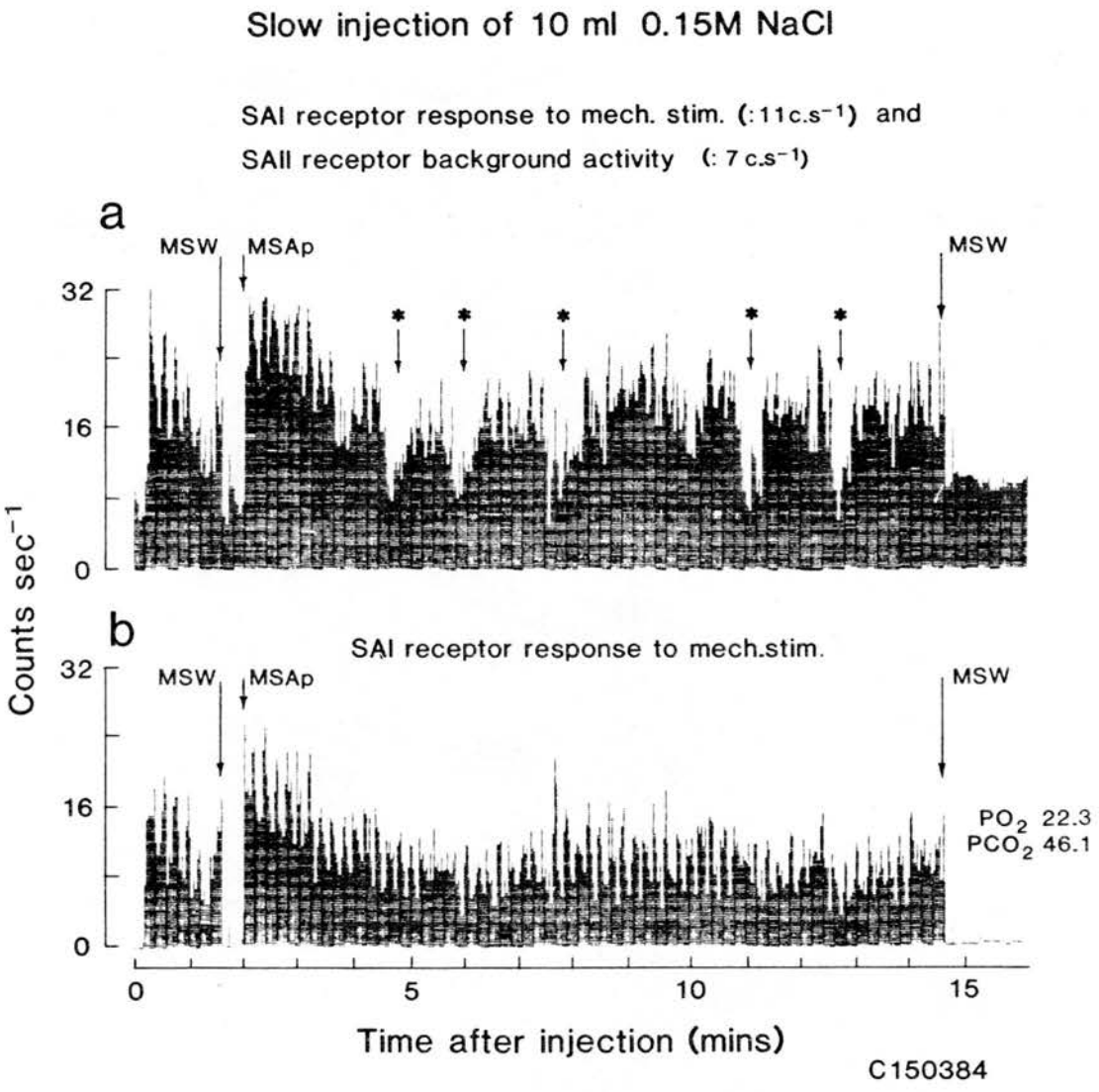
FIGURE 4.2

Experiment C150384

a. The responses produced by mechanical stimulation of an SAI receptor and the background activity of an SAII receptor are shown. The fluctuating response was produced by the background activity of the SAII receptor periodically falling almost to zero (*) and then recovering again.

b. The SAII background activity has been filtered out leaving only the response of the SAI receptor to mechanical stimulation. Also shown are the PO_2 and PCO_2 levels in the limb circulation 15 minutes after the injection.

(MSW - mechanical stimulator withdrawn;
MSAp - mechanical stimulator applied to SAI receptor).


Slow injection of 10 ml 0.15M NaCl
SAI receptor response to mech. stim. (:11c.s⁻¹) and
SAII receptor background activity (: 7 c.s⁻¹)
a
MSW
MSAp
MSW
32
16
0
Counts sec⁻¹
b
SAI receptor response to mech.stim.
MSW
MSAp
MSW
32
16
0
PO2 22.3
PCO2 46.1
0
5
10
15
Time after injection (mins)
C150384

SAII receptor. The response of the SAI receptor to mechanical stimulation and the spontaneous discharge of the SAII receptor were recorded together. It is this data, plotted as a histogram by the analysis program RATE 1, which is presented in Figure 4.2a. With the use of the spike discriminator during data analysis it was possible to remove the SAII response and the resultant histogram of the SAI receptor responses to mechanical stimulation is shown in Figure 4.2b. The response of the SAII receptor throughout the experiment was calculated by subtracting the SAI receptor response from the combined response of both the SAI and SAII receptors.

Throughout the control period of the experiment, before the saline was injected, the SAII background response was steady varying only slightly about its average discharge rate of 7 spike counts per second (c/s) (see Figure 4.3a). However after the injection into the localised limb circulation the SAII background response increased initially to 150% of its control response then began to fluctuate sometimes falling almost to zero. This is reflected in the significant increase in the variability ($p < 0.01$, 2-tailed F-test for variances) of the SAII background response at 12 minutes compared to the variability at 90s after the injection (Table 4.1). Figure 4.3b shows clearly the SAII background response falling to 10% of its control firing rate after 8 minutes 40 seconds and to about 35% of its control firing rate after 10 minutes 10 seconds. However the SAII receptor response always recovered quickly and, at the end of this experiment had a

FIGURE 4.3

Experiment C150384 - Same data as presented in Figure 4.2, but with the responses plotted as a percentage of the control responses.

a. Both the response of the SAI receptor to mechanical stimulation (●) and the background activity of the SAII receptor (O) are increased for a few minutes immediately after the injection.

b. Clearly shown is the fluctuating background response of the SAII receptor. At approximately 8.5 minutes after the injection the response has fallen below 10% of its control response whereas within a further 20 - 30s it has increased to almost 150% of its control response.

The SAI receptor response to mechanical stimulation is relatively constant responding, 11 minutes after the injection, at approximately 75% of its control level.

The PO_2 and PCO_2 levels at this time are indicated.

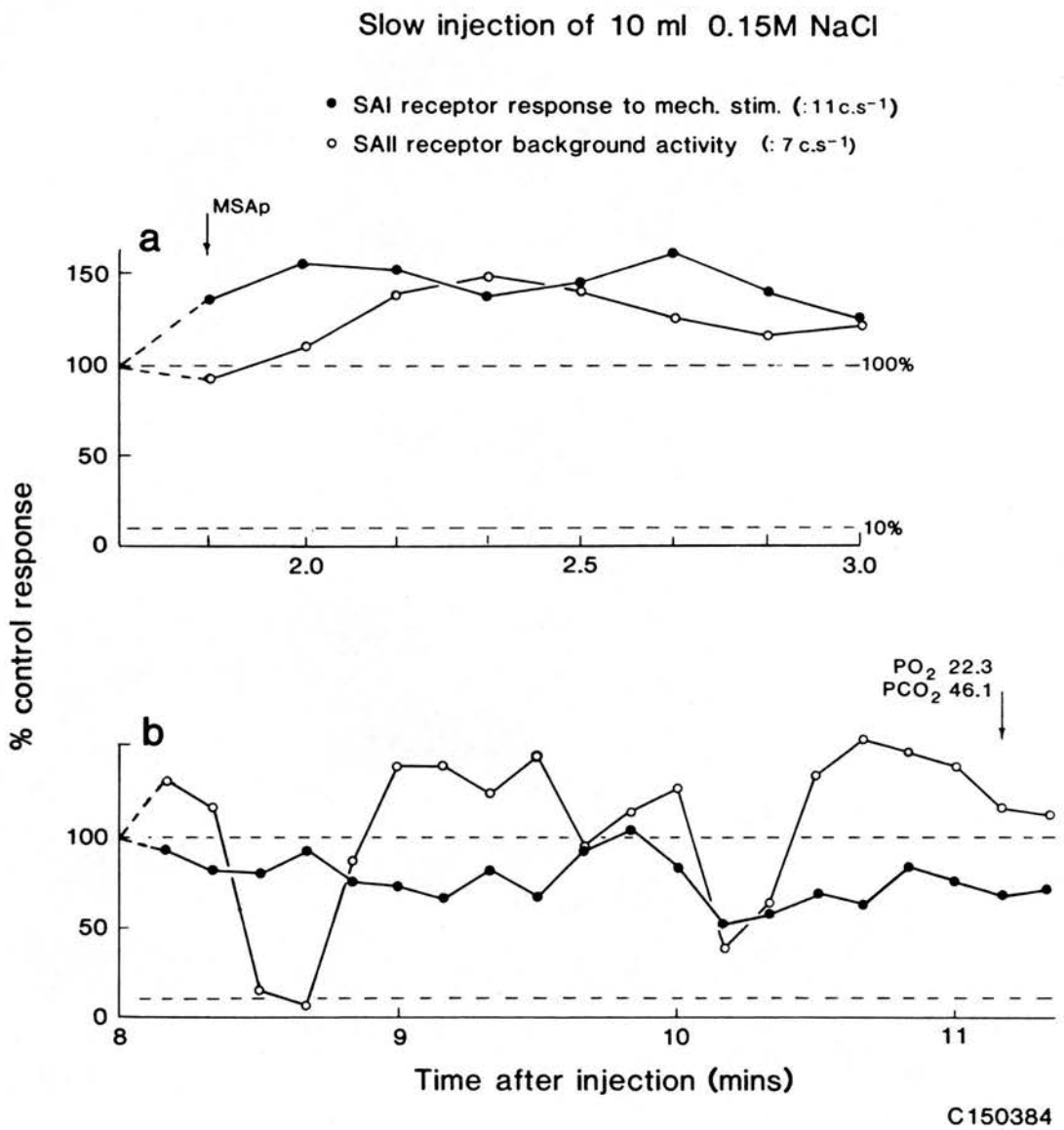

Slow injection of 10 ml 0.15M NaCl
• SAI receptor response to mech. stim. (: 11 c.s⁻¹)
○ SAII receptor background activity (: 7 c.s⁻¹)
MSAp
a
150
100
50
0
100%
10%
2.0
2.5
3.0
PO2 22.3
PCO2 46.1
b
100
50
0
8
9
10
11
% control response
Time after injection (mins)
C150384

TABLE 4.1

Experiment C150384. A summary table of the responses of both the SAI and SAII mechanoreceptors at different times after the slow injection of 0.15M saline (physiological saline). Also shown are the Po_2 and PCO_2 levels 12 minutes after the injection.

TABLE 4.1

C150384 - SUMMARY

Time after injection	SAI + SAII		SAI		SAII		PO_2	PCO_2
	Response (Counts/s)	% Control Response	Response (Counts/s)	% Control Response	Response (Counts/s)	% Control Response		
CONTROL	18.5 ± 1.4 (n = 8)	-	11.5 ± 1.2 (n = 8)	-	7.0 ± 0.3 (n = 8)	-	-	-
1.5 min	25.1 ± 0.8 (n = 9)	135.6	15.8 ± 0.9 (n = 6)	137.4	8.8 ± 0.3 (n = 6)	125.7	-	-
12 min	16.4 ± 0.8 (n = 20)	88.6	8.8 ± 0.3 (n = 20)	76.5	7.54 ± 0.7 (n = 8)	107.6	22.3	46.1
13 min	-	-	-	-	9.3 ± 0.91 (n = 8)	133		

(mean ± SE)

discharge rate of 9.3 c/s which is 133% of its control response.

In contrast to the response of the SAII receptor the SAI receptor response, after also increasing to approximately 150% of its control firing rate, declined over the remainder of the experiment to a steady firing rate of about 76% of the receptor's control response. At no time did the response approach the 10% failure point. The difference in the variability in the response with time was also not significant ($p < 0.05$ 2-tailed F test).

The PO_2 and PCO_2 levels in the limb circulation at the end of the experiment, when the receptor response was at 76% of its control rate, were 22.3 and 46.1 mm Hg respectively.

Table 4.1 summarises the data from this experiment. The results of all experiments in which 10 ml of physiological saline was injected into the limb circulation of the cat are shown in Figure 4.4. Also shown in this figure are the PO_2 and PCO_2 levels in the limb circulation at various times through the experiment. Only SAI receptor responses were recorded in these experiments. In one experiment (C130582) the receptor was stimulated for 40 minutes and in another (C061283) for 35 minutes after the injection of 0.15M NaCl. These figures indicate that over a period of time the responses of SAI receptors to mechanical stimulation varied from time to time, but never approached the 10% failure level. In experiment C130582, the receptor response was approximately 75% of the receptor's control response ten minutes after

the saline injection. Thirty minutes later the response had increased to 120% of the control firing rate. A summary of the responses at different times throughout the experiment are given in Table 4.2.

From the results of unit C061283 (Table 4.3), there is evidence to suggest that the levels of PO_2 and PCO_2 measured in the limb circulation had little significant effect on the response of the receptor to mechanical stimulation. Ten minutes after the saline injection, PO_2 and PCO_2 levels were 38.0 and 61.0mm Hg respectively. The response at this time was down to approximately 58% of the control. However 35 minutes after the injection when the PO_2 level had dropped to 21.5mm Hg and the PCO_2 level had risen to 62.9mm Hg, the response of the receptor was back to 79% of its control response. In the hypoxic experiments of Chapter 3 receptor failure had occured in all experiments in less than 10.5 minutes with similar PO_2 and PCO_2 levels (Table 3.1).

Intradermal injection of 0.15M NaCl (Physiological saline)

In all experiments in which intradermal injections were carried out, there was a normal arterial blood supply to the limb.

Electrophysiology Figure 4.4 shows the response of SAI mechanoreceptors to mechanical stimulation before and after 100µl of saline had been injected intradermally just beneath each SAI receptor. The response of each touch dome after the injection was similar to the response of the touch dome after the injection of saline into the limb circulation - the receptor

TABLE 4.2

Experiment C130582. Slow injection of 0.15M NaCl - summary of results.

TABLE 4.2

C130582 - SUMMARY

Time after injection	Response (Counts/s)	% Control Response
CONTROL	8.42	-
+ 6 min	10.02 ± 0.3 (n = 8)	119
+ 10 min	6.36 + 0.2 (n = 8)	75.5
+ 20 min	7.96 ± 0.5 (n = 8)	94.5
+ 40 min	10.1 ± 0.4 (n = 7)	120

(mean ± SE)

TABLE 4.3

Experiment C061283. Slow injection of 0.15M NaCl - summary of results including PO_2 and PCO_2 levels throughout the experiment.

TABLE 4.3

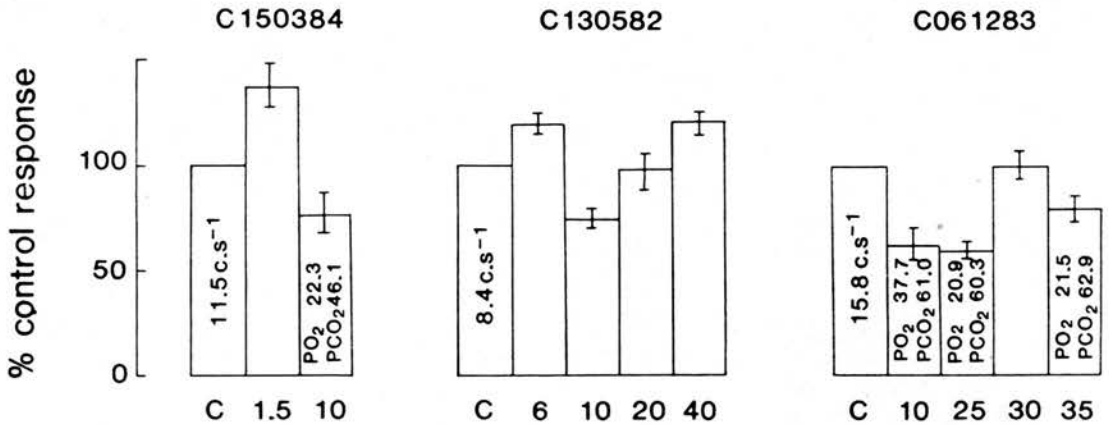
Time after injection	Response (Counts/s)	% Control Response	PO_2 (mm Hg)	PCO_2 (mm Hg)
CONTROL	15.8	-	98.9	32.1
10min	9.1 ± 1.2 (n = 7)	57.6	37.7	61.0
25min	9.7 ± 0.3 (n = 6)	61.7	20.9	60.3
30min	15.6 ± 1.3 (n = 6)	100.0	-	-
35min	12.5 ± 0.8 (n = 5)	79.1	21.5	62.9

(mean ± SE)

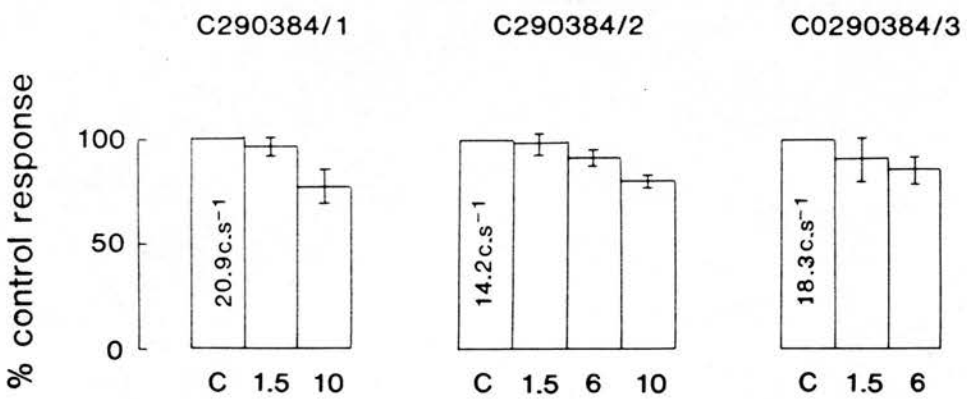
FIGURE 4.4

Illustrated are histograms of the % control response against time for all experiments in which saline was either slowly or intradermally injected. In the first column of each histogram (C) is the control response of the touch dome for that experiment. Also shown, where these were measured, are the PO_2 and PCO_2 levels at the times indicated at the bottom of the columns.

Slow injection of 10 ml 0.15M NaCl



Intradermal injection of 0.15M NaCl



Time after injection (min)

response varied slightly throughout each experiment showing an overall small decrease in response with time. The response however never approached the 10% failure point. A summary of the results showing the percentage of the control response against time for each experiment in which 0.15M NaCl was intradermally injected is set out in Table 4.4.

Table 4.5 summarises the results from all experiments in which saline was either slowly injected into the limb circulation or was intradermally injected under SAI mechanoreceptors.

Analysis of ISI's of the adapted response in experiment C150384 revealed an exponential distribution both in the control period prior, to the injection of 10ml of 0.15M NaCl, and at approximately 10 minutes after the injection (Table 4.6). However comparison of the ISI distribution 10 minutes after the the injection with the normalised control response showed a signifance difference ($p < 0.05$, X^2 test) in the mean interval length between the 2 distributions. Similar results were found in experiment C290384/2. Exponential distributions of ISI's were found during the control period and at 5 and 10 minutes after the intradermal injection of saline. However the mean ISI's at these times were again significantly different ($p < 0.05$, X^2 test) from the mean ISI of the normalised control response.

Histology Figure 4.5a. is of a Merkel cell taken from a mechanically stimulated touch dome after the injection of 0.15M NaCl. The appearance of this cell is similar to that of a normal Merkel cell, the

TABLE 4.4

Units C290384/1, /2 and /3. Summary of the results of experiment C290384 in which 0.15M NaCl was intradermally injected under touch domes

TABLE 4.4

EXPERIMENT Number	Control Response (spike Counts/s)	% CONTROL RESPONSE		
		1-2 min	5-6 min	9-10 min
C290384/1	20.9 ± 2.0 (n = 12)	87	-	73
C290384/2	14.25 ± 0.9 (n = 8)	98	83	75
C290384/3	18.3 ± 1.2 (n = 6)	90	86	-

(mean ± SE)

TABLE 4.5

Summary table showing the normalized responses of touch domes in all experiments in which 0.15M NaCl was injected.

TABLE 4.5

% OF CONTROL RESPONSE

	EXPERIMENT NO.	CONTROL (spike counts/s)	1 - 2 (mins)	5 - 6 (m)	10 - 12 (m)	20 - 25 (m)	35 - 40 (m)
SLOW INJECTION	C150384	11.3	137	-	76	-	-
	C130582	8.42	-	119	75	94	119
	C061283	15.8	-	-	58	62	79
INTRADERMAL INJECTION	C290384/1	20.9	87	-	73	-	-
	C290384/2	14.2	98	83	75	-	-
	C290384/3	18.3	90	86	-	-	-

TABLE 4.6

Analysis of the distribution of ISI's before and after the injection of physiological saline. The times shown are the approximate times in minutes from the start of the injection.

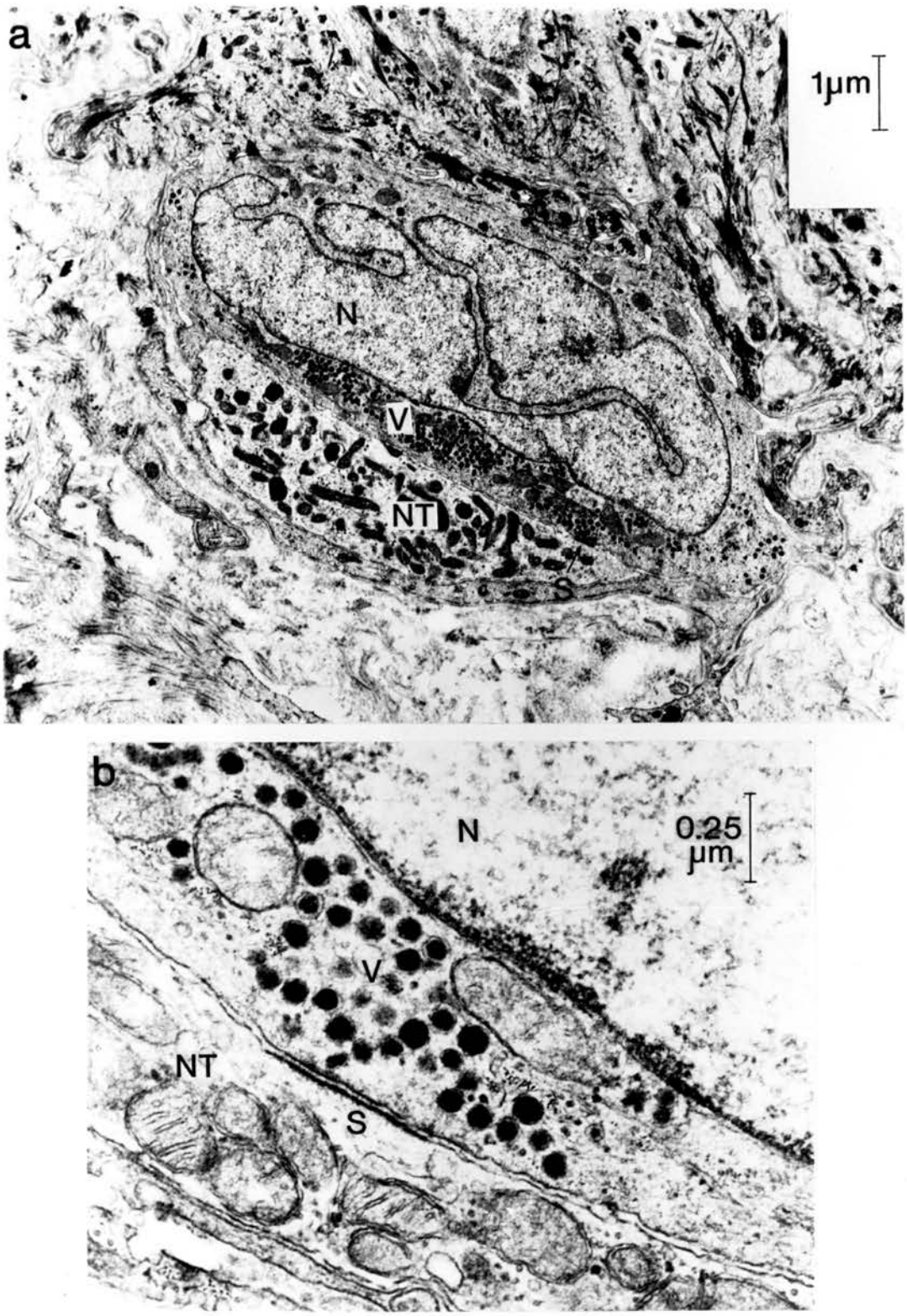
TABLE 4.6

	COMPARISON WITH EXPONENTIAL			COMPARISON WITH NORMALISED CONTROL			HISTOGRAM TRUNCATED AT
C150384	X^2	d.f.	p.	X^2	d.f.	p.	(ms)
Control	23.2	24	>0.05	-----			38.0
+10m	41.1	43	>0.05	214.0	8	<0.05	34.0
C290384/2							
Control	24.4	23	>0.05	-----			41.0
+5m	15.1	13	>0.05	220.8	13		84.0
+10m	43.2	50	>0.05	245.0	13	<0.05	21.0

FIGURE 4.5

a. A Merkel cell and nerve terminal from a touch dome after the injection of 0.15M NaCl. Both structures are similar in appearance to that of a normal Merkel cell and nerve terminal (Figure 3.8). The Merkel cell has a multilobulated nucleus (N) and numerous dense-cored vesicles (V) concentrated mainly between the Merkel cell nucleus and the nerve terminal (NT). Also present but not clearly seen is a 'synapse-like' structure (S).

b. A high power micrograph of a typical 'synapse-like' structure (S) between the Merkel cell and nerve terminal. In this example the dense-cored vesicles (V) are around but not adherent to the region of membrane specialization. (N - Merkel cell nucleus; NT - nerve terminal).


a
1µm
N
V
NT
S
b
N
0.25
µm
V
NT
S

structure of which is described in Chapter 3. There is no significant difference ($p > 0.1$ Student,s 2-tailed 't' test) in the vesicle numerical density of Merkel cells taken from stimulated receptors after the injection of 0.15 NaCl ($12.1 \pm 1.5/um^2$) and the vesicle density in normal Merkel cells ($14.6 \pm 0.97/\mu m^2$).

Also shown in Figure 4.5b is a high power electron micrograph of a synapse-like structure between a Merkel cell and its adjacent nerve terminal. In a total of 13 randomly selected sections of Merkel cells taken from receptors after saline injections, there was an average of 0.6 ± 0.2 synapses per section (S.E.M., n=13)

Slow Intra-arterial Injection of $CoCl_2$

Electrophysiology The changes in the response of SAI receptors when mechanically stimulated in the presence of $CoCl_2$ occurred consistently from one experiment to another although the timing of the events, in relation to the start of the injection, varied considerably between experiments.

In experiment C190184, two of the afferent fibres being recorded from innervated three touch domes (Figure 4.6a). The action potential amplitude of the fibre which innervated two of the touch domes, 1a and Ib, was greater than the amplitude of the fibre which innervated the single touch dome, touch dome 2. Both these action potential amplitudes were in turn different from that of hair follicle afferents. When 10ml of 10mM $CoCl_2$ was slowly injected into the limb circulation of the cat (Figure 4.7) there occurred, within 30s of the start of the injection, a transient increase in afferent fibre activity to approximately

FIGURE 4.6

Experiment C190184

a. Oscilloscope traces of action potentials contributing to the activity recorded in the nerve strand :

1 - response of touch dome 2 to mechanical stimulation.

2 - action potentials contributing to the increased activity which occurred within 60s of the $CoCl_2$ injection. The action potentials illustrated were recorded in-between stimulation of the touch dome.

3 - response of touch dome Ia to mechanical stimulation.

4 - response of touch dome Ib to mechanical stimulation.

5 - response to hand stimulation of hair follicles.

6 - action potential produced by mechanical stimulation of touch dome Ia 20 minutes after the restoration of the general circulation to the limb.

By comparing spike height and duration it can be seen that the increased activity produced by injection of $CoCl_2$ occurred only in fibres innervating SAI receptors.

b. Trace I : Shown is the response of touch dome Ia when stimulated 6 minutes after the injection of $CoCl_2$.

Trace 2 : Output from the displacement transducer which can be related to the responses presented in Traces 1, 3 and 4.

Trace 3 : Activity in the afferent fibre 14 minutes after the $CoCl_2$ injection when the mechanical stimulator was withdrawn.

Trace 4 : Response of touch dome Ia when mechanically stimulated 20 minutes after restoration of the general circulation. The only response present is that produced by mechanical stimulation.

c. Trace 1 : The response of touch dome Ib approximately 10 minutes after the rejection of 10 mils of 15mM $CoCl_2$.

Trace 2 : Displacement transducer output.

Trace 3 : The response to mechanical stimulation of hair follicles when the SAI afferent fibre activity had ceased.

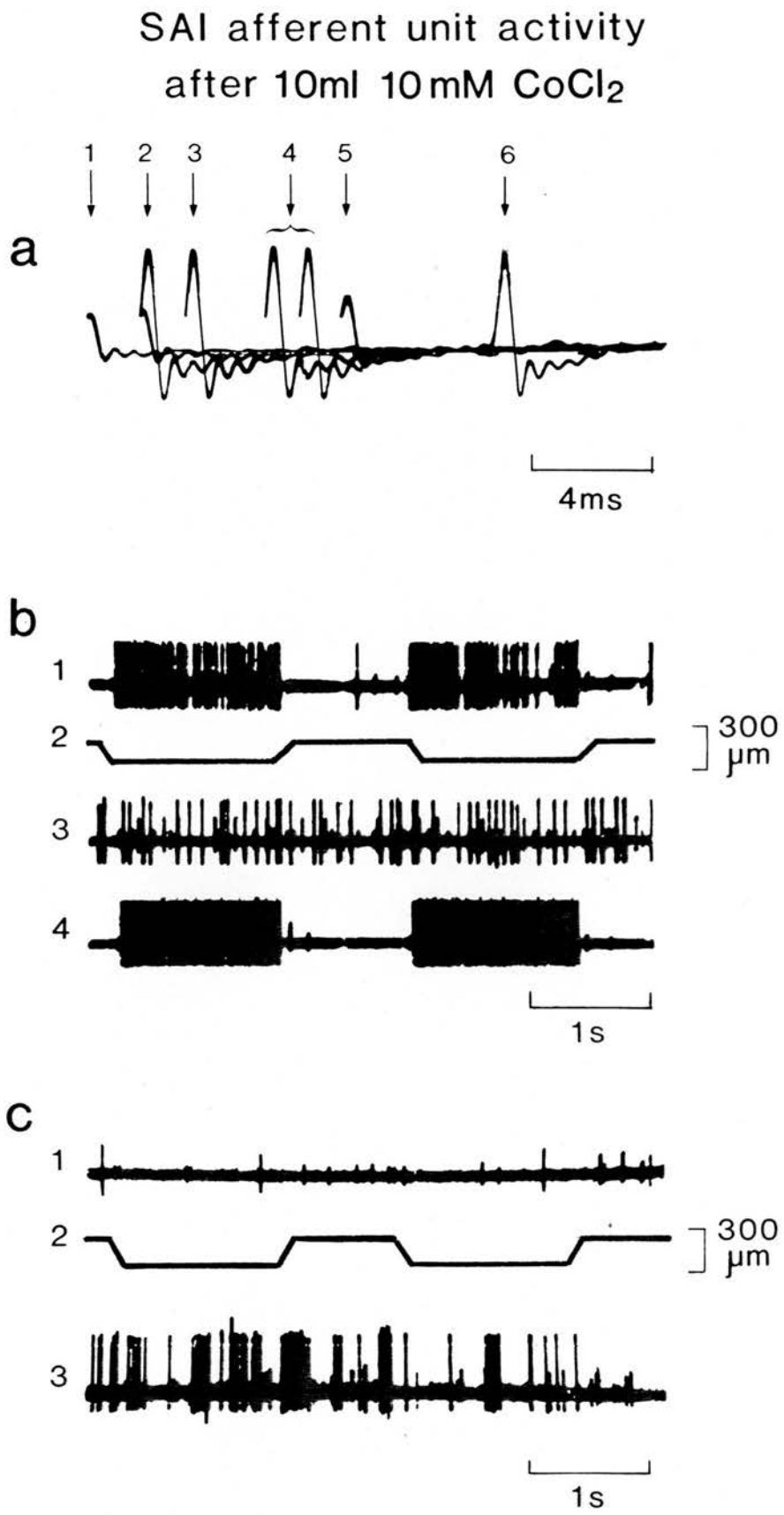
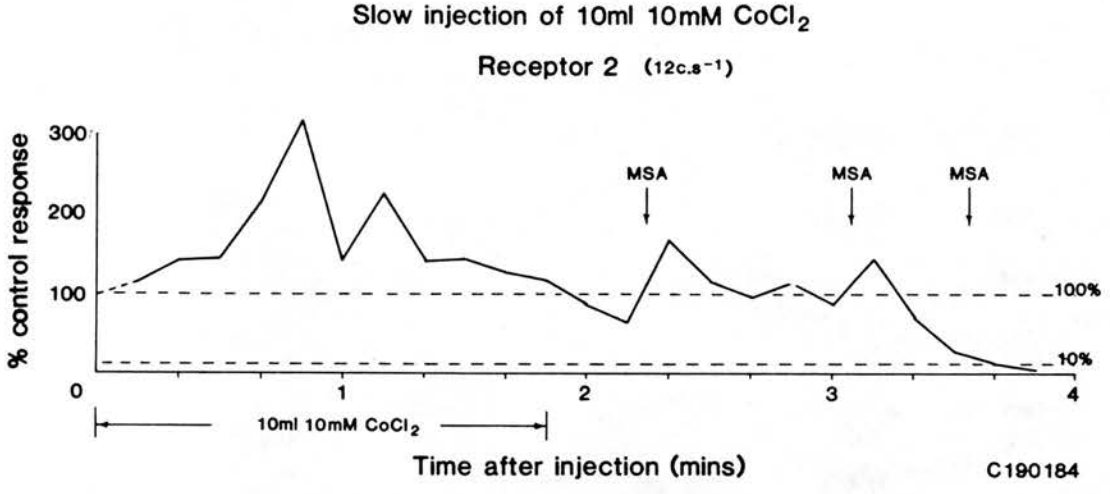

SAI afferent unit activity
after 10ml 10mM $CoCl_2$
a
1
2
3
4
5
6
4ms
b
1
2
3
4
300 µm
1s
c
1
2
3
300 µm
1s
C190184

FIGURE 4.7

Experiment C190184

Normalized response of receptor 2 showing the effects of the injection of 10mls 10mM $CoCl_2$ into the limb circulation. The response from the beginning of the injection until receptor failure is shown. The large rise in the touch dome fibre activity, which occurred within the first minute from the start of the injection, is clearly seen.

(MSA - mechanical stimulator adjusted).


Slow injection of 10ml 10mM $CoCl_2$
Receptor 2 (12c.s^{-1})
% control response
300
200
100
0
MSA
MSA
MSA
100%
10%
1
2
3
4
10ml 10mM $CoCl_2$
Time after injection (mins)
C190184

300% of the control response of the receptor which was unrelated to mechanical stimulation. This activity then declined to control levels before falling rapidly to 10% of the control firing rate in approximately 3.8 minutes from the start of the injection.

At approximately six minutes after the injection touch dome 1a was stimulated mechanically (Figure 4.8). As the response of this receptor declined with time there was a slow but steady increase in the background activity in the afferent fibres. When the stimulator was withdrawn 14 minutes into the experiment the response in the unit continued at 4.9 c/s. When the stimulator was reapplied to the touch dome there was only a small increase in the response indicating that the SAI receptor had effectively ceased to respond to mechanical stimulation and that most of the activity was background activity. Figure 4.6b shows the action potentials which contributed to this increased activity. Although receptors 1a and 2 had ceased to respond to mechanical stimulation there was still activity in the fibres innervating these receptors. Because receptor 1a was first stimulated after the injection of $CoCl_2$ there was no control response recorded from this receptor with which subsequent changes in response could be compared.

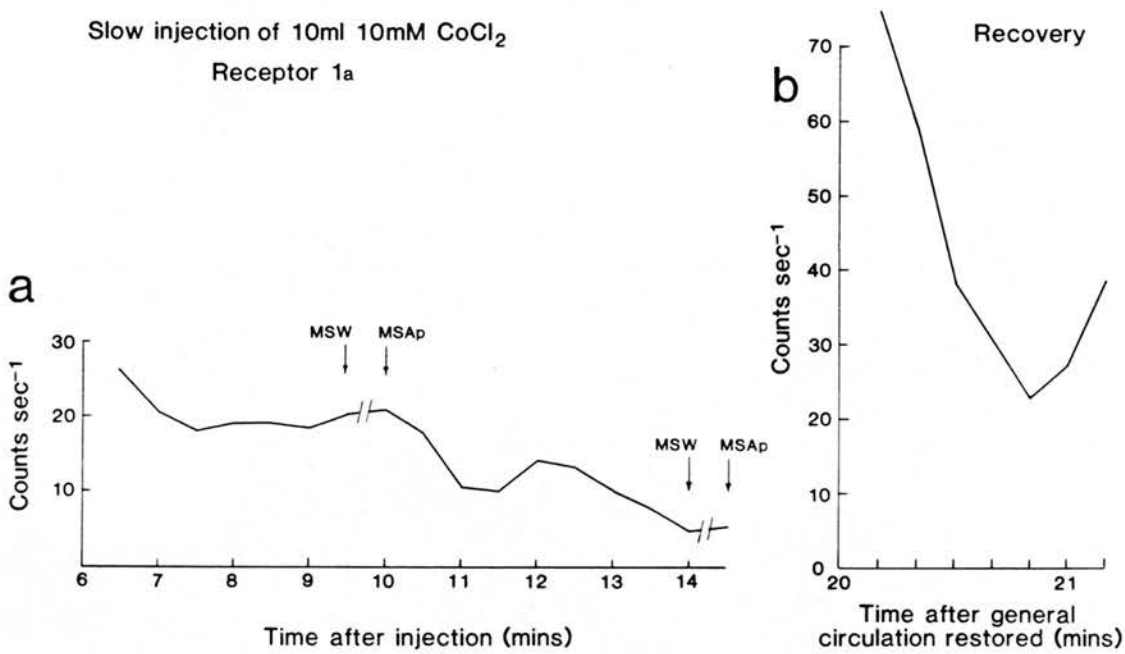
The restoration of the general circulation to the limb produced a gradual return of the receptor response to mechanical stimulation. This is illustrated in Figures 4.6a & b and Figure 4.8 which shows the response of receptor 1a to mechanical stimulation 20 minutes after the circulation had been restored to the

FIGURE 4.8

Experiment C190184

a. Receptor Ia response when stimulated for the first time 6 minutes after the start of the $CoCl_2$ injection. Withdrawal (MSW) and re-application (MSAp) of the stimulator to the touch dome, 9-10 minutes and 14 minutes after the start of the injection had no significant effect on the response. This indicates that the touch dome is no longer responding to mechanical stimulation but is reacting to the $CoCl_2$ injection. (see also Figure 4.6b).

b. The response of touch dome Ia 20 minutes after the restoration of the general circulation to the limb. The response is greatly increased when compared to the response 14 minutes after the injection and is produced by mechanical stimulation only. (see also Figure 4.6b).


Slow injection of 10ml 10mM $CoCl_2$
Receptor 1a
a
Counts sec^{-1}
30
20
10
MSW
MSAp
MSW
MSAp
6
7
8
9
10
11
12
13
14
Time after injection (mins)
b
Recovery
Counts sec^{-1}
70
60
50
40
30
20
10
0
20
21
Time after general circulation restored (mins)

C190184

limb. The response between the last minute of stimulation before the circulation was restored to the limb and 20 minutes after its restoration was significantly increased ($p < 0.002$ Student's 2-tailed 't' test).

The response of touch dome 1a after the restoration of the general circulation to the limb was used as the control response for this receptor when it was stimulated after the slow injection of 10 ml of 15mM $CoCl_2$ (Figure 4.9a). Afferent fibre activity was increased for a short time, but this increase was not statistically significant ($p > 0.1$ Student's 2-tailed 't' test) nor was it as great as occurred during the previous trial when 10 ml of 10 mM $CoCl_2$ was injected. The response had returned to control levels within 40s and continued to decline until it failed at approximately 3.5 minutes after the start of the injection. There was no transient increase in other afferent fibre activity nor did activity slowly appear with time as it had done during the previous trial on the same preparation.

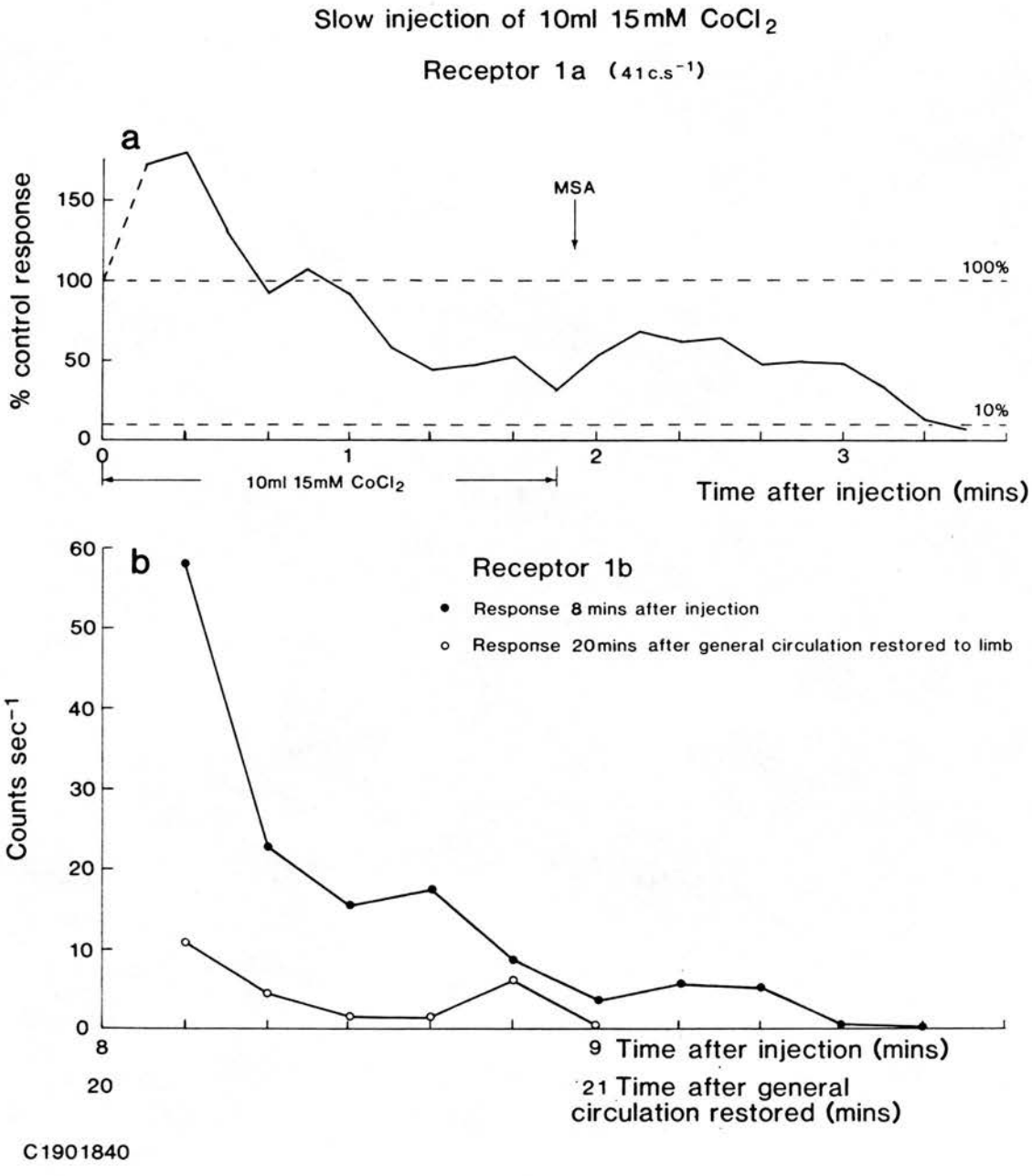
Touch dome 1b when stimulated at approximately 8 minutes after the injection of 15mM $CoCl_2$, failed in about 90s (Figure 4.9b). At the moment of receptor failure the PO_2 and PCO_2 levels in the limb circulation were 24.9 and 63.0mm Hg respectively. This is comparable to the values of PO_2 and PCO_2 measured in the limb circulation 35 minutes after the slow injection of saline when the response of the receptor to mechanical stimulation was still at 80% of its control firing rate (see Table 4.3). Manual

FIGURE 4.9

Experiment C190184

a. Normalized response of touch dome both during and after the injection of 10ml of 15mM $CoCl_2$. After an initial transient increase there is a steady decline in the response to mechanical stimulation until 3.5 minutes after the injection it is less than 10% of its control firing rate. No other activity arose in the afferent fibre after the CoCl injection.

b. Response of touch dome Ib beginning 8 minutes after the start of the $CoCl_2$ injection (●). The response is down to zero count/s within 1.5 minutes from the start of mechanical stimulation.
The restoration of the general circulation to the limb for 20 minutes (O) produced no significant recovery.


Slow injection of 10ml 15mM $CoCl_2$
Receptor 1a (41 c.s^{-1})
a
% control response
150
100
50
0
MSA
100%
10%
0
1
2
3
10ml 15mM $CoCl_2$
Time after injection (mins)
b
Receptor 1b
Response 8 mins after injection
Response 20 mins after general circulation restored to limb
Counts sec^{-1}
60
50
40
30
20
10
0
8
9 Time after injection (mins)
20
21 Time after general circulation restored (mins)
C1901840

stimulation of hair follicles at this time still produced afferent fibre activity (Figure 4.6c).

When the receptor was stimulated 20 minutes after the restoration of the general circulation to the limb there was no significant recovery ($p > 0.05$ Student's 1-tailed 't' test) in the receptor response.

Table 4.7 shows the concentration in the serum of Co^{2+} and Ca^{2+} measured in the blood samples removed from the limb circulation at different times throughout the experiment. When 10ml of 10mM $CoCl_2$ was injected the Co^{2+} levels rose quickly from 0.1mM to a measured maximum of 1.4mM then fell gradually throughout the rest of the experiment to a minimum of 1.1mM at 25 minutes after the injection. The Co^{2+} concentration was not measured in this experiment after the restoration of the circulation to the limb and therefore it is not known to what extent the Co^{2+} levels had returned to control levels. The Ca^{2+} concentration throughout this part of the experiment remained steady at 2.0mM. After the injection of 10ml of 15mM $CoCl_2$, the rise in the serum Co^{2+} levels was accompanied by a fall in the Ca^{2+} levels. As the Co^{2+} concentration began to fall with time the Ca^{2+} concentration, which had fallen from 2.0mM to a low of 1.5mM, remained at 1.6mM.

When 10ml of 5mM $CoCl_2$ was injected into the limb circulation the effects on the response of the touch dome were similar to the effects produced by the injection of 10mM $CoCl_2$ except that the time scale over which these events took place was extended. The results of such an experiment, number C260184/1, are

TABLE 4.7

Experiment C190184. Cobalt and calcium concentrations in the serum of the limb circulation after the slow injection of 10ml of 10mM and 15mM $CoCl_2$.

TABLE 4.7

C190184

		COBALT Concentration in serum	CALCIUM Concentration in serum
	Time after injection (min)	mM	mM
10 ml 10 mM $CoCl_2$	CONTROL	0.1	2.0
	5	1.4	2.0
	10.2	1.35	2.0
	25	1.1	2.0
10 ml 15 mM $CoCl_2$	3.7	2.8	1.5
	5.83	2.05	1.7
	24.67	2.05	1.6
	37.17	1.1	1.6

illustrated in Figure 4.10.

In this trial as the $CoCl_2$ was injected the mechanical stimulator was withdrawn so that the effects of the injection alone could be more clearly seen. Within 20s of the start of the injection the activity in the afferent fibre started to increase and by 40s was at 85% of the control response of the receptor to mechanical stimulation (Figures 4.10a & 11a). This is clear evidence that the increase in afferent fibre activity just after the start of the injection of $CoCl_2$ is unrelated to mechanical stimulation of the receptor. This increase in activity then fell quickly and steadily until just after the end of the injection the mechanical stimulator was reapplied to the touch dome. The increase in afferent fibre activity was less evident in this experiment than in the experiment in which 10mM $CoCl_2$ was injected although, in the latter case, the touch dome was being stimulated whilst the $CoCl_2$ was being injected. The response of the receptor declined with time until at just over 11 minutes into the experiment the receptor failed. The PO_2 and PCO_2 levels at the time of receptor failure were 38.8 and 69.0mm Hg respectively. There was then a period of time when activity in the afferent fibre, unrelated to mechanical stimulation of the touch dome, increased gradually. This is illustrated clearly at 20 minutes after the injection (Figure 4.10c), where the stimulator was applied to the touch dome with no increase in fibre activity. This spontaneous activity continued for a further 2-3 minutes before eventually declining. At 30 minutes after the injection there was

FIGURE 4.10

Experiment C260184/1

a. 10mJ 5mM $CoCl_2$ was injected over a period of 1 minute during which time the mechanical stimulator was withdrawn from the touch dome. Within 20s of the start of the injection there is an increase in afferent fibre activity. This activity is at a maximum after 40s after which it starts to decline. Application of the mechanical stimulator to the touch dome (MSAp) after the $CoCl_2$ injection produces a slowly declining response with time.

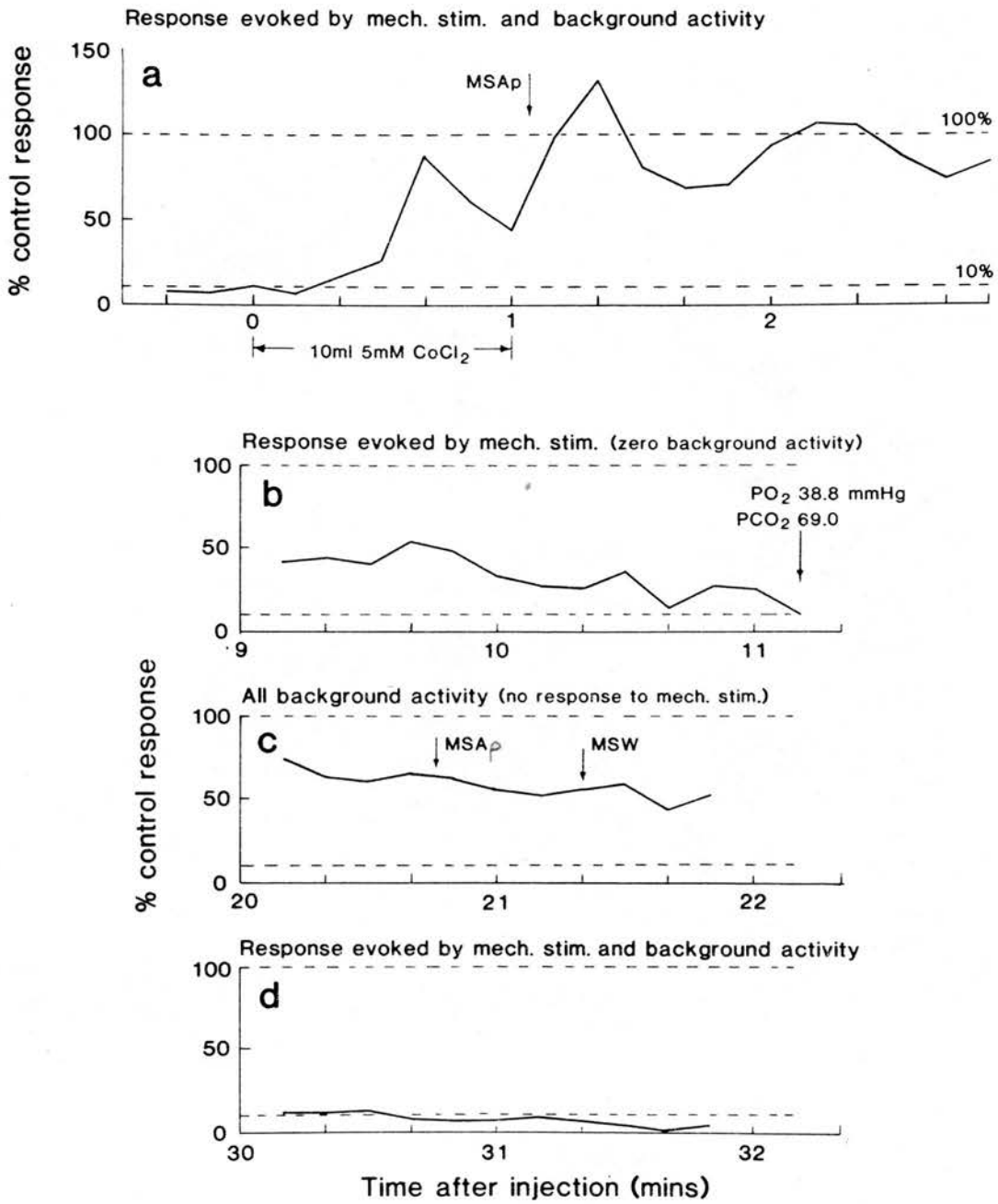
b. Response of touch dome to mechanical stimulation beginning 9 minutes after the start of $CoCl_2$ injection. Also shown are the PO_2 and PCO_2 levels in the blood at the time when the response falls to 10% of its control level. There was, at this time, no background activity.

c. 20 minutes after the injection and all the activity present is that produced by the injection of $CoCl_2$. Application (MSAp) and withdrawal (MSW) of the mechanical stimulator produces no change in the response.

d. 30 minutes after the injection there is very little activity present in the afferent fibre. The mechanical stimulator was applied to the touch dome throughout the period shown.

Slow injection of 10ml 5mM $CoCl_2$,activity recorded from SAI fibre

(control response :16c.s^{-1})



C260184/1

FIGURE 4.11

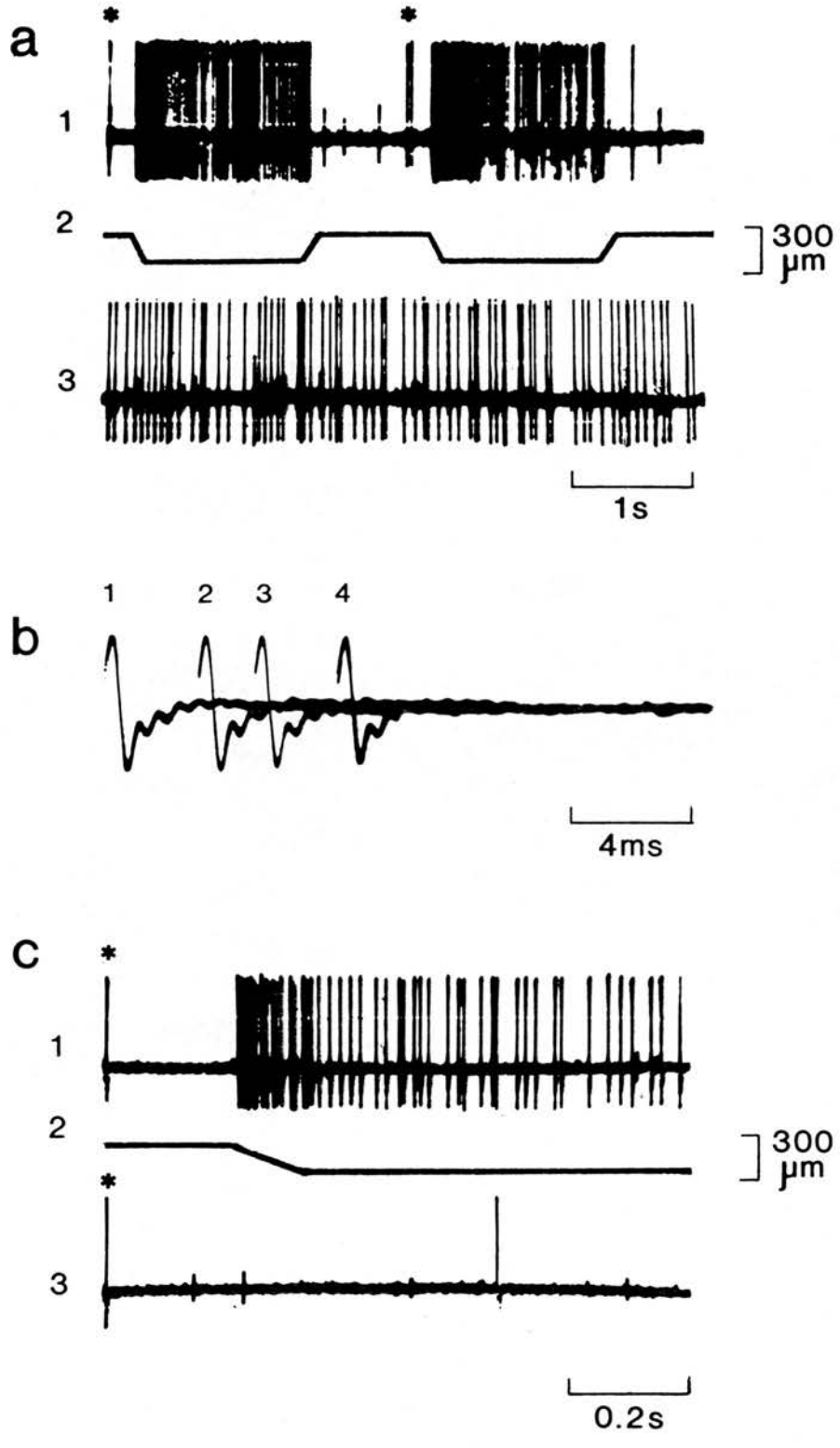
Experiment C260184

a. 1. Control responses produced by electrical stimulation of the afferent fibre (*) and mechanical stimulation of the touch dome.
   2. Mechanical displacement.
   3. Increased activity approximately 30s after the start of the $CoCl_2$ injection. This response was recorded before the mechanical stimulator was applied to the touch dome.

b. 1. Action potential produced by electrical stimulation of the afferent fibre close to the touch dome before the injection of $CoCl_2$.
   2. Response produced by mechanical stimulation of the touch dome before $CoCl_2$ injection.
   3. Action potential from the period of increased activity 30s after the start of the injection before the application of the mechanical stimulator to the touch dome.
   4. Action potential making up the activity 20 minutes after the injection when the response to mechanical stimulation was effectively zero.

c. 1. Response to electrical stimulation of the afferent fibre (*) and mechanical stimulation of the touch dome before the $CoCl_2$ injection.
   2. Mechanical displacement.
   3. Approximately 12 minutes after the injection. Electrical stimulation of the afferent fibre close to the touch dome still produces a response when there is no response to mechanical stimulation.

SAI afferent unit activity
after 10ml 5mM $CoCl_2$



C260184/1

little afferent fibre activity.

Throughout the experiment electrical stimulation of the afferent fibre innervating the receptor was carried out and the action potential produced by this is illustrated in Figure 4.11b. This figure shows the action potentials produced by both electrical and mechanical stimulation during the control period before the injection of $CoCl_2$. Two other spikes are shown, one from the increased activity at approximately 30s after the start of the injection before mechanical stimulation of the receptor and the other from the period of time, at about 20 minutes, when activity in the unit was again increased and when the mechanical stimulator was withdrawn from the receptor. As can be seen, the action potentials are similar in amplitude and duration confirming that the increased activity in the afferent fibres as a result of $CoCl_2$ injections was occurring in fibres which innervated SAI receptors only. Although hair follicle afferents were present in the strand of fibres being recorded from, they never responded spontaneously after the injection of $CoCl_2$ although they could still be mechanically stimulated by hand (see Figure 4.6c).

Figure 4.11c shows the response of the receptor to electrical and mechanical stimulation before and after the injection of $CoCl_2$ into the limb circulation. It indicates that when the response of the receptor to mechanical stimulation had ceased an action potential could still be initiated by electrical stimulation of the afferent fibre innervating the receptor. Evidence of recovery is presented in Figures 4.12a and b which

FIGURE 4.12

Experiment C260184/1

a. Response to mechanical stimulation of the touch dome beginning 15 minutes after the restoration of the general circulation to the limb.

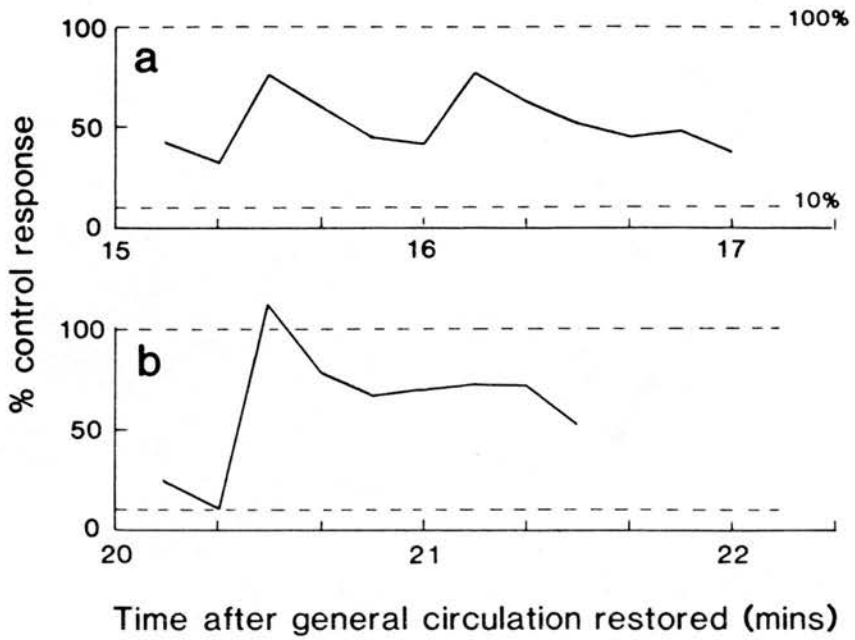
b. Response to mechanical stimulation of the touch dome beginning 20 minutes after the restoration of the general circulation to the limb.

The responses shown in both a and b are significantly greater ($p < 0.002$) than the response 30 minutes after the $CoCl_2$ injection (c.f. Figure 4.10d).

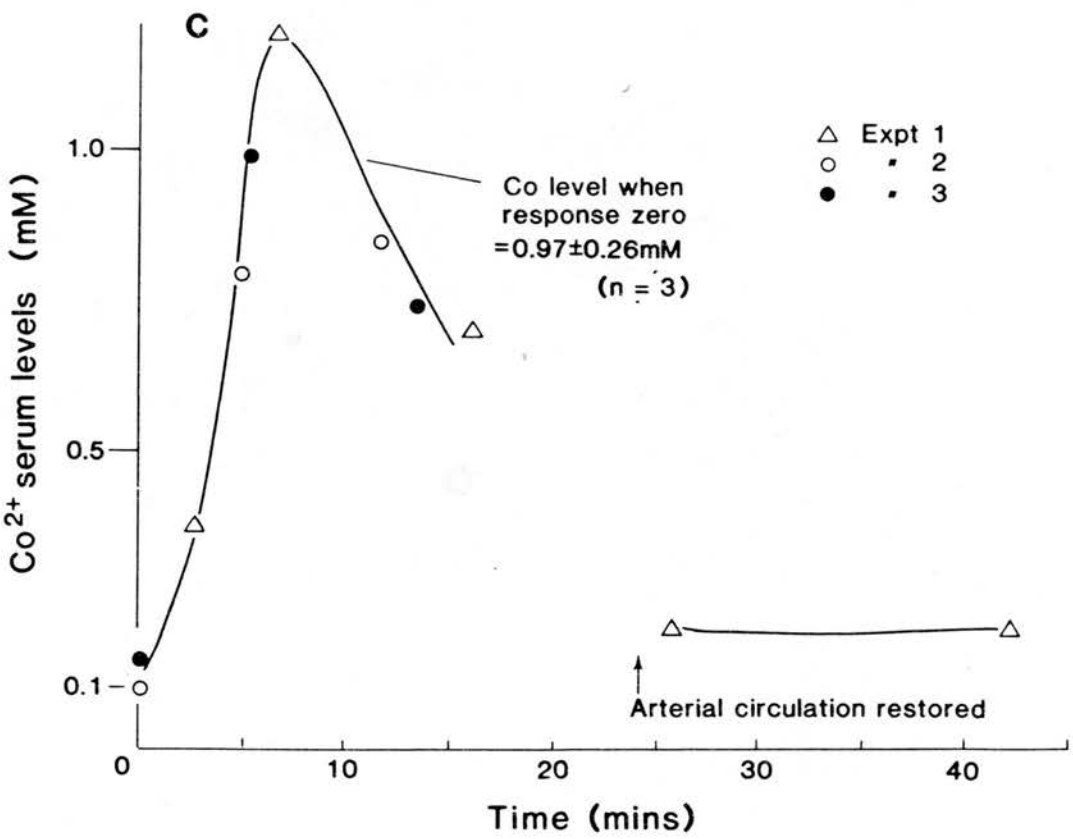
c. Shown is a graph of the serum Co^{2+} levels in mM against time for 3 different experiments. Using this graph the average Co^{2+} level in the serum at the moment of receptor failure for all 3 experiments was obtained and found to be 0.97 ± 0.26 mM (mean $\pm$ S.E.).

In one experiment (Δ) the Co^{2+} levels were measured after the restoration of the general circulation to the limb. As can be seen the Co^{2+} levels were almost back to control values and this reduction coincided with a recovery of the response to mechanical stimulation.

Recovery after slow injection of 10ml 5mM $CoCl_2$,
activity recorded from SAI fibre (control response : 16c.s^{-1})



C260184/1



show the response of the receptor when mechanically stimulated 15 and 20 minutes after the circulation was restored to the limb. At 15 minutes the response was back to 52% of the control response; after 20 minutes the response had increased to 76% of the control response. The increase in the response between 30 minutes after the injection when afferent fibre activity had ceased and the recovery responses at 15 and 20 minutes after the restoration of the circulatiuon to the limb is significant ($p < 0.005$ Student's 2-tailed 't' test).

Co^{2+} and Ca^{2+} levels were measured throughout this experiment and are presented, along with the values for experiments C260184/2 and C310184, in Table 4.8. The Co^{2+} values for all three experiments are plotted against time in Figure 4.12c and from this graph the mean serum Co^{2+} concentration in the circulation at the time of receptor failure was found to be 0.97 ± 0.26mM (S.E.M., n=3). In experiment C260184/1 the Co^{2+} concentration in the circulation had returned to normal after the circulation had been restored to the limb. Coincident with the drop of Co^{2+} levels there was recovery of the receptor response described above.

It would appear from Table 4.8 that the Ca^{2+} concentration varies inversely with the Co^{2+} concentration. As Co^{2+} levels in the limb circulation rise the Ca^{2+} levels fall. However the recovery of Ca^{2+} back to control concentrations lags behind the return of Co^{2+} in the circulation to control concentrations. Within 20 minutes of restoring the general circulation to the limb the Co^{2+} concentration

TABLE 4.8

Cobalt and calcium concentrations in the serum of the limb circulation for all experiments in which 10ml of 5mM $CoCl_2$ was slowly injected.

TABLE 4.8

Serum Co^{2+} levels after the slow injection of 10 ml of 5 mM $CoCl_2$
" Ca^{2+} " " " " " " " " " " "

	TIME AFTER INJECTION (min)	COBALT mM	CALCIUM mM
C260184/1	CONTROL	0.1	2.4
	6.75	1.2	1.7
	16.22	0.7	1.9
	25.83	0.2	1.7
	42.23	0.2	2.0
C260184/2	CONTROL	0.2	2.2
	5	0.8	1.6
	11.67	0.85	1.9
C310184	CONTROL	0.15	1.9
	5.1	1.0	1.0
	13	0.75	1.0

was almost at control values, whereas 90 minutes after the original injection into the limb circulation and almost 50 minutes after the circulation had been restored to the limb, the Ca^{2+} concentration was still depressed. This effect however is not evident at higher concentrations of $CoCl_2$ (Table 4.7). With 10mM $CoCl_2$ the Ca^{2+} concentration in the serum remained unaltered from control values throughout the sampling period. When 15mM $CoCl_2$ was injected there was in fact a slight increase in the Ca^{2+} concentration over the control value in contrast to the effects described above with lower concentrations of $CoCl_2$.

Figure 4.13a shows the effects of a 10ml injection of 5mM $CoCl_2$ on the response of an SAI receptor and on the background discharge of an SAII receptor. As described in Chapter 2.11 the background activity of the SAII receptor was calculated by subtracting the SAI receptor response from the combined activity of both receptors. During the control period and before the receptor was mechanically stimulated, the SAII receptor had a background firing rate of approximately 11 spike counts/second (c/s). As soon as the mechanical stimulator was applied to the SAI receptor the background activity of the SAII receptor began to vary.

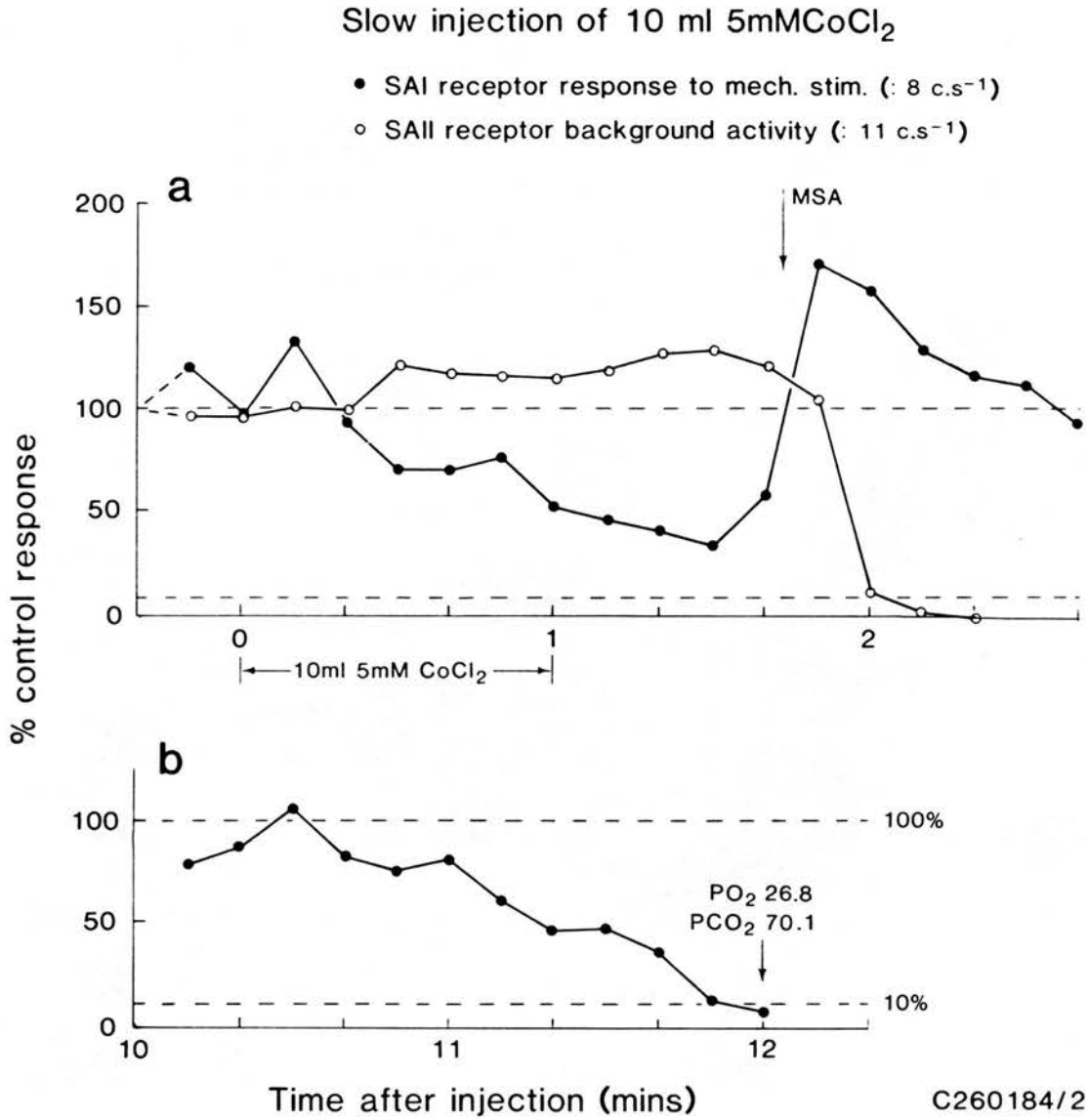
When the $CoCl_2$ was injected there was a gradual decline in the response of the SAI receptor whereas the firing rate of the SAII receptor increased. When the mechanical stimulator position was checked and adjusted at about 1.5 minutes after the injection (see Figure 4.13a) there was a marked increase in the SAI receptor response. Coincident with the adjustment of the

FIGURE 4.13

Experiment C260184/2

a. Shown is the response of an SAI mechanoreceptor (●) to mechanical stimulation and the background discharge of an SAII receptor (O). As the SAI response slowly declines after the injection, the SAII response rises to approximately 125% of its control rate where it remains until the position of the mechanical stimulator probe on the touch dome is adjusted. This produces a transient increase in the SAI response whereas the SAII background discharge fails almost immediately.

b. Shown is the time when the response of the SAI mechanoreceptor falls to <10% of its control firing rate. Also shown are the PO_2 and PCO_2 levels in the limb circulation at the moment of receptor failure.


Slow injection of 10 ml 5mMCoCl2
• SAI receptor response to mech. stim. (: 8 c.s-1)
○ SAII receptor background activity (: 11 c.s-1)
a
MSA
200
150
100
50
0
0
1
2
10ml 5mM CoCl2
% control response
b
100
50
0
100%
10%
PO2 26.8
PCO2 70.1
10
11
12
Time after injection (mins)
C260184/2

stimulator there was almost an immediate failure of SAII receptor activity. The SAI receptor continued to respond to mechanical stimulation for a further 12 minutes when it too failed. The SAI receptor was removed for histological examination at the moment of receptor failure.

The PO_2 and PCO_2 levels at the end of the experiment were 26.8 and 70.1 mm Hg respectively, which again are comparable with the levels found in saline experiments when the response to mechanical stimulation was still present.

Further evidence to that shown in Figure 4.11 that the increase in afferent fibre activity after the injection of $CoCl_2$ is produced in fibres innervating SAI receptors only is given in Figures 4.14 and 4.15. Figure 4.14a shows the response of a touch dome to mechanical stimulation in experiment C310184 before and after the injection of 10ml of 5 mM $CoCl_2$. There was a transient increase in activity in the afferent fibre followed by a steady decline in the mechanically evoked response of the SAI receptor to the point of failure at about 7 minutes after the start of the injection (Figure 4.14b). Before the receptor failed there was a gradual appearance of other activity in the afferent fibres being recorded from. The activity that arose within seconds of the $CoCl_2$ injection was in the same afferent fibre that innervated the receptor being stimulated (Figure 4.15a). Although hair follicle afferents were present and could be recorded from, there was never a spontaneous increase in activity in these afferent fibres when $CoCl_2$ was injected. The

FIGURE 4.15

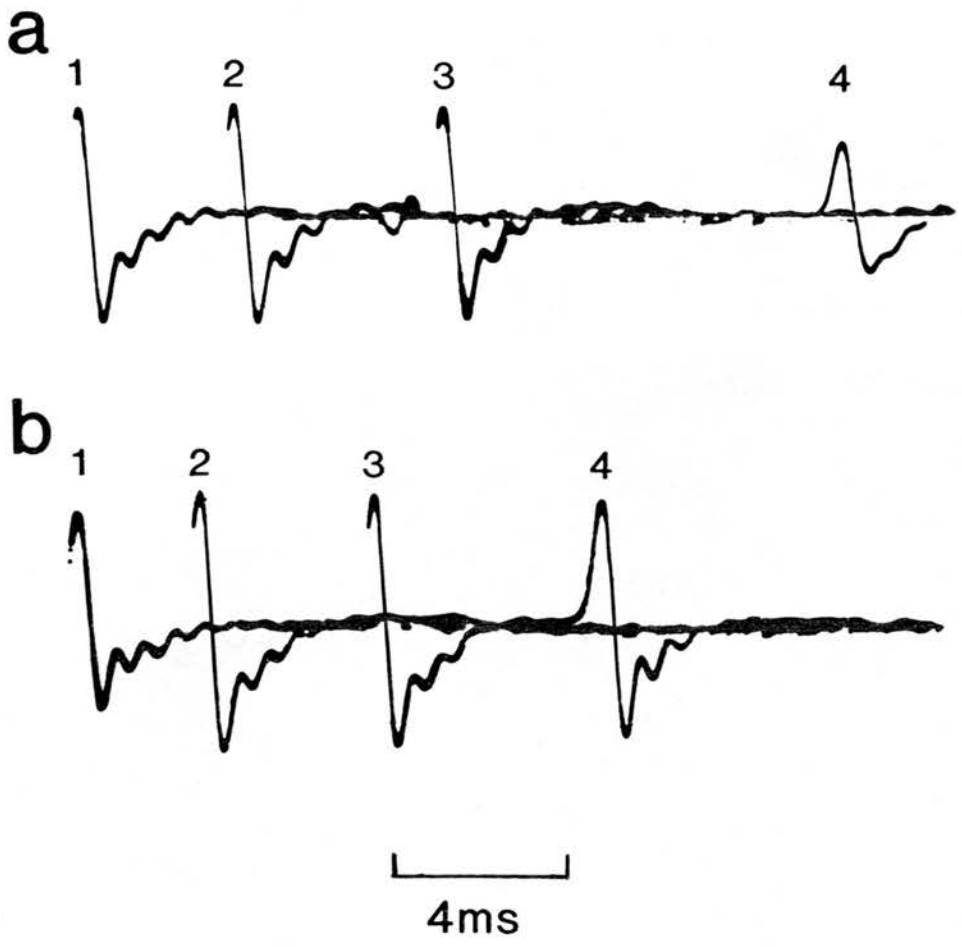
Experiment C310184

a. Spike 1 - action potential produced by electrical stimulation of the afferent fibre close to touch dome 1 before the $CoCl_2$ injection.
Spike 2 - action potential produced in the afferent fibre by mechanical stimulation of touch dome 1 before the $CoCl_2$ injection.
Spike 3 - action potential contributing to the increased activity between stimulations of the touch dome within 30s of the $CoCl_2$ injection.
Spike 4 - action potential produced by mechanical stimulation of hair follicles.

Action potentials 1, 2 and 3 are all of similar amplitude and duration indicating activity in the same afferent fibre whereas the action potential of the hair follicle afferent fibre is smaller.

b. Spike 1 - action potential produced by electrical stimulation of touch dome 1 afferent fibre.
Spike 2 - action potential producing increased activity when there was no evoked response from touch dome 1.
Spikes 3 and 4 - action potentials produced by mechanical stimulation of touch domes 2a and b respectively.

SAI afferent unit activity
after 10ml 5mM $CoCl_2$,



C310184

activity that arose when the response of the receptor to mechanical stimulation had failed was found to be occurring in an afferent fibre which innervated two other touch domes (Figure 4.15b).

Within four minutes of the restoration of the limb circulation the response of the touch dome had recovered to 26% of its control firing rate. This is a significant increase ($p < 0.002$ Student's 2-tailed 't' test) over the response of the touch dome to mechanical stimulation in the two minutes immediately before receptor failure. When the circulation had been restored for 11 minutes the response had returned to 41% of the control response (Figure 4.14c).

There was a linear relationship between the recovery response, plotted as the natural log of the % of the control response, and the time after the restoration of the circulation. This is illustrated in Figure 4.16.

Intradermal Injection of $CoCl_2$

Electrophysiology A total of three units were investigated in 1 experiment in which different quantities of $CoCl_2$ were intradermally injected under SAI mechanoreceptors (Figures 4.17 and 4.18) The limb was prepared in the same way as for the slow injection of substances but in this case the general circulation was maintained to the limb at all times.

When 25µl of 5mM $CoCl_2$ was injected the initial response of the touch dome to mechanical stimulation was only slightly increased over the control response (Figure 4.17a). The response then declined steadily until after almost 6.5 minutes it had fallen to 25% of

FIGURE 4.16

Illustrated is a graph of the recovery response, from 2 different experiments, plotted as the natural log of the % control response against the time after the restoration of the general circulation to the limb.

Recovery of SAI receptor response after restoration of general circulation.

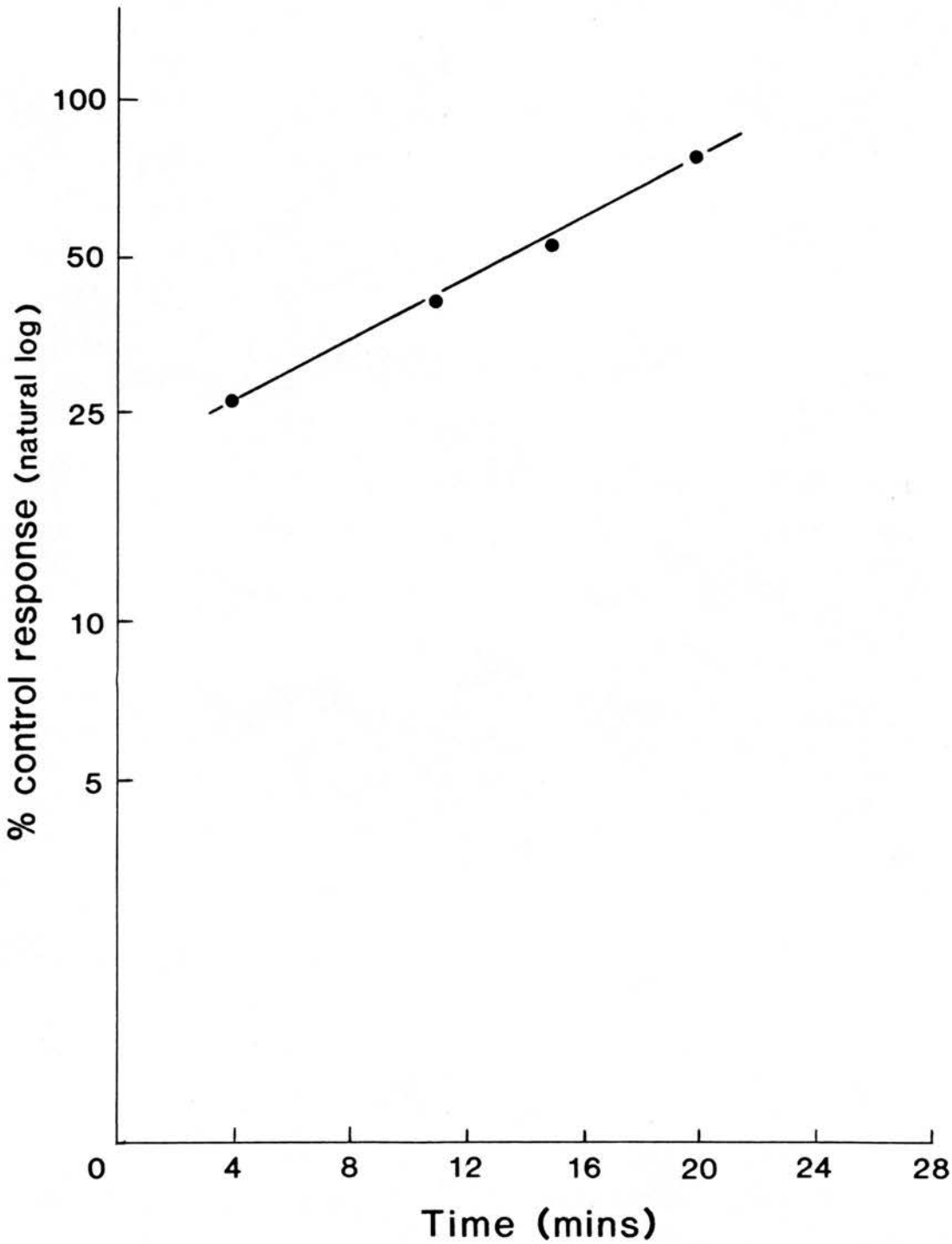


FIGURE 4.17

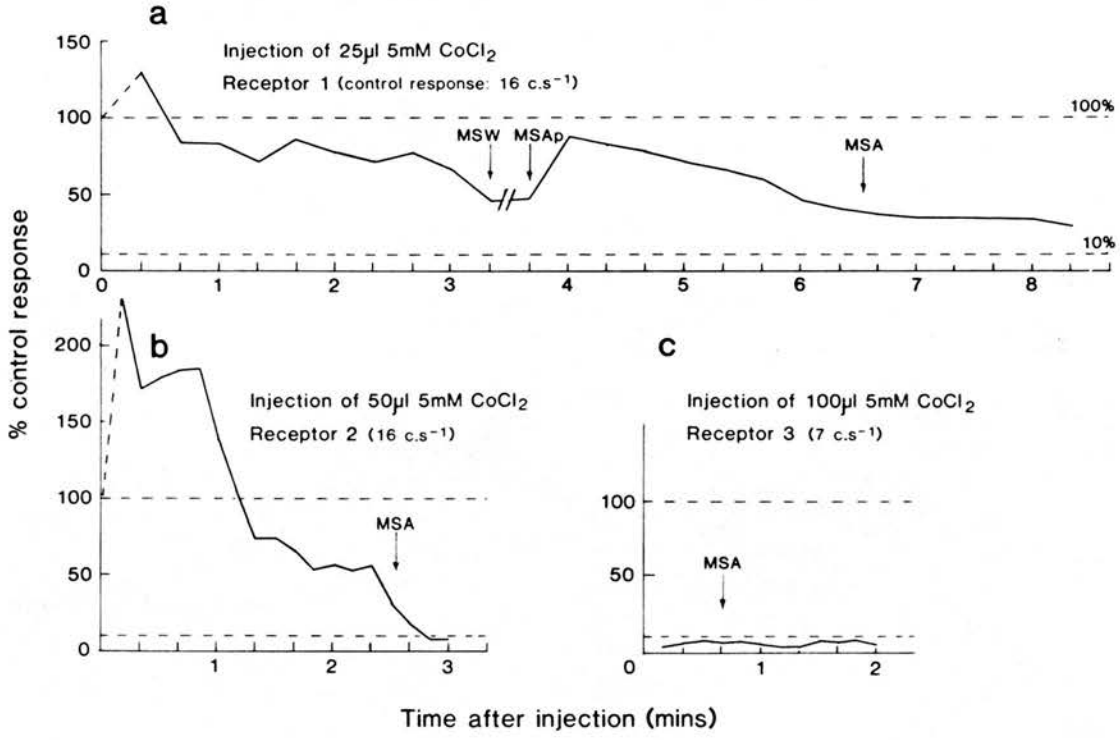
Experiment C290384

a. The intradermal injection of 25µℓ of 5mM $CoCl_2$ produces only a slight transient increase in the response of the touch dome to mechanical stimulation before it gradually declines to about 25% of the control response. When the stimulator was withdrawn at approximately 3.5 minutes (MSW) the response continued indicating the appearance of spontaneous activity in the afferent fibre. (MSAp - mechanical stimulator applied; MSA - mechanical stimulator adjusted).

b. 50µℓ of 5mM $CoCl_2$ produces a large transient increase in the response to mechanical stimulation followed by a steady decline to receptor failure within 3 minutes.

c. The injection of 100µℓ of 5mM $CoCl_2$ produces immediate receptor failure.

SAI receptor response after intradermal injections of 5mM $CoCl_2$



C290384

FIGURE 4.18

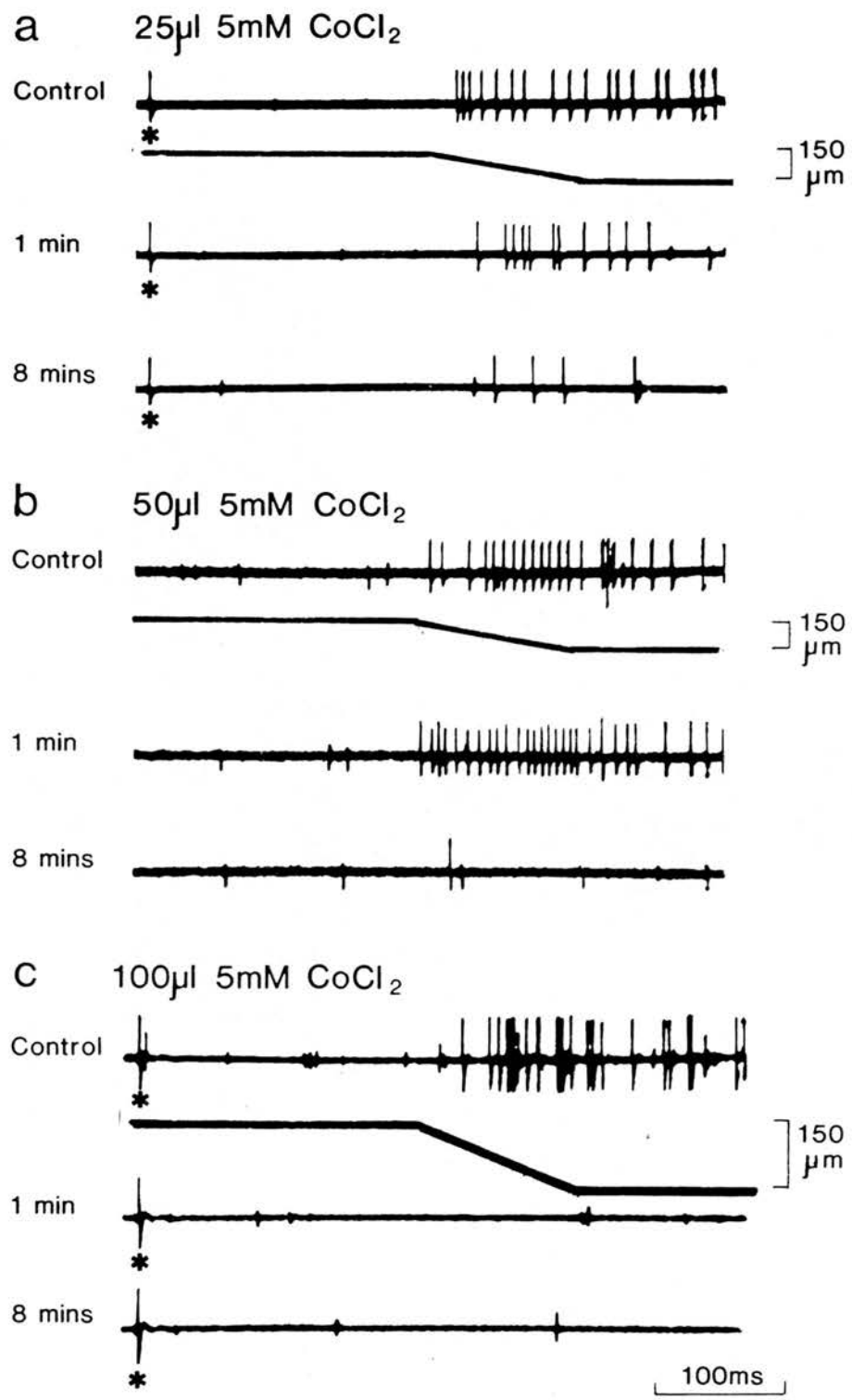
Illustrated are the responses to electrical and mechanical stimulation before and after the intradermal injections of different quantities of 5mM $CoCl_2$.

a. 25µℓ of 5mM $CoCl_2$ causes a reduction in the response to mechanical stimulation at 1 minute and a further reduction at 8 minutes. Not shown is the time when there is an increased spontaneous activity unrelated to mechanical stimulation.

b. 50µℓ of 5 mM $CoCl_2$ produces a slight increase in the response to mechanical stimulation at 1 minute but failure of the response at 8 minutes. In the example shown there is no electrical stimulation(*) of the afferent fibre.

c. 100µℓ of 5 mM $CoCl_2$ causes receptor failure within 1 minute although even after 8 minutes there is still a response in the afferent fibre to electrical stimulation.

Intradermal injection of $CoCl_2$



C290384/5

the control response. It remained at this level, with only minor variations, for the remainder of the experiment. At about 2.5 minutes after the injection there was an increase in activity in the fibre innervating the touch dome unrelated to mechanical stimulation, which lasted for 2-2.5 minutes. This is seen in Figure 4.17a where, at approximately 3.5 minutes after the injection, the mechanical stimulator was withdrawn and the response continued at about 50% of its control firing rate.

The injection of 50µl of 5 mM $CoCl_2$ produced a transient increase in the response of the touch dome to mechanical stimulation (Figure 4.17b). The response then declined quickly until at about three minutes after the injection the receptor failed. Between two minutes after the injection and receptor failure there was an increase in afferent fibre activity which was identified as arising in the same afferent fibre that innervated the SAI receptor.

This receptor, which failed within three minutes of the injection of 50µl of 5mM $CoCl_2$ was the same receptor which had been previously stimulated after the intradermal injection of 10ul of saline the results of which are illustrated in Figure 4.4. In the latter case the receptor was still responding at 73% of its control response, 10 minutes after the saline injection.

When 100µl of 5 mM $CoCl_2$ was intradermally injected (Figure 4.17c), the touch dome failed within 25s, this being the time that it took to adjust the stimulator probe onto the receptor for mechanical stimulation.

Electrical stimulation of the afferent fibre however, still initiated an action potential eight minutes after the injection. Figure 4.18 shows the responses of both electrical and mechanical stimulation of the SAI receptors before, at one minute and at eight minutes after the injection of the different quantities of $CoCl_2$.

The ISI distributions of the adapted responses were examined in unit numbers C260184/1, C261084/2, and C310184 both before and after the slow injection of 10ml of 5mM $CoCl_2$ (Table 4.9). In both units C260184/2 and C310184 the mean rates of discharge in the control responses were low - 8 counts/s and 9 counts/s respectively. Consequently as the response declined with time, longer sampling periods were necessary to gather sufficient numbers of intervals for analysis. Over the prolonged periods of data collection used, in some cases up to 2 minutes, stationarity of the response became a problem.

In only unit C260184/1 were there sufficient intervals initially, and a rate of decline of response suitable for analysis to be carried out after the injecton of $CoCl_2$. In this unit the ISI distribution of the response of the receptor to mechanical stimulation was analysed during the control period and at 10 minutes after the start of the injection of $CoCl_2$, just before receptor failure occurred. At both of these times the distribution was exponential although the difference between the mean interval length just before receptor failure was significantly different ($p < 0.05$, X^2-test) from that of the control

TABLE 4.9

Analysis of the ISI distribution before and after the slow injection of $CoCl_2$. The times shown are the approximate times in minutes from the start of the injection.

TABLE 4.9

	COMPARISON WITH EXPONENTIAL			COMPARISON WITH NORMALISED CONTROL			HISTOGRAM TRUNCATED AT
C260184/1	X^2	d.f.	p.	X^2	d.f.	p.	(ms)
Control	30.9	26	>0.05	-------			25.3
*‡$CoCl_2$ injected	54.6	54	>0.05	N.A.			----
+10min	16.5	16	>0.05	552.5	8	<0.05	48.0
‡+20min (B.A. only)	69.4	75	>0.05	N.A.			115.0
C260184/2							
Control	19.2	16	>0.05	-------			51.0
+11min	TOO FEW INTERVALS						
C310184							
Control	27.3	24	>0.05				42.0
+6min	TOO FEW INTERVALS						

* Non-Stationary Response ‡ Background Activity only; Mech.Stim. withdrawn
N.A. Not Applicable

period.

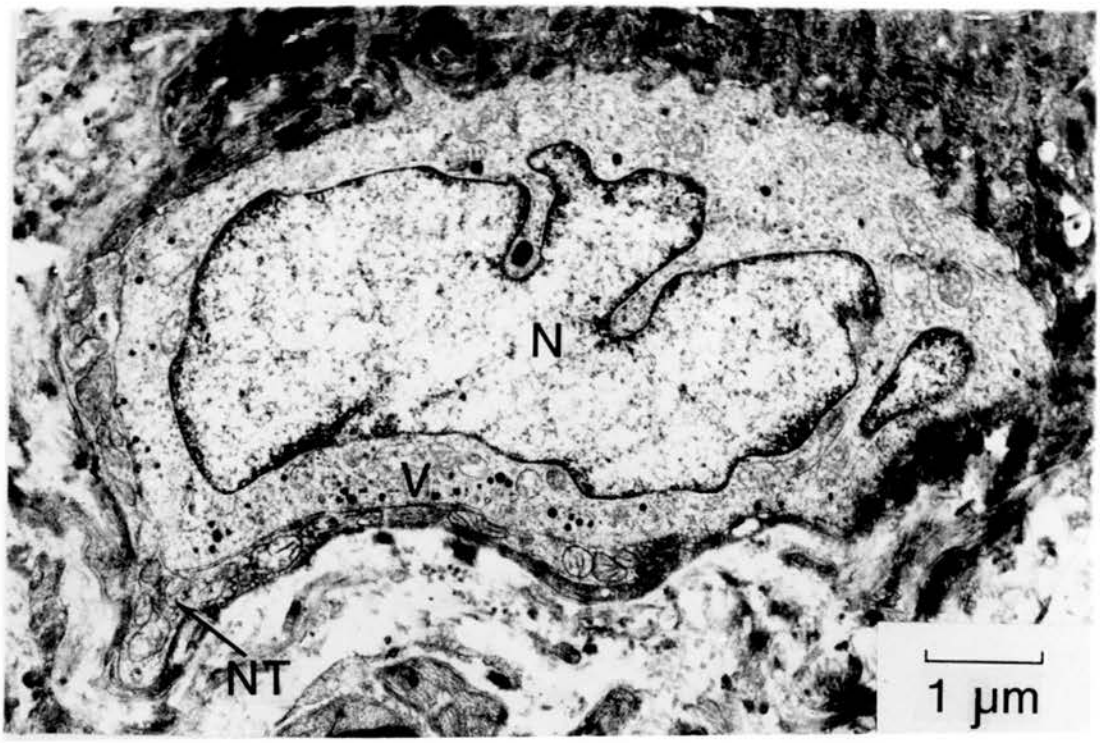
The ISI distribution during the periods of background activity was also examined; first when the $CoCl_2$ was being injected into the limb circulation and then at aproximately 20 minutes after the start of the injection when the mechanical stimulator was withdrawn from the touch dome (see Figure 4.10a and 4.10c). In the first case, even though the response was non-stationary during the sampling period the ISI distribution was exponential. However the background activity present 20 minutes after the start of the $CoCl_2$ injection was stationary and the ISI distribution at this time was not significantly different from an exponential distribution for intervals >115ms.

In units C260184/1 and C310184 the ISI distributions during the control periods were exponential. Thereafter, for the reasons stated earlier further analysis was impossible.

Histology Figure 4.19 shows a Merkel cell taken from an SAI receptor at the moment of receptor failure after the slow injection of 10ml of 5mM $CoCl_2$. The most striking change is a significant depletion ($p < 0.001$. Student's 2-tailed 't' test) in the number of Merkel cell granules present in the cytoplasm adjacent to the nerve terminal. The remaining vesicles were generally found closer to the cell membrane than in control Merkel cells where there was a gap of approximately 200nm between the vesicles and the Merkel cell membrane. This can be seen if Figure 4.5 and Figure 4.19 are compared. The average number of 'synapse-like' structures found in 13 randomly selected sections

FIGURE 4.19

A Merkel cell and nerve terminal from a touch dome after the slow injection of 10mls of 5mM $CoCl_2$ when all activity, both spontaneous and that produced by mechanical stimulation, has ceased. The most striking change to affect the Merkel cell is the almost total depletion of the dense-cored vesicles (V) (c.f. Figure 3.8) (N - Merkel cell nucleus; NT - nerve terminal).


N
V
NT
1 µm

was 2.1 ± 0.4 per section (S.E.M., n=13). This is a highly significant ($p < 0.001$. Student's 2-tailed 't' test) increase when compared to the number of synapse-like structures present in saline treated receptors (0.6 ± 0.25/section, S.E.M., n=13)

Slow Intra-arterial injection of Verapamil Electrophysiology One experiment was carried out in which 10ml of 100μM verapamil were injected into the localised limb circulation of the cat. The results of this experiment, C150384, are presented in Figure 4.20.

Evoked activity from an SAI receptor and background activity of an SAII receptor were present in the afferent fibres being recorded from. Within 30s of the start of the injection of verapamil there was a significant increase in the response of the SAI receptor to mechanical stimulation ($p < 0.01$ Student's 2-tailed 't' test). There was no other increase in the afferent fibre activity between stimulations (Figure 4.21a) as occurred when $CoCl_2$ was injected (Figure 4.11a). Also shown in Figure 4.20 is the regular background discharge of the SAII receptor.

At about six minutes after the injection, the SAII background activity suddenly stopped although it continued to respond to manual stimulation throughout the rest of the experiment (Figure 4.21b). The SAI response declined gradually to approximately 25% of its control response, the level around which it fluctuated for the remainder of the experiment.

Intradermal Injection of Verapamil Electrophysology When 100μl of 100μM verapamil was

FIGURE 4.21

Experiment C150384

a. 1. Shown is the response of an SAI mechanoreceptor to mechanical stimulation plus the regular background discharge of an SAII mechanoreceptor before the injection of verapamil. The SAII response is present at all times and has a smaller spike height than that of the SAI receptor.

2. Trace of the displacement transducer output. A downward deflection reflects displacement of the probe tip.

3. Responses of both SAI and SAII receptors 10s after the injection of verapamil. There is no noticeable change in the SAI receptor response to mechanical stimulation nor in the rate of the background discharge of the SAII receptor.

4. Response in the afferent fibres 40s after the verapamil injection. There is still no increase in activity in either receptor system both during and between stimulations of the touch dome (c.f. Figure 4.11a).

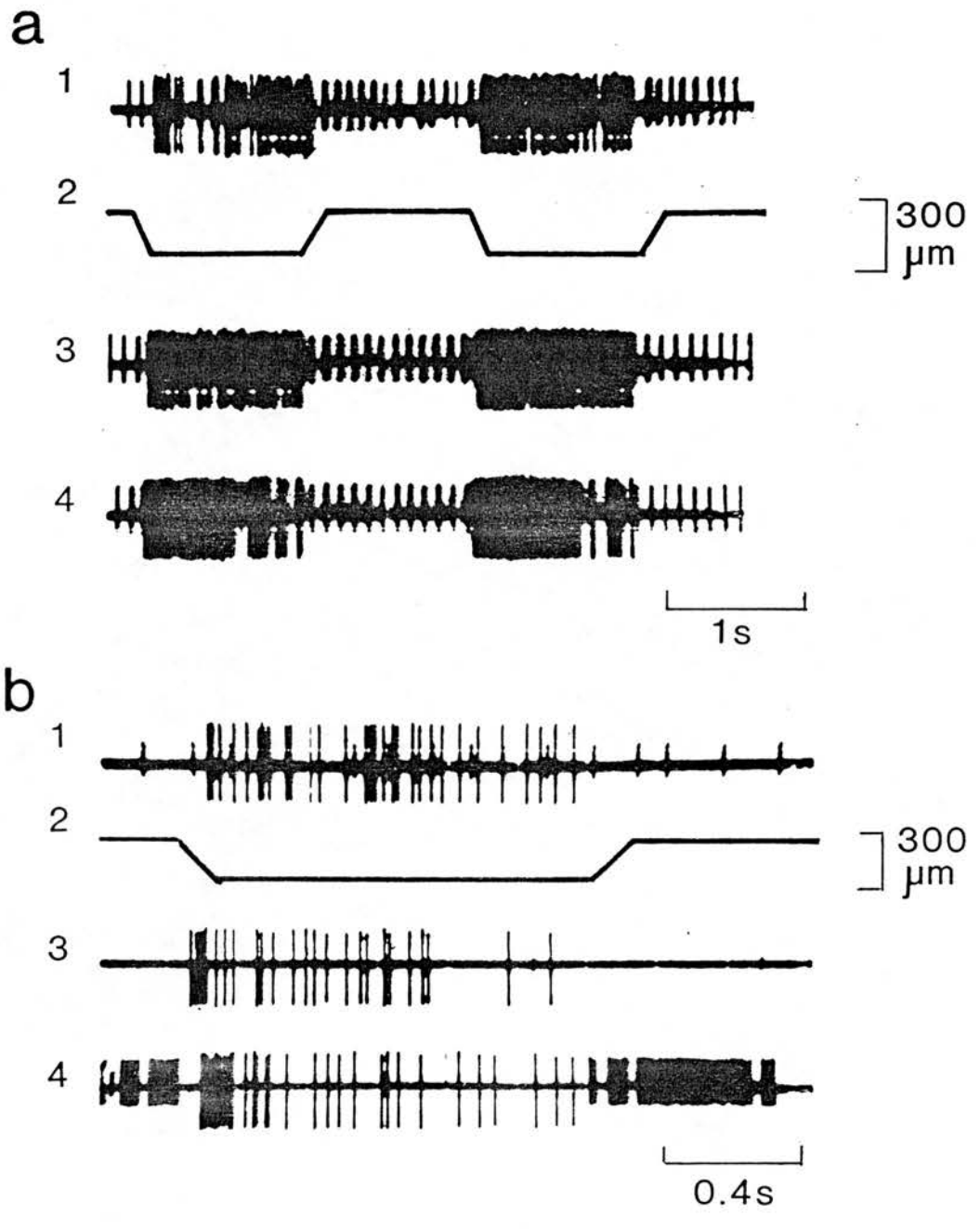
b. 1. Expansion of the top trace presented in Figure 4.21a. It shows the response of both the SAI receptor to mechanical stimulation and the regular background activity of the SAII receptor.

2. Trace of displacement transducer output.

3. Response of touch dome to mechanical stimulation when the background discharge of the SAII receptor has ceased. (approximately 8 minutes after verapamil injection).

4. Response of SAI receptor to mechanical stimulation plus the response of the SAII receptor to mechanical stimulation. This indicates that although the background response of the SAII receptor has ceased, a response can still be produced by mechanical stimulation.

SAI and SAII receptor responses after 10ml 100 μM Verapamil



C150384

injected intradermally under SAI receptors, the response of the receptors to mechanical stimulation failed at times varying between 90 and 125 seconds with a mean time of 105 $\pm$ 10.4s (S.E.M., n=3). There was no increase in inter-stimulus activity nor was there an enhancement of the response to mechanical stimulation. Electrical stimulation of the afferent fibre innervating the receptors still initiated an action potential in the fibre even although the response to mechanical stimulation had ceased (Figure 4.22). The results of the three experiments in which verapamil was intradermally injected are presented in Figure 4.23.

The effects of intradermal injections of verapamil on hair follicle responses are illustrated in Figure 4.24. It was evident that verapamil injections, of the same quantity that caused SAI receptor failure, had little effect on the response of hair follicle afferents. However, because the method of stimulation could not be measured, quantifying the response was not possible. There was no other activity produced in the afferent fibre by the verapamil injection as can be seen by the absence of activity in the afferent fibre between stimulations.

The ISI distribution was examined in unit C150384, the one experiment in which verapamil was slowly injected into the limb circulation of the cat (Table 4.10). The low mean firing rate of the control responses in the 3 experiments in which verapamil was intradermally injected prevented their use in this analysis. In unit C150384 the ISI distribution of the adapted response was exponential during the control

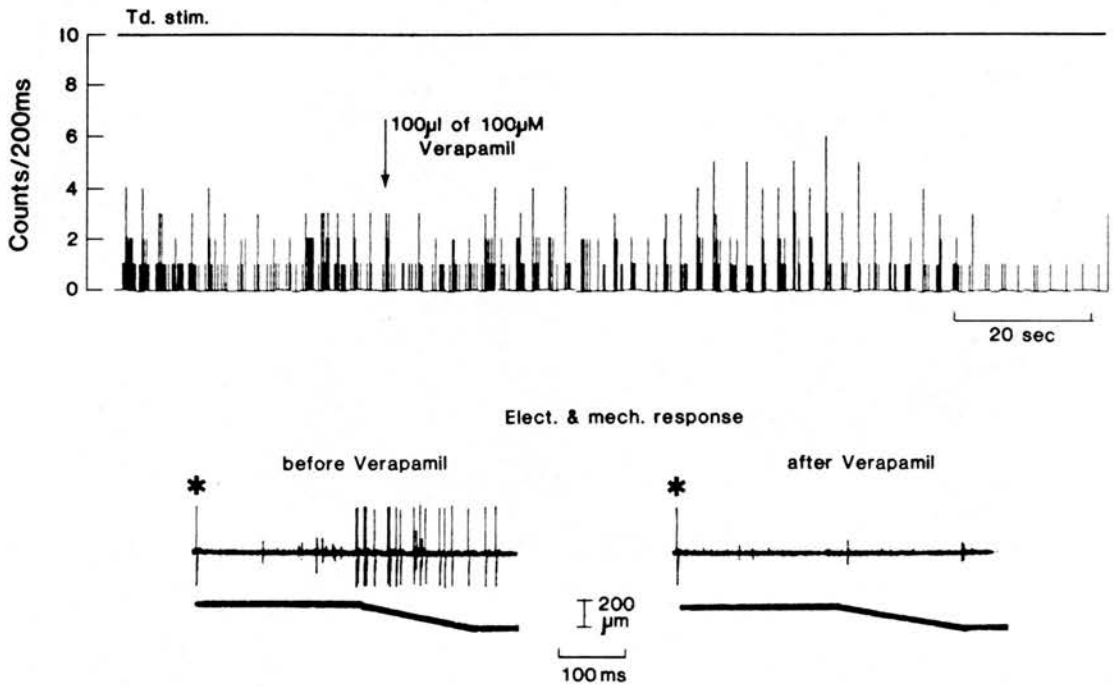
FIGURE 4.22

Experiment R230284

The histogram shows the response of the touch dome to mechanical stimulation falling to zero within 90s of the intradermal injection of 100µℓ of 100 µM verapamil. Electrical stimulation of the afferent fibre throughout the experiment continues to produce a response when the response of the touch dome to mechanical stimulation has ceased. This is seen over the last 20s of the histogram where the regular response is that produced by electrical stimulation.

The two oscilloscope traces show the responses produced in the afferent fibre to electrical (*) and mechanical stimulation of the touch dome. As can be seen, when the response of the touch dome to mechanical stimulation has ceased,there is still a response to electrical stimulation.

Intradermal injection of Verapamil Hydrochloride



R230284

FIGURE 4.23

Shown are the results of 3 experiments in which 100µℓ of 100µM verapamil was intradermally injected. As can be seen, receptor failure occurred in all 3 cases. In experiment C150384/2 (c) the touch dome was stimulated for the first time after the injection of verapamil. Consequently there was no control response with which to compare the response after the verapamil injection.

SAI receptor response to intradermal injections of 100µl 100 µM Verapamil

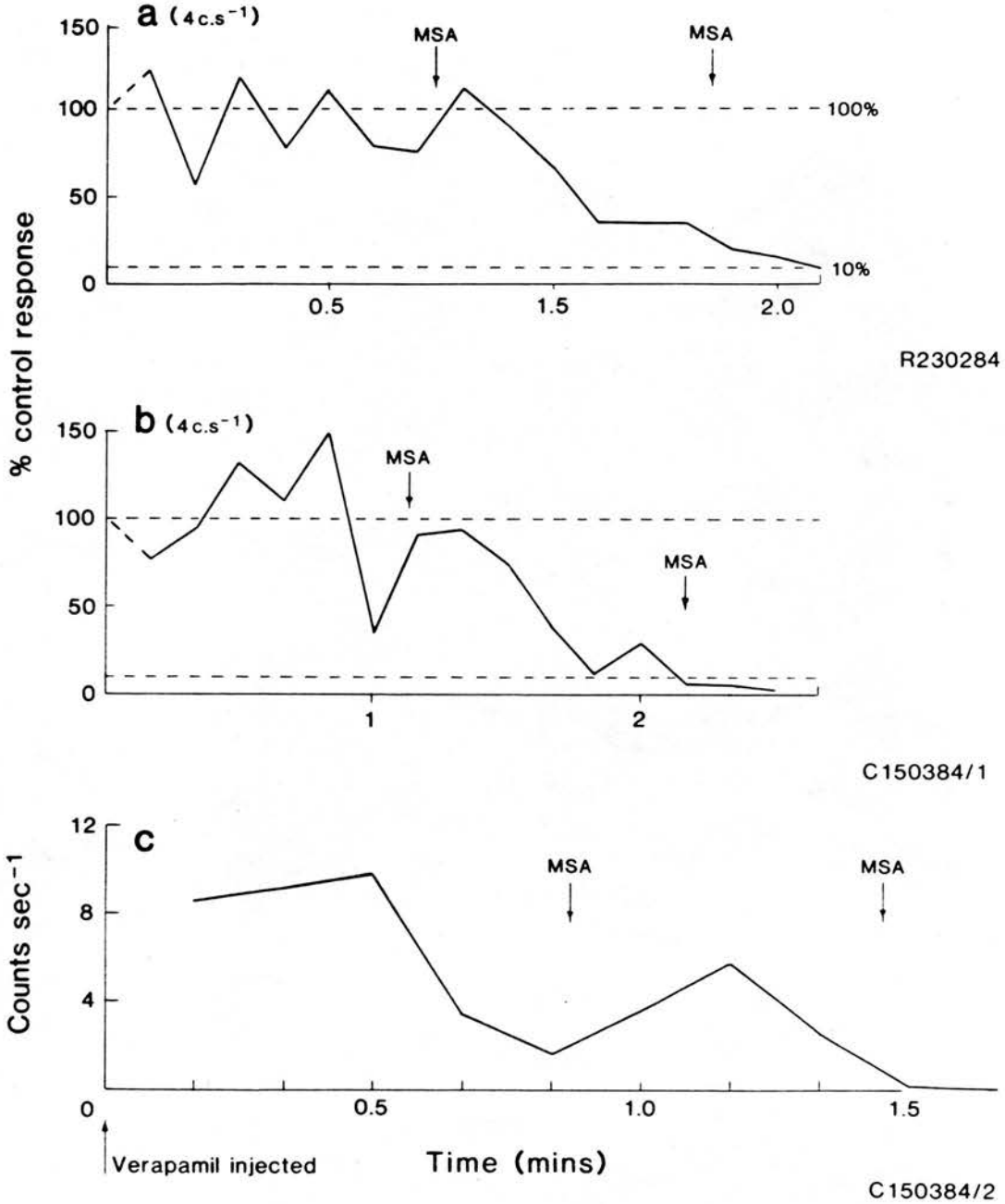


FIGURE 4.24

The injection of verapamil, in quantities which caused SAI receptor failure, has no effect on the response of hair follicles when mechanically stimulated. An additional injection of 100µℓ of 100µM verapamil 5 minutes after the original injection has still no effect.

Hair follicle response to intradermal injections of 100µl 100µM Verapamil

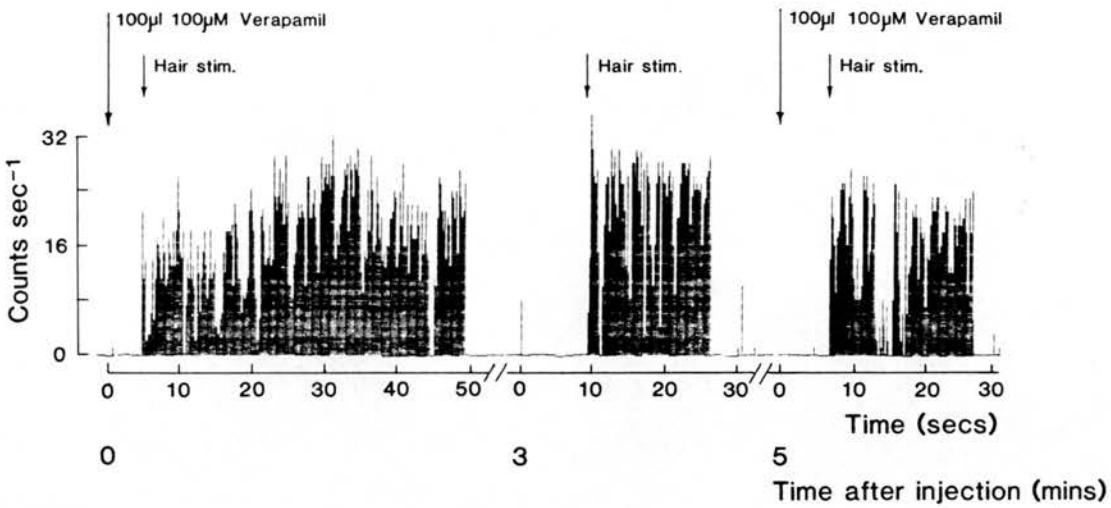


TABLE 4.10.

Analysis of the ISI distriution before and after the injection of verapamil. The times shown are the approximate times in minutes from the start of the injection.

TABLE 4.10

	COMPARISON WITH EXPONENTIAL			COMPARISON WITH NORMALISED CONTROL			HISTOGRAM TRUNCATED AT
C150384	X^2	d.f.	p.	X^2	d.f.	p.	(ms)
Control	18.6	22	>0.05	-----			39.0
+6min	21.3	18	>0.05	208.6	9	<0.05	45.0
+16min	TOO FEW INTERVALS						

period and also 6 minutes after the start of the verapamil injection. However the mean ISI length at 6 minutes was significantly different ($p < 0.05$, X^2-test) from that of the normalised control distribution. Sixteen minutes after the injection there were too few intervals for analysis.

Histology When 100µl of 100µM verapamil was intradermally injected under an SAI receptor (Figure 4.25a), the number of vesicles present in the Merkel cells at the moment of receptor failure was 6.8 ± 0.8 vesicles/μm^2 (S.E.M., n=18). This is a significant reduction ($p < 0.001$: Student's 2-tailed 't' test) in vesicle numerical density when compared to the number of vesicles in Merkel cells of saline treated receptors (12.1 ± 1.5 vesicles/μm^2, S.E.M., n=13). There was no significant difference in vesicle numerical density between $CoCl_2$ and verapamil treated receptors. Where vesicles were present in the Merkel cell they were found adjacent to the Merkel cell membrane. Figure 4.25b. is an enlargement of the boxed area in Figure 4.25a. and shows an accumulation of dense-cored vesicles adjacent to the Merkel cell membrane.

Table 4.11 summarises the histological data obtained from the experiments presented in this chapter.

Part 4.4. Summary of Results

When 0.15M NaCl was slowly injected into the localised limb circulation of the cat, the response of the touch dome to mechanical stimulation never failed even after 40 minutes of stimulation. When 0.15M NaCl

FIGURE 4.25

a. A Merkel cell from a touch dome after the intradermal injection of 100μM verapamil. The touch dome from which this Merkel cell was taken had ceased to function to mechanical stimulation. As was the case when the touch dome failed after the injection of $CoCl_2$ (Figure 4.19) there is almost a total loss of dense-cored vesicles (V) from the Merkel cell. Those that remain are down close to the Merkel cell membrane and in places are clustered around the synapse-like structure (S).
(N - Merkel cell membrane;
NT - nerve terminal).

b. Enlargement of boxed area in a. showing a close-up view of the synaptic region (S). The vesicles (V) are clustered together against the Merkel cell membrane at this region.
(NT - nerve terminal).

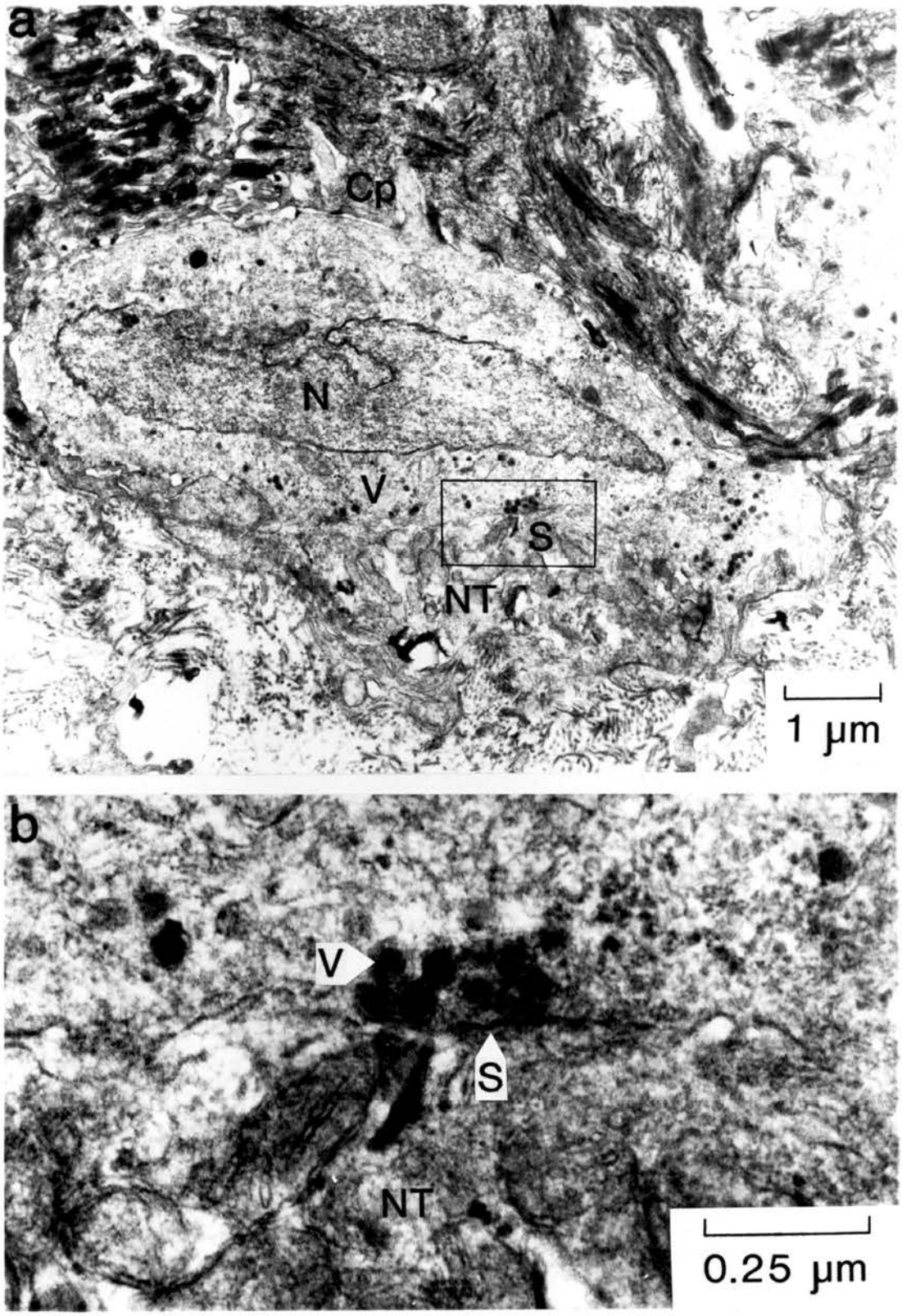

a
Cp
N
V
S
NT
1 µm
b
V
S
NT
0.25 µm

TABLE 4.11

Table showing the vesicle density and the number of 'synaptic-like' structures in Merkel cells after the injection of 0.15M NaCl, 5mM $CoCl_2$ and 100uM verapamil.

TABLE 4.11

Injection of: (Control)	Granule density (nos./μm^2)	Synapses (nos./section)
0.15M NaCℓ	12.1 ± 1.5 (n = 13)	0.6 ± 0.25 (n = 13)
5mM $CoCℓ_2$	4.3 ± 0.8 (n = 17)	2.1 ± 0.4 (n = 13)
100μM verapamil	6.8 ± 0.8 (n = 18)	-

(mean ± S.E.)

was injected intradermally under SAI receptors, again the response to mechanical stimulation continued for as long as the touch domes were stimulated.

The injection of $CoCl_2$ into the localised limb circulation eventually caused the SAI receptor to fail to respond to mechanical stimulation. The time to receptor failure was correlated to the concentration of $CoCl_2$ injected - the greater the concentration, the quicker the receptor failed. When the general circulation was restored to the limb, receptor recovery occurred after a period of time.

$CoCl_2$ also produced an increase in background activity in afferent fibres which innervated SAI receptors. This increased activity was unrelated to mechanical stimulation of the receptor. There was no increase in SAII or hair follicle afferent fibre activity. The background activity of SAII receptors did however cease sometime after the injection of $CoCl_2$ although the receptor continued to respond to mechanical stimulation.

The intradermal injection of different quantities of $CoCl_2$ under touch domes produced effects which suggested dose dependency. The smallest quantity injected produced a reduction in the receptor response to mechanical stimulation but not failure, whereas the highest quantity injected produced receptor failure immediately. In all experiments electrical stimulation of the afferent fibre close to the Merkel cell produced an action potential even when the response to mechanical stimulation had ceased.

Verapamil hydrochloride in the concentration used

caused a significant reduction in the SAI receptor response to mechanical stimulation but did not cause receptor failure. It did however abolish the background activity of an SAII receptor. Verapamil, when intradermally injected in both rats and cats caused SAI receptors to fail although the afferent fibre innervating the receptor still responded to electrical stimulation.

Where analysis was possible, the ISI distribution of the adapted response both during the control period and after the injection of any one of the solutions used, was exponential, although the mean ISI's were different.

Correlated with receptor failure in the presence of $CoCl_2$ and verapamil was a reduction in the number of dense-cored vesicles present in the Merkel cell. There was also a significant increase in the number of 'synapse-like' structures seen in $CoCl_2$ experiments.

Part 4.5 Discussion

The changes observed in the structure and function of Merkel cells after the injections of various solutions may be the result of oedema produced by the injections. On the basis of 70ml blood/Kg bodyweight, the injection of 10ml of a solution into the restricted circulation of a cat's hind limb would effectively double the circulating volume within the limb. This might have resulted in cell swelling with consequent alteration or redistribution of intracellular structures including the dense-cored vesicles. A

change in function may also result from a change in the skin mechanics produced by oedema both within and around the Merkel cells. However at all times the effects of the injection of $CoCl_2$ or verapamil on the structure and function of SAI receptors were compared with those of physiological saline, any effects of which did not significantly alter the appearance of the receptors. After saline injections, responses to mechanical stimulation continued for as long as receptors were tested and histological examination of Merkel cells taken after mechanical stimulation showed no significant change in the numbers of dense-cored vesicles/μm^2 from normal Merkel cells (see Tables 3.6 and 4.11).

The effects of Co^{2+} on the evoked response of SAI mechanoreceptors to mechanical stimulation is similar to that in the frog sciatic nerve-sartorius muscle preparation (Weakly,1973; Kita and Van der Kloot, 1973). The effects were dose dependent and reversible, except with the highest concentration used (15mM $CoCl_2$) when receptor recovery failed to occur. Weakly (1973) found that increasing concentrations of Co^{2+} produced a progressive decrease in the compound end-plate potential (e.p.p.) until, with bathing solutions containing 1mM $CoCl_2$, the compound e.p.p. had almost disappeared. This is the same as the Co^{2+} concentration (0.97 $\pm$ 0.26mM) measured in the blood serum when the SAI receptor response to mechanical stimulation had ceased. The Co^{2+} concentration at the Merkel cell-neurite complex when evoked activity had ceased was probably less than this as the blood sample,

from which the serum was taken, was from the main blood supply to the limb and not the cutaneous circulation which supplied the touch dome. It does indicate however that the Co^{2+} concentration required to block evoked activity in both the Merkel cell-neurite complex and the neuromuscular junction is in approximately the same range.

By showing that the reduction in evoked transmitter release could be reversed by washing the preparation in a Co^{2+} free solution and antagonized by elevating the external Ca^{2+} concentration, Weakly (1973) suggested that the inhibition of neuromuscular transmission by Co^{2+} was a result of interference in the process linking presynaptic depolarization to transmitter release. He concluded that the data indicated competitive antagonism of Ca^{2+} by Co^{2+} leading to failure of transmitter release. In the Merkel cell-neurite complex no attempt was made at elevating the Ca^{2+} levels in the limb circulation after the evoked response had ceased but reversal of the effects of Co^{2+}, by returning the general circulation to the limb, was demonstrated on several occasions. The only time that some degree of receptor recovery did not occur was when 15mM $CoCl_2$, the highest concentration used, was slowly injected into the limb circulation. This is similar to the results reported by Heuser and Miledi (1971) who studied the effects of lanthanum (La^{3+}) on the nerve-sartorius preparation of frogs. They found that with low concentrations of La^{3+} the blockage of transmitter release was readily reversed. However after treatment with high

concentrations of La^{3+} no recovery was observed even after repeated washing in normal Ringer. These similarities between the effects of Co^{2+} in the Merkel cell-neurite complex and the effects of Co^{2+} and La^{3+}, which are now generally regarded as Ca^{2+} channel blockers, in the neuromuscular junction suggest that Co^{2+} may be antagonizing Ca^{2+} channels on the Merkel cell membrane preventing the release of transmitter from the Merkel cell.

Because the localized limb circulation procedure was used in these experiments, the PO_2 levels in the limb slowly but steadily fell with time. However because the PO_2 levels measured in the limb circulation at the moment of receptor failure were higher than those in control experiments (injections of physiological saline) when the receptor still responded in excess of 70% of its control response, it is unlikely that hypoxia contributed to receptor failure. It has also been shown in the hypoxic experiments in Chapter 3 that in order for receptor failure to occur, even when animals were ventilated with N_2, the external environment around the limb had also to be hypoxic. If this was not the case, then receptor failure did not occur, certainly not in the relatively short time that it takes for the receptor to fail after the administration of $CoCl_2$.

Cobalt slowly injected into the limb circulation produced an increase in activity, unrelated to mechanical stimulation, in the afferent fibres of SAI mechanoreceptors only. In the neuromuscular junction Co^{2+} has been found to cause an increase in miniature

end plate potentials (m.e.p.p.'s) produced by an increase in spontaneous transmitter release (Kita and Van der Kloot, 1973; Weakly, 1973). Lanthanum has also been shown to increase spontaneous transmitter release in the neuromuscular junction (Heuser and Miledi, 1971; Kajimoto and Kirpekar, 1972). In the SAI mechanoreceptor the increased activity was in the form of action potentials recorded in the afferent fibre innervating the touch dome. If Co^{2+} caused an increase in spontaneous transmitter release from each Merkel cell-neurite complex in the touch dome, and there are in the region of 50 Merkel cell-neurite complexes in a single touch dome, then sufficient transmitter may be released to initiate the action potentials recorded in the afferent fibre.

Weakly (1973) found that concentrations of Co^{2+} of 20-60 times that required to decrease the compound e.p.p. were necessary to significantly increase the spontaneous transmitter release. With the highest concentrations used, it was impossible to evoke transmitter release. The increase in spontaneous transmitter release observed by Weakly (1973) was unaffected by the presence or absence of Ca^{2+} in the bathing solution. However Kita and Van der Kloot (1973) using a similar preparation found that Co^{2+}, in the presence of Ca^{2+}, depressed spontaneous transmitter release. Heuser and Miledi (1971) used Ca^{2+} free solutions in their study on spontaneous transmitter release with La^{3+}. They do not however state whether this was a prerequisite for spontaneous transmitter release.

In a quite different experimental situation Hensel (1981) demonstrated that a decrease in Ca^{2+} concentration produced by the chelating agent EDTA could, on its own, produce an increase in the discharge rate and pattern of firing in afferent fibre activity of cold receptors. Orchardson (1978) also found that a reduction in Ca^{2+} concentration produced an increase in the number of action potentials evoked in nerve fibres dissected out from the cauda equina of rats. It therefore appears that variations in Ca^{2+} concentration, particularly a decrease, is capable of increasing spontaneous nerve fibre activity. Two possible explanations for the effects Ca^{2+} on nerve fibres were proposed by Frankenhauser and Hodgkin (1957). One was that Ca^{2+} was absorbed at the outer surface of the membrane which resulted in a redistribution of other charged particles inside the membrane without changing the overall membrane potential. The second explanation was that depolorization removed Ca^{2+} ions from sites in the membrane allowing the entry of Na^{+} into the cell, so that in low Ca^{2+} concentrations the permeability of the cell membrane to Na^{+} was increased. If this explanation is correct then, in the Merkel cell-neurite complex, it is possible that Co^{2+} acted initially to block Ca^{2+} channels on the Merkel cell membrane but that the later increase in spontaneous afferent fibre activity was due to the reduced Ca^{2+} concentration having a direct effect on the nerve fibre. The normal Ca^{2+} concentration in cat plasma or serum is approximately 2.5mM. In the present experiments

control Ca^{2+} concentrations varied from 1.9mM in experiment C310184 to 2.4mM in experiment C260184 prior to the stimulation of unit 1. As Ca^{2+} is involved in all but 2 of the steps in the blood clotting process it may be that the observed variation in control Ca^{2+} concentrations in the serum arose through different amounts of Ca^{2+} being used in clot formation. The decrease in the serum Ca^{2+} concentration after the injection of 10ml of 5mM $CoCl_2$ solution (Table 4.8) could have arisen by the dilution effect of adding a volume of a solution sufficient to effectively double the circulating volume within the limb. In one experiment however, experiment C190184, the Ca^{2+} levels remained unchanged throughout the experiment and yet there was still an increase in afferent fibre activity after the injection of $CoCl_2$. The Ca^{2+} concentrations in the serum samples from this experiment were rechecked and the same values obtained. If this result is valid, then, in the Merkel cell-neurite complex, the increase in spontaneous transmitter release cannot be attributed to a fall in Ca^{2+} concentrations.

The frequency of m.e.p.p.'s at the frog neuromuscular junction has been shown to increase with time of exposure to both Co^{2+} (Weakly, 1973; Kita and Van der Kloot, 1973) and La^{3+} (Heuser and Miledi, 1971; De Bassio, Schnitzler and Parsons, 1972). Heuser and Miledi (1971) found that after 45 minutes exposure to La^{3+} the frequency of m.e.p.p's had increased but thereafter gradually declined until after several hours, the spontaneous transmitter release had all but ceased. Related to the decrease in spontaneous

m.e.p.p's was a decrease in the number of synaptic vesicles observed in the nerve terminals. This is similar to the observations in the Merkel cell-neurite complex although the timing of events was minutes as opposed to hours in the neuromuscular junction. The increase in spontaneous activity in the afferent fibres innervating SAI mechanoreceptors, which occurred after 10-20 mintes, could have resulted from exposure of the Merkel cells or their nerve endings to Co^{2+} for an appropriate length of time. This increase in activity was transient and touch domes removed from the skin when there was no longer activity in the afferent fibre, evoked or otherwise, contained Merkel cells which were significantly depleted in dense-cored vesicles. Electrical stimulation of the afferent fibre innervating the touch dome still produced an action potential even when all other activity in the afferent fibre had ceased. As Heuser and Miledi (1971) point out, such a depletion in vesicles and the absence of mechanically evoked as well as spontaneous activity in the nerve fibre may be taken as a necessary corollary, but not definite proof, that transmitter substances are contained in the vesicles.

If the only action of Co^{2+} is to block the entry of Ca^{2+} into the Merkel cell, then receptor failure should occur with numerous dense-cored vesicles still present in the Merkel cells. As described above the opposite was found to be the case. In control experiments in which 0.15M NaCl was injected, there was no significant decrease in the number of dense-cored vesicles present in the Merkel cell cytoplasm even

after 40 minutes of mechanical stimulation. This suggests that Co^{2+}, as well as stopping the evoked response to mechanical stimulation, also has an effect on the synthesis or transport of transmitter substances within the cell. Lavoie and Bennett (1983) have recently shown that in dorsal root ganglion cells of the bullfrog, fast axonal transport is inhibited by bathing the neuron in Co^{2+} containing or Ca^{2+} free solutions. Protein synthesis was not affected and it was suggested that Ca^{2+} is required for proteins to leave the Golgi region in transit for the fast axonal transport system. It is conceivable that Ca^{2+} in secretory cells has more than one function. Its possible primary function could be the initiation of transmitter release and its secondary function the mobilization of transmitter substances from their point of synthesis to their place of requirement.

The method by which Co^{2+} brings about spontaneous transmitter release is not clear. If Co^{2+} acts to prevent transmitter release by blocking Ca^{2+} channels, in what way can the increase in m.e.p.p. produced by Co^{2+} be explained? It could be that short exposure to Co^{2+} prevents evoked release of transmitter from the Merkel cell by Co^{2+} occupying Ca^{2+} channels on the membrane preventing the entry of Ca_2 into the cell, but not itself entering through the membrane. After several more minutes exposure the levels of Co^{2+} around the Merkel cell may be such that it starts to diffuse passively through the membranes to activate the release mechanism directly and thereby increase transmitter release. This would explain why the evoked response

produced by mechanical stimulation of the touch dome failed before the late increase in Co^{2+} produced afferent fibre activity occurred.

This however would not explain the rapid initial rise in spontaneous activity which occurred within 20-30s of the $CoCl_2$ injection. It is unlikely that it was produced by changes in pH, osmolality or temperature as these were all practically unaltered from the values measured in the control solution (0.15M NaCl). This was found to have little effect on the SAI receptor response to mechanical stimulation. Cobalt evoked activity could occur, however, if it causes an inward displacement of Ca^{2+} ions from bound sites in the membrane or the mobilisation of other Ca^{2+} stores either within or around the Merkel cell. Storage and sequestration of Ca^{2+} within mammalian presynaptic nerve terminals is described by Blaustein, Kendrick, Fried and Ratzlaff (1977) in a study of rat synaptosomes.

Occasionally afferent fibres which innervated both SAI and SAII mechanoreceptors were present in the nerve bundle being recorded from. The structure and function of SAII receptors are fully described by Chambers et al. (1972). Unlike the Merkel cell of the SAI mechanoreceptor, SAII receptors (Ruffini endings) do not possess specialized accessory cells and it is thought that the neurone itself functions as a mechano-electric transducer. The characteristic regular discharge observed in these receptors is believed to arise from summation of the depolarization at active transducer sites which are scattered over the receptor

core (Chambers et al. 1972). Because the resting discharge is not produced by transmitter release from a 'transducer' cell the Ruffini ending was used as an other receptor system with which the effects of Co^{2+} on the SAI receptor response could be compared. When the control solution (0.15M NaCl) was injected, the touch dome response fluctuated slightly but remained generally constant throughout the duration of the experiment. The resting discharge of the Ruffini ending however fluctuated considerably falling almost to zero at one moment before rising again sometimes to 150% of its control level. The background activity of the Ruffini ending started to fluctuate at approximately five minutes after the saline injection. A possible explanation for this effect could be the development of hypoxia in the limb circulation of the animal. However,regardless of the cause, the varying background activity of the SAII mechanoreceptor indicates that it is more susceptible to environmental changes than the SAI receptor.

When $CoCl_2$ was injected the resting discharge of the Ruffini ending fell to zero after about two minutes whereas the SAI receptor continued to respond for a further 10 minutes. At first sight it appeared that the failure of the resting discharge was in someway related to the adjustment of the stimulator probe which was stimulating the touch dome (Figure 4.13). However as the SAII resting discharge failed in an equally short time after the injection of verapamil, when no adjustment of the stimulator was carried out, it would appear that the background discharge of the SAII

receptor failed as a direct result of the effects of Co^{2+} and verapamil. It is possible that the spontaneous discharge in the Ruffini ending is also a Ca^{2+} dependent process and therefore susceptible to Ca^{2+} channel blockers.

As shown in Figure 4.21 manual stimulation of the SAII receptor still produced a response sometime after the background discharge of the receptor had ceased. However unlike touch domes, the Ruffini ending was not regularly stimulated after the injection of $CoCl_2$. Consequently the ability to produce a response from the SAII receptor by intermittent mechanical stimulation is insufficient evidence to claim that the SAII receptor was still functional when the SAI receptor response was considerably reduced. It does however show that an evoked response could still be obtained from the Ruffini ending when the background resting discharge had ceased. This suggests that in the Ruffini ending there may be two separate transduction processes involved, one for the production of the background discharge and another for the production of the response to mechanical stimulation. Alternatively it may be that the sensitivity of the receptor had decreased in some way causing failure of the background discharge and leaving only the receptor response to mechanical stimulation.

Where there were sufficient intervals for analysis the ISI distribution both before and after the injection of either physiological saline, $CoCl_2$ or verapamil, was exponential. As described in Chapter 3 an exponential distribution of intervals implies random

activity and if this activity is produced at individual generator sites then as these sites fail, the response of the receptor to mechanical stimulation would be expected to become more regular. In unit C260184/1, 10 minutes after the start of the injection of $CoCl_2$ when the response of the receptor was <50% of its control firing rate (Figure 4.10b), the ISI distribution was still exponential (Table 4.9) for intervals >25.0ms. This was also the case for unit C150384, 6 minutes after the injection of 10ml 100μM verapamil (Figure 4.20b). The response at this time was approximately 50% of the control firing rate for the unit but the ISI distribution was still exponential for intervals >45.0ms (Table 4.10). This suggests that rather than individual Merkel cell-neurite complexes giving rise to a more regular discharge as they failed, these complexes still act as individual independent generator sites producing random activity in the nerve fibre. However the rate of production of this activity is reduced. If, as is postulated, Ca^{2+} is necessary for the release of a transmitter substance from the Merkel cell then as Ca^{2+} is hindered in its entry into the cell the entire process of transmitter release would be delayed. This could account for the persistence of random activity in the afferent fibre of SAI receptors even though the firing rates of the receptors were greatly reduced. The ISI distribution of the background activity in unit C260184/1 approximately 20 minutes after the injection of $CoCl_2$ (Figure 4.10c) was exponential for intervals >115ms (Table 4.9).

There was, in all cases, a significant difference

between the mean interval length of the adapted response after the injection of a solution, and that of the normalised control.

For all ISI distributions, including those in Chapter 3, which did not vary significantly from an exponential, the mean interval length of the adapted response at which the histograms were truncated was 41.5 $\pm$ 15.6 (mean $\pm$ S.D., n = 13). Iggo and Muir (1969) found that the adapted response of an SAI receptor fitted an exponential pattern for intervals greater than 45.0ms which is similar to the value obtained in these experiments.

In addition to the depletion of vesicles in the Merkel cell there was an increase in the number of synaptic-like structures seen between the Merkel cell and the nerve terminal. This is possibly a further indication of transmitter release from the Merkel cells. Heuser and Miledi (1971) argued that it was quite conceivable that agents such as La^{3+} could make previously inactive membrane areas capable of releasing transmitter. This result requires further study by a systematic examination of many more $CoCl_2$ treated Merkel cells.

Verapamil was used to verify the results obtained with $CoCl_2$. It was chosen as it is a known calcium channel blocker and does not have the excitatory effects of Co^{2+}. When injected into the limb circulation, 10ml of 100µM verapamil caused a significant reduction in the response of the touch dome to mechanical stimulation but did not produce receptor failure. This result is consistent with the effects of

verapamil on cardiac muscle (Kohlhardt et al. 1972) and in the snail neurone (Kostyuk et al. 1977). Both of these studies found that verapamil reduced the inward Ca^{2+} current but did not block it altogether.

Intradermal injections of verapamil caused receptor failure to mechanical stimulation within three minutes in all experiments although an action potential could still be evoked in the afferent fibre by electrical stimulation of the fibre close to the touch dome. As was the case when $CoCl_2$ was used the pH, osmolality and temperature of the solutions injected were all unaltered from that of the control solutions. Because the control solutions did not significantly affect the touch dome response to mechanical stimulation it is probable that receptor failure resulted as a direct effect of verapamil on the Merkel cell-neurite complex.

At receptor failure there was a significant decrease in the number of dense-cored vesicles present in the Merkel cells of stimulated touch domes. This is similar to the effects of Co^{2+} on the Merkel cell-neurite complex and could possibly arise as a result of a similar mode of action - verapamil, by blocking Ca^{2+} channels on the Merkel cell membrane, causes a reduction in the amount of Ca^{2+} entering the Merkel cell. As this occurs the dense-cored vesicles would continue to release their contents using what Ca^{2+} was available to them. Coincident with this would be a gradual breakdown in the transport of transmitter substances to their active sites within the cell eventually producing receptor failure due to the lack

of transmitter substance.

Part 4.6. Conclusions

The effects of Co^{2+} and verapamil on the response of SAI mechanoreceptors to mechanical stimulation were similar to the effects produced by these substances in other preparations where they are known to function as Ca^{2+} channel blockers. It may be inferred from this that both Co^{2+} and verapamil act in the Merkel cell-neurite complex by blocking the entry of Ca^{2+} into the cell and hence prevent transmitter release from the Merkel cell.

At receptor failure, electrical stimulation of the SAI afferent fibre still produced activity in the fibre. At the same time Merkel cells taken from failed receptors were significantly depleted in dense-cored vesicles. This suggests that the Ca^{2+} channel blockers Co^{2+} and verapamil may have had an indirect effect on the transport of transmitter substances to their active sites as well as preventing the release of transmitter substances from the Merkel cell. It is difficult, however, to reconcile the effects of Co^{2+} and verapamil with a similar mode of action. Cobalt produces an immediate, plus a later rise in afferent fibre discharge as well as blocking the evoked response to mechanical stimulation whereas verapamil blocks the evoked response only.

The depletion in dense-cored vesicles at receptor failure leads to the conclusion, as it did in the hypoxic experiments of Chapter 3, that the dense-cored vesicles of the Merkel cell are a prerequisite for

normal functioning of SAI mechanoreceptors possibly by the Ca^{2+} dependent release of a neurotransmitter substance from the vesicles.

CHAPTER 5

General Discussion and Conclusions

GENERAL DISCUSSION and CONCLUSIONS

The results presented in both Chapter 3 and 4 suggest that, in the Merkel cell-neurite complexes of SAI mechanoreceptors, the dense-cored vesicles of the Merkel cells are necessary for the normal functioning of the receptor. There is also evidence that the transduction process in the touch dome may involve the secretion of a neurotransmitter substance which is possibly stored in the dense-cored vesicles. When Merkel (1875) first observed specialized epidermal cells in the basal layer of the epidermis and the close association of these cells with nerve fibres, he postulated that they were, in some unknown way, involved in the conversion of physical stimuli applied to the skin to neural activity in the adjacent nerve ending.

Morphologically the Merkel cell-neurite complex has features which suggest a neurosecretory function. However, past attempts to verify this have proved fruitless. Smith and Creech (1967) tested the effects of numerous drugs on the response of SAI mechano-receptors and found none that would initiate spontaneous activity in the afferent fibre. Nictone produced a transient increase in the response to mechanical stimulation before the response was finally blocked. Lobeline was also found to block the response to mechanical stimulation. Iggo and Muir (1969) treated some animals with sufficient reserpine to deplete any catecholamines that may be present. They

found however that after several days treatment neither the vesicle number nor vesicle content were altered.

Because of its appearance the Merkel cell has been classed as a part of the diffuse neuroendocrine system or APUD cell system by Winkelmann (1977). Cells of the APUD system, according to Pearse (1969) share several cytochemical and ultrastructural characteristics but their sole common function is the secretion of low molecular weight polypepties. Another newly described common characteristic for the components of the APUD system is the presence of neuron-specific enolase (NSE). NSE is an enzyme which was originally regarded as being present only in neurons but which has now been shown to be present in cells of the APUD system (Schmechel, Marangos and Brightman, 1978). Recently, low molecular weight polypeptides have been demonstrated in Merkel cells (Hartschuh et al. 1979, 1983; Weber, Hartschuh, Feurle and Weihe, 1980) as well as NSE (Gu, Polak, Tapia, Marangos and Pearse, 1981). These findings all support the earlier suggestion that Merkel cells are members of the APUD system and if so have a secretory function.

Using immunohistochemical techniques, Hartschuh et al. (1979, 1983) observed a met-enkephalin-like immunoreactivity in the Merkel cells of rats and a VIP immunoreaction in the Merkel cells of other animals. At the light microscopic level the immunoreaction appeared to be restricted to the area of the Merkel cell of greatest vesicle density. To eliminate the possibility that the reaction could be occurring in the expanded nerve ending adjacent to the Merkel cell,

touch domes were denervated. Subsequent electron microscopic examination of Merkel cells from the touch domes revealed a total absence of nerve endings although the immunoreaction at the light microscopic level was still present. Electron microscopic immunohistochemistry requires to be done to determine if the immunoreaction is localized to the vesicles themselves. Both VIP and met-enkephalin are now generally regarded as neurotransmitters (Snyder, 1980) and their presence within Merkel cells, particularly in the cytoplasm adjacent to the nerve terminal, may be taken as further evidence for a neurosecretory function of Merkel cells in the transduction process.

Gottschaldt and Vahle-Hinz (1982) disputed the possibility of chemosynaptic transmission from Merkel cells to their adjacent nerve terminal and carried out experiments in which naloxone, a known antagonist of met-enkephalin (Sawynok,Pinsky and La Bella, 1979), was intravenously injected into a cat. They found that naloxone had no effect at all on the response of SAI mechanoreceptors to mechanical stimulation. They concluded that these negative findings were evidence against the hypothesis that Merkel cells function as neuroreceptor cells. The point to be noted is that the met-enkephalin immunoreaction reported by Hartschuh *et al*. (1979) was restricted to the Merkel cells of rats only and that in cats the immunoreaction was to VIP. As Gottschaldt and Vahle-Hinz (1982) used naloxone in a cat it may well be they were using the wrong animal species. However, H.O. Handwerker (unpublished observation) has obtained the same negative result when

using naloxone in rats. VIP has, at present, no effective inhibitors (Said, 1980) although in the oesophageal sphincter of the opossum responses to VIP were reduced by almost 50% by using VIP antisera (Goyal, Said and Ratton,1979). This is one approach that could be tried in the Merkel cell-neurite complex in an attempt to determine what effects VIP antisera might have on the evoked response of a touch dome to mechanical stimulation.

Previous to the work described above, Gottschaldt and Vahle-Hinz (1981) studied the ability of Merkel cell-neurite complexes, in the sinus hair follicles of the cat, to follow high frequency stimulation. They found that these complexes could follow, in a 1:1 relationship, frequencies of up to 1500 Hz. Beyond this frequency the afferent fibre was unable to conduct impulses within the refractory period. They also determined the receptor delay by measuring the time difference between the response produced by electrical stimulation of the afferent fibre and mechanical stimulation of the sinus hair follicle and found this to be about 0.3ms (300μs). The speed of events they claimed was too fast for chemosynaptic transmission to occur. However it has been shown by the use of voltage clamp techniques on the squid giant synapse (Llinás, Walton and Hess, 1976; Llinás, 1977) that the delay between Ca^{2+} entry into the postsynaptic terminal and the appearance of the excitatory postsynaptic potential is in the order of 200μs. Hubbard and Schmidt (1963) also measured a similar synaptic delay in the rat neuromuscular junction. The similarity in the synaptic

delay between these other systems, in which it is known that chemosynaptic transmission occurs, and the Merkel cell-neurite complex is evidence for rather than against the possibility of transmitter release from Merkel cells.

It is possible that at high frequencies the nerve ending does respond directly to mechanical stimulation whereas with static displacements it is chemosynaptic transmission in the Merkel cell-neurite complex that produces the characteristic slowly adapting response. Evidence for this is presented by Kasprzak et al. (1970) who demonstrated in kittens that ingrowing nerve endings could follow frequencies of 200 Hz with a 1:1 relationship although no Merkel cells were present. Brown and Iggo (1963) found similar results in degeneration/regeneration studies on touch domes. Mechanical stimulation of the ingrowing nerve tip produced a non-specific response. In both of the above investigations it was only after nerve endings innervated Merkel cells that a sustained response to mechanical stimulation was obtained.

The presence of 'synaptic-like' structures between Merkel cells and their adjacent nerve terminals has been disputed for some time. Some investigators described these structures in detail (Chen et al. 1973; Mihara et al. 1979; Hartschuh and Weihe, 1980) whereas others found no evidence suggesting synaptic junctions (Munger,1965; Hashimoto, 1972). Hartschuh and Weihe (1980) studied these regions of membrane specialization using a fixation procedure specifically designed to show up these structures. With this technique they

described these structures as common features in all animal species studied. However it was suggested by Gottschaldt and Vahle-Hinz (1981) that the junctions observed by Hartschuh and Weihe (1980) were possibly desmosome-like attachment points between Merkel cells and nerve endings and that the Merkel cells functioned as passive abutments for the deformation of the mechanosensitive nerve endings. Smith in 1970 stated that synaptic thickenings of membranes between the Merkel cell and its neural terminal were never seen, irrespective of the mammalian species studied. However in 1977 he withdrew that statement (Smith, 1977) and stated that there was little doubt that synaptic specialization of Merkel cell-neurite membranes existed and the observation of Merkel cell vesicles fusing with these specialized regions was evidence of synaptic transmission. From the results presented in Chapter 4 there is evidence, based on preliminary observations, that the number of 'synaptic-like' junctions may vary depending on the 'stimulation' applied to the Merkel cell-neurite complex. There was a significantly greater number of these structures observed after treatment with Co^{2+} than there was in 'control' Merkel cells. If this result is confirmed in future experiments, it may go someway towards explaining the contradictory results observed in previous investigations.

A feature not present at the presynaptic membrane of the Merkel cell and which is generally regarded as a characteristic feature of synapses are clear synaptic vesicles. However the functional significance of these

vesicles is still uncertain (Gray, 1977; Tauc, 1982) and it is possible, as suggested by Hartschuh and Weihe (1980), that the dense-cored vesicles of the Merkel cell function as synaptic vesicles. In cat motoneurons several different types of synapse have been described (Conradi, 1969). It may therefore be expected that in a unique receptor system such as the SAI mechanoreceptor, slight variations from the generally accepted structure of a synapse may be present.

A fundamental problem associated with the hypothesis of vesicular release of peptide transmitters from Merkel cells is the transport of transmitter substance to their active site at a rate sufficient to maintain a static response to sustained mechanical stimulation. According to Hökfelt, Johansson, Ljungdahl, Lundberg and Schultzberg (1980) there is no apparent re-uptake of peptide transmitters in nerve endings and as a consequence every single peptide molecule released has to be replaced by axonal transport. When compared with classical neurotransmitters the synthesis and storage of peptide transmitters is an inefficient and slow mechanism. Based on the measurements of Llinás (1977) that Ca^{2+} ions take, at the most, only 200μs from their time of entry into a nerve to effect the release of neurotransmitters, Parsegian (1977) calculated that little vesicular movement could occur in such a short time and that Ca^{2+} was probably acting very close to its point of entry. However when considering the response of SAI mechanoreceptors to mechanical stimulation it is the summed activity of the touch dome that is observed and

not the response of individual Merkel cell-neurite complexes. In a single cat touch dome there are approximately 50 Merkel cell-neurite complexes. It is quite conceivable, if not probable, that activity in the afferent fibre may be initiated by transmitter release from only a proportion of the Merkel cells present in the touch dome at any one time. As some Merkel cells fail through transmitter depletion, others start to function as more transmitter substance becomes available for release. Using this hypothesis Merkel cells would remain inactive for the time that it takes for sufficient vesicles to pass from the Golgi region to their site of activity. If only one Merkel cell-neurite complex could be stimulated then it would respond to mechanical stimulation with a regular discharge the frequency of which would be determined by the time taken for sufficient new transmitter to be synthesized and transported to its active site. Horch et al. (1974), by using small diameter probes, stimulated small areas of touch domes and found that the smaller the portion of the dome stimulated the more regular was the response. They also confirmed the earlier findings of Iggo and Muir (1969) that the response of a touch dome to sustained mechanical stimulation fitted an exponential pattern for long intervals(>45ms) but failed to do so for shorter intervals. Horch et al. (1973) suggested that some mechanism suppressed the number of shorter intervals and that such a mechanism was possibly a recovery cycle. It is possible that this recovery cycle is that described above which is the time taken for

neurotransmitter synthesis and transport to its active site within the Merkel cell.

GENERAL CONCLUSIONS

From previous work it has been shown that Merkel cells are necessary for the characteristic electrophysiological response of SAI mechanoreceptors to mechanical stimulation. The work presented in this thesis indicates that this response is an O_2 dependent process - in the absence of O_2 the response eventually fails with a coincident depletion in the number of dense-cored vesicles present within the Merkel cell. The calcium channel blockers, cobalt and verapamil hydrochloride also produce receptor failure which is correlated to a reduction in dense-cored vesicles.

In both of the above experimental conditions, direct electrical stimulation of the afferent fibre still produces an action potential within the fibre showing that the nerve is still functional. This suggests that SAI mechanoreceptor failure is as a result of vesicle depletion from the Merkel cells, supporting the concept that touch domes respond to sustained mechanical stimulation by the secretion of a neurotransmitter from the Merkel cell. This hypothesis is strengthened when taken in conjunction with the recent evidence that Merkel cells appear to contain the neurotransmitters met-enkephalin and VIP. It still remains to be shown, however, if these substances are contained within the vesicles and if so, that they are responsible for the transduction process.

It is possible that the experimental approach used

in this work not only affected the Merkel cell but also the adjacent nerve ending. As stated previously in chapter 3, the resolution of this problem awaits an experimental procedure which will, in some way, allow electrophysiological separation of the Merkel cell and nerve ending.

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ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I would like to thank my supervisors Professor A. Iggo and Dr E.J. Cooksey for their guidance and assistance throughout the past 3 years.

Grateful thanks are also due to Colin Warwick and Ian Mackay for their patient assistance and advice in the production of figures for this thesis and for all other art work that I have required in the past and to Lilian Manson and my wife for their efficient typing of the script.

Sincere thanks are also due to my wife and children for their understanding, support and encouragement throughout these past few years.

APPENDIX

APPENDIX

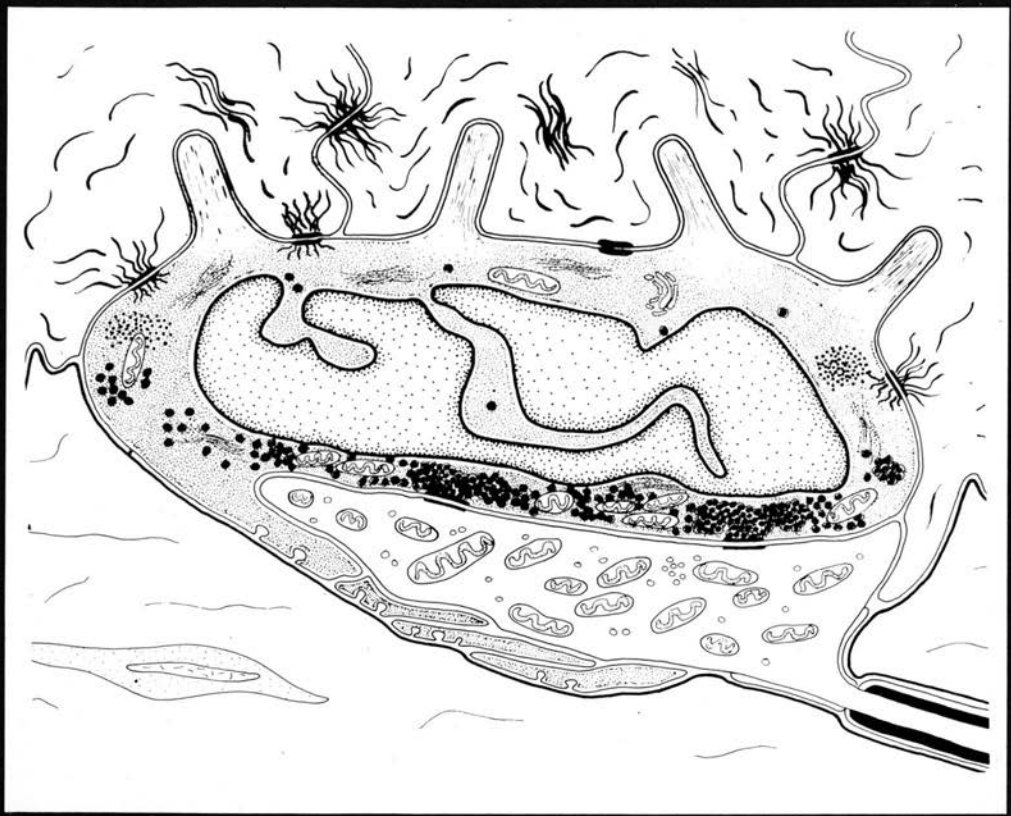
Some of the work reported in this thesis has been published in:

Sensory Receptor Mechanisms. ed. Hammann,W. & Iggo,A. pp117-131. Singapore:World Scientific.

and communicated to meetings of the Physiological Society in September 1983 and July 1984.

SENSORY RECEPTOR MECHANISMS

Edited by: W Hamann & A Iggo



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Correction

Page 126 FIGURE 4(B): Graph of the response of an SAI mechanoreceptor to repeated mechanical stimulation after the onset of hypoxia, caused by ventilation of the anaesthetised cat with N_2. The graph shows the effect of exposing the surface of the limb to atmospheres of N_2 and O_2, a result similar to that previously reported by Anand *et al.* (1979). Similar results were obtained when the arrangement shown in (A) was used to make the limb hypoxic. The inset table shows the arterial PO_2 and PCO_2 levels in control and hypoxic conditions using the latter arrangement.

SENSORY RECEPTOR MECHANISMS
edited by W. Hamann & A. Iggo

A REVIEW OF MERKEL CELL MECHANISMS

A. Iggo and G.S. Findlater

Department of Physiology
Royal (Dick) School of Veterinary Studies
University of Edinburgh
Edinburgh EH9 1QH
U.K.

ABSTRACT

The various roles ascribed to the Merkel cell in the Merkel cell-neurite complexes of mammals are reviewed. This complex, in whatever receptor system it is found, is always associated with a slowly adapting response to sustained mechanical stimulation. Embryological and degeneration/regeneration studies are considered but the results are found to be contradictory. Analysis of the discharge pattern produced by mechanical stimulation of the receptor suggests that it could be produced by chemosynaptic transmission across, what appear to be, synaptic-type junctions. The presence of numerous granular vesicles in the Merkel cell adjacent to the nerve terminal plus recent evidence that Merkel cells in some species show an immunoreaction to VIP supports this concept. Experiments to determine the effects of hypoxia on Merkel cell-neurite complexes show that the afferent fibre still conducts during hypoxia after the response to mechanical stimulation has ceased. Associated with the loss of response under hypoxic conditions is a depletion in the number of Merkel cell granules. From the available evidence and by making comparisons between certain structural features of Merkel cells and auditory sensory hair cells, an hypothesis is presented suggesting a possible process in the Merkel cell-neurite complex.

1. INTRODUCTION

The Merkel cell in mammals is an integral component of the SAI cutaneous mechanoreceptors and is found in other slowly adapting receptors of hairy and glabrous skin. Tastzellen (touch cells) were first introduced to the scientific literature in 1875 by Merkel (1) who described specialized epidermal cells in the skin of several animal species which he investigated. These cells, which have come to be known as Merkel cells, were distinguishable from the surrounding epidermal cells by their large size and pale cytoplasm. When a neurite was found adjacent to the 'Tastzelle' the complex was called a 'Tastkörperchen' - a touch corpuscle. As the neurite frequently appeared to be expanded when adjacent to the cell it was subsequently termed a

Merkel's disc by many later authors. The terms Tastzellen and Tastkörperchen used by Merkel reflected his belief that these cells and their accompanying neurites were responsible in an unspecified way for converting physical stimuli applied to the skin to neural activity in the adjacent nerve ending. Merkel's hypothesis however did not receive universal support and as recently as 1955 Weddel et al. (2) suggested that Merkel's cells were in fact the same structures described as dendritic cells by Billingham (3) and that the Merkel's discs were artefacts produced by the histological methods used by earlier workers.

Since then the dispute over the reality or fiction of Merkel cell-neurite complexes has been resolved largely through the use of improved histological methods and of the electron microscope. The first EM description in 1962, was by Cauna (4) and then in 1963 by Iggo and Muir (5) in Merkel cell-neurite complexes in the touch domes or SAI mechanoreceptors of hairy skin followed by descriptions in the glabrous skin of the opossum snout by Munger (6) and in the outer root sheath of sinus hair follicles by Andres (7). In 1963, using the method of single unit recording, Iggo (8) demonstrated a morpho-functional correlation between the Merkel cell-neurite complexes found in 'touch spots' or cat skin and the activity produced in the afferent fibres innervating these 'touch spots'. From the responses obtained these touch spots were classed as slowly adapting mechanoreceptors. Although prior to this, slowly adapting responses had been obtained from afferent fibres, originally by Adrian and Zotterman (9), and were later associated with mechanically sensitive spots on the skin, no attempt had been made to identify the underlying structures responsible (10, 11). The findings of Iggo (8) were confirmed by Tapper (12) and in 1969, Iggo and Muir (13) published a comprehensive report in which they described the unique structure of the receptor, now called a SAI mechanoreceptor, with its equally distinctive physiological response. In other locations where Merkel cell-neurite complexes were found, slowly adapting responses were obtained (14, 15) and it is now generally accepted that Merkel cells underlie the characteristic responses of SAI mechanoreceptors as a class.

The functional role of the Merkel cell in the Merkel cell-neurite complex has been the focus of attention for some time and several hypotheses, based largely on morphological information, have been proposed. Some investigators have suggested, as did Merkel (1), that it is a primary receptor cell (16) responding to mechanical deformation by the secretion of a chemical transmitter (7, 13, 17, 18). An alternative view is that Merkel cell-neurite complexes are abutments making mechanical stimuli efficient in deforming the mechanosensitive nerve ending (19, 20).

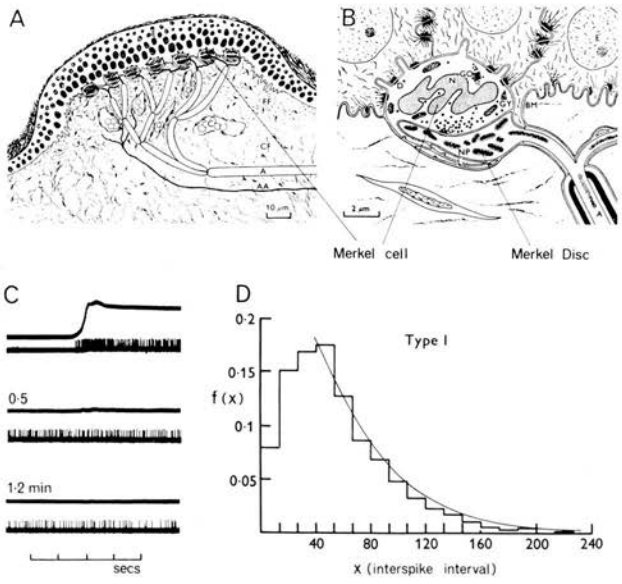

FIGURE 1. Slowly adapting Type 1 (SAI) mechanoreceptor. (A), A diagrammatic cross section of the receptor showing the myelinated axon branching in the dermis to end in Merkel cell-neurite complexes at the base of the epidermis. (B), Detailed schematic diagram of a single Merkel cell-neurite complex. (C), Discharge of afferent impulses (lower traces) from a SAI mechanoreceptor during prolonged indentation (upper traces) of the skin. (D), Interspike interval distribution of the adapted discharge of an SAI unit showing the characteristics Poisson-like frequency distribution. [(A), (B), (D) from Iggo and Muir, 1960; (C) from Iggo, 1963]

2. Origin of Merkel Cells

By looking at investigations into the developmental origin of Merkel cells, some insight into their function may be gained. There is however no agreement in the literature on this point. Winkelmann (21) by drawing comparisons between Merkel cells and cells of the APUD system (22) believed them to be neural crest migrants to the epithelium. This is a view supported by Breathnach (23) and Hashimoto (24) who have both found Merkel cells present within the dermis and passing into the epidermis in tissue from human foetuses. At no time did these cells ever resemble keratinocytes (23). English (25, 26) on the other hand described cells in the epidermis of cats and rats which were transi-

tional in appearance between Merkel cells and keratinocytes. Lyne and Hollis (27) found Merkel cells present within sheep epidermis during foetal development but only in the oldest foetuses were these cells associated with neurites. Because the Merkel cells had desmosomal contact with adjacent epidermal cells, they concluded that they were modified epidermal cells. Kasprzak et al. (28) describe a reverse order to this of receptor development in SAI receptors. In newborn kittens mechanically sensitive spots were innervated by type I afferent fibres even though very few Merkel cells were present. The Merkel cells appeared after neural innervation had occurred. This supports the findings of Symonowicz (29) who suggested that Merkel cells differentiated from epithelial cells after the arrival of nerve fibres. From this brief review it is evident that uncertainty still surrounds the cellular origin of Merkel cells, and a more detailed account is given by K.B. English in this symposium proceedings.

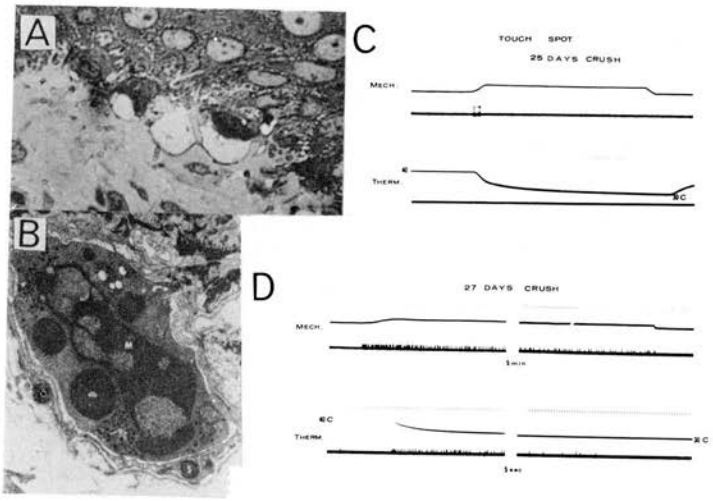

FIGURE 2. Degeneration/Regeneration experiments. (A), Four days after nerve crush. Light micrograph of the dermal-epidermal junction in a SAI receptor. The Merkel cells (M) arranged along the base of the epidermis show degenerative changes. (B), 25 days after nerve transection. Electron micrograph of denervated Merkel cell (M). The cytoplasm is dark and contains large dense bodies (db). The Schwann cell (s) remains after the nerve has degenerated. (C), 25 days after nerve crush. Mechanical stimulation and cooling of the receptor fails to produce the characteristic responses normally obtained. (D), 27 days after nerve crush. Responses to both mechanical stimulation and cooling of the receptor have returned. [(A), (B), (D) from Brown and Iggo, unpublished; (B) from English; 1977]

3 Degeneration/Regeneration

Regardless of the precise function of Merkel cells in Merkel cell-neurite complexes, they are essential for the characteristic slowly adapting response obtained on mechanical stimulation of these complexes (30). After nerve crush, the SAI mechanoreceptor with its associated Merkel cell-neurite complexes showed degenerative changes. At various stages of regeneration distal to the crush, mechanical stimulation could still produce nonspecific responses from the tips of the regenerating nerve. It was only when new fibres reformed Merkel cell-neurite complexes that the typical slowly adapting response was obtained. This conclusion was supported by the experiments of Kasprzak et al. (28) who report similar physiological results in developing slowly adapting receptors of new born kittens. It was only when numerous Merkel cells appeared in the epidermis that the receptor developed a capacity for sustained response to a constant mechanical indentation. Denervation experiments in other sensory receptor systems have produced similar effects, both morphologically and physiologically. Transection of the glossopharyngeal nerve in rats produced a generalized degeneration of the gustatory epithelium including the taste buds. Regeneration of the nerve was followed by the reappearance of taste buds (31). In the carotid body, osmosensory activity was lost after nerve crush. With time however, the response returned and ultrastructural examination revealed that the reappearance of nerve endings adjacent to the glomus-sustentacular cell complex coincided in time with re-establishment of chemosensory activity (32). This result suggested that the regenerating nerve tips within the carotid body were unable to respond to chemical stimuli before contacting the glomus cells. The dependence of Merkel cell integrity upon neural innervation has been investigated by Burgess et al. (33) and English (34) who found a progressive degeneration of Merkel cells and epithelial cells of SAI receptors in cats following denervation. It was assumed that the type I afferent fibres were the 'trophic' neurones as they were juxtaposed to the dermal aspect of the Merkel cell. An effect similar to that of nerve transection can be produced by colchicine or vinblastine; - Merkel cells are decreased in number and show degenerative changes interpreted by Chelyshev and Vinter (35) as evidence suggesting that axonal blockade prevents the secretion of trophic chemical factors from the nerve terminal which are necessary to maintain Merkel cell integrity. Hartschuh and Weihe (36) however found that nerve transection in the cat had no effect on the number of Merkel cells present in SAI receptors or sinus hair follicles or on the ultrastructure or these cells regardless of the survival time. They concluded that there was no evidence to indicate that Merkel cells in cats were more dependent upon their sensory innervation for their morphological integrity than Merkel cells in rats (37). In a study on the reinnervation of receptors, Burgess et al. (33) showed that both crushed and transected type I nerve fibres regenerated preferentially to old receptor sites. In experiments to determine why this should be, Horch (38) found that the reappearance

of type I receptors at old receptor sites following nerve transection appeared to be primarily due to intrinsic properties of the receptor sites rather than to guidance of regenerating axons along Schwann tubes in the distal stump. This result suggests that Merkel cells may in fact be acting as target cells for afferent fibres, an hypothesis proposed by Scott et al. (39).

4 Analysis of Discharge Pattern

A feature of the adapted discharge of the SAI mechanoreceptor is an exponential distribution of impulses, which would arise if the impulses were generated at sites that were independent of each other (13). The structure of the Merkel cell-neurite complex is consistent with the concept of separate generators - each Merkel cell is innervated by the single expanded nerve ending of a branch of the main axon which is myelinated to within a few microns of the Merkel cell. Horch et al. (17), on the assumption that SAI receptors did in fact contain multiple impulse generating sites, proposed two models based on the morphological characteristics of the receptor. One model assumed that each Merkel cell-neurite complex acted as an independent oscillator which generated a regular discharge and that the irregular discharge of SAI receptors arose from a mixing of regular discharge patterns. The main assumption in this model was the independence of individual oscillators; - impulses produced at one generator site did not influence the generation process of the other oscillator sites. In the alternative model each terminal impulse-generating site was assumed to have an irregular discharge. Reset of one generating site by another may or may not occur. It was suggested in this model that the irregularity of the discharge generated by each terminal could result from variability in transmitter release by the Merkel cells. From investigations carried out to test these models, it was found that the second model - the irregular oscillator model - best described the characteristic discharge pattern of the SAI mechanoreceptors. Horch et al. (17) concluded that the most likely cause of such a discharge pattern was transmitter release from the Merkel cell. They also suggested that the unique relationship between Merkel cells and the adjacent expanded nerve terminal was functionally related to the equally distinctive discharge pattern of SAI mechanoreceptors. Gottschaldt and Vahle-Hinz (20) disputed this idea of transmitter release from Merkel cells on the grounds that Merkel cell-neurite complexes in the sinus hair follicle of the cat were able to follow, in a 1:1 relationship, vibration frequencies of up to 1500 Hz. This they argued was far too fast for chemosynaptic transmission to occur. They also determined the receptor delay by mechanically stimulating the sinus hair shaft and electrically stimulating the afferent nerve fibre and found this to be about 0.3 ms. Again this was believed to be too fast for chemo-synaptic transmission to occur. This argument is thus related to the speed with which synaptic transmission occurs. In a quite different system, the mammalian neuromuscular junction, the interval between the maximum rise of the intracellular spike potential and the beginning of transmitter release was in the region of 0.2 ms (40). This finding was later

confirmed by Llinas (41). By the use of an improved voltage clamp technique, he found that the time elapsed between Ca^{2+} entry in a stimulated terminal of the squid giant synapse and the start of the postsynaptic potential could be as short as 200 μs. This is less time than the time proposed by Gottschaldt and Vahle-Hinz (20) as being too short for chemosynaptic transmission to occur, so that their assertion of non-involvement of Merkel cells in transduction loses force.

5 Neurosecretion

Ultrastructurally the Merkel cell and its adjacent sensory fibre have features suggestive of a neurosecretory function. In particular they contain numerous osmophilic granules which are most abundant between the nucleus of the Merkel cell and the subjacent expanded nerve terminal. These granules are sometimes found to be concentrated at closely apposed specialized regions of the Merkel cell membrane (7, 13). Chen et al. (42) described in detail these junctions and the fusion of the Merkel cell granules with these areas of Merkel cell membrane. These results have been confirmed by Smith (19), Mihara et al. (43) and Hartschuh and Weihe (18). This contrasts with the observations of other authors who found no evidence suggesting synaptic junctions between the Merkel cell and the adjacent nerve terminal (6, 21, 24, 44). The presence of these structures in several different species of animal led Hartschuh and Weihe (18) to suggest that the methodology used in the preparation of the tissue for electron microscopy was critical if these junctions were to be visualized. Smith (19), who had earlier reported a lack of membrane specializations (44) stated on the basis of new evidence that there was no doubting the existence of such specialized junctions and that the appearance of Merkel cell granules fusing with these regions was evidence of synaptic transmission. A characteristic not present at the presynaptic membrane of the Merkel cell, and which is generally regarded as a main feature of synapses, are clear synaptic vesicles. However, the functional significance of clear synaptic vesicles is still uncertain (45, 46) and it is possible as suggested by Hartschuh and Weihe (18) that the Merkel cell granules function as synaptic vesicles. Munger (6) found Merkel cells to be PAS positive particularly on the dermal aspect of the nucleus, suggesting the presence of carbohydrates whereas Smith (19) reported them to be PAS negative, and suggested that the positive reaction found by Munger was to the glycogen stores within the neurite terminal rather than a reaction to the Merkel cell granules. However the PAS positivity observed by Munger was diastase resistant and therefore unlikely to have been produced by glycogen wherever the reaction occurred. More recently Hartschuh et al. (47) demonstrated, at the light microscopic level, a met-enkephalin-like immunoreactivity in the Merkel cells of rats. As the strongest immunoreaction was observed in those parts of the Merkel cell with the highest granule density, they speculated that the granules were the site of the met-enkephalin immunoreaction. This, they suggested, supported the concept that the Merkel cell was a member of the paraneuronal cell system and therefore a potential neuroreceptive cell. In an experiment to test the hypothesis that met-enkephalin acted

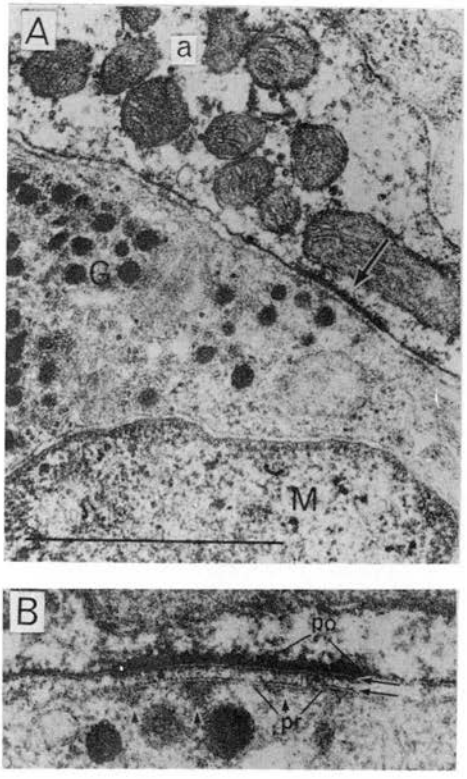

FIGURE 3. (A), Electron micrograph of part of a Merkel cell and Merkel disc, showing the stained membranes of the Merkel cell (M) and the axon (a). The arrow indicates the densely stained postsynaptic membrane of the synaptic junction. (B), High power micrograph of the synapse in 3(A). Presynaptic (pr) and postsynaptic (po) membranes are indicated. Arrowheads point to dense projections at the presynaptic membrane. (From Hartschuh and Weihe, 1980).

as a neurotransmitter, Gottschaldt and Vahle-Hinz (48) made an intravenous injection of naloxone, a known antagonist of met-enkephalin, while mechanically stimulating the sinus hair follicles of the cat. Irrespective of the dose administered, there was no alteration in the response obtained from the type I afferent unit to sustained stimulation from which they concluded that Merkel cells did not function as neuroreceptor cells. The point to be noted here is that Gottschaldt and Vahle-Hinz (48) carried out their investigation in a cat whereas Hartschuh et al. (47) used rats. In an update of their previous work,

Hartschuh et al. (in press) now report that the met-enkephalin immunoreaction was restricted to the Merkel cells of rodents and this reaction could not be demonstrated in Merkel cells of cat, dog, pig or man. This might explain the negative results obtained by Gottschaldt and Vahle-Hinz (48) as they used naloxone in the cat, a species in which it has now been shown the Merkel cell does not exhibit an immunoreaction to met-enkephalin. H.O. Handwerker (unpublished observation) has however obtained the same negative result in rats. Hartschuh et al. (in press) found in all the species investigated except rodents that Merkel cells in different locations were VIP-immunoreactive whereas the associated sensory nerve endings gave no such reaction. They suggest that the met-enkephalin-like' material shown to exist in the Merkel cells of rodents is a characteristic of those species only and possibly indicates a separate evolutionary line. Now that a VIP-like substance has been demonstrated in Merkel cells, it remains to be demonstrated what effects this substance has on the response to standard mechanical stimulation of mechanoreceptors containing Merkel cells when it is applied topically or inter-arterially. Smith and Creech (49) found no drugs that could produce spontaneous action potentials in the afferent fibre innervating SAI mechanoreceptors. The response to standard mechanical stimulation was transiently increased by nicotine before the receptor was finally blocked. Lobeline also blocked the response but never caused stimulation. When extracts from SAI mechanoreceptors of the cat were injected into other similar receptors, they did not change the response to touch nor did they produce spontaneous action potentials (19). Iggo and Muir (13) treated some animals for several days prior to recording electrophysiologically from them with sufficient reserpine to deplete catecholamine stores. Again they failed to modify the behaviour of the receptors.

In a different approach to determine the involvement of the osmophilic granules in the transduction process, Anand et al. (50) tested the effects of extreme hypoxia on granule number and distribution and on the response of receptors to standard mechanical stimuli. Cats were ventilated with 99.9% N_2 while at the same time N_2 was passed over with limb containing the SAI mechanoreceptor which was being mechanically stimulated. Immediately upon receptor exhaustion the limb was rapidly perfused with fixative. Subsequent histological examination of the SAI receptor revealed an almost total loss of granules from the Merkel cells. As the author's point out, this result shows a lability of the granular vesicles but does not however, resolve the role of Merkel cells in the transduction process. In an extension of this work Anand et al. (unpublished) found that the effects of hypoxia were reversed by replacing the N_2 around the limb with O_2. When this was done the response returned in less than 30s. Once the response to mechanical stimulation had returned to 'normal', N_2 was reapplied to the limb and the response quickly faded again. This process could be repeated several times for up to 30 minutes after circulatory arrest.

This work has been repeated in our laboratory in a preparation in

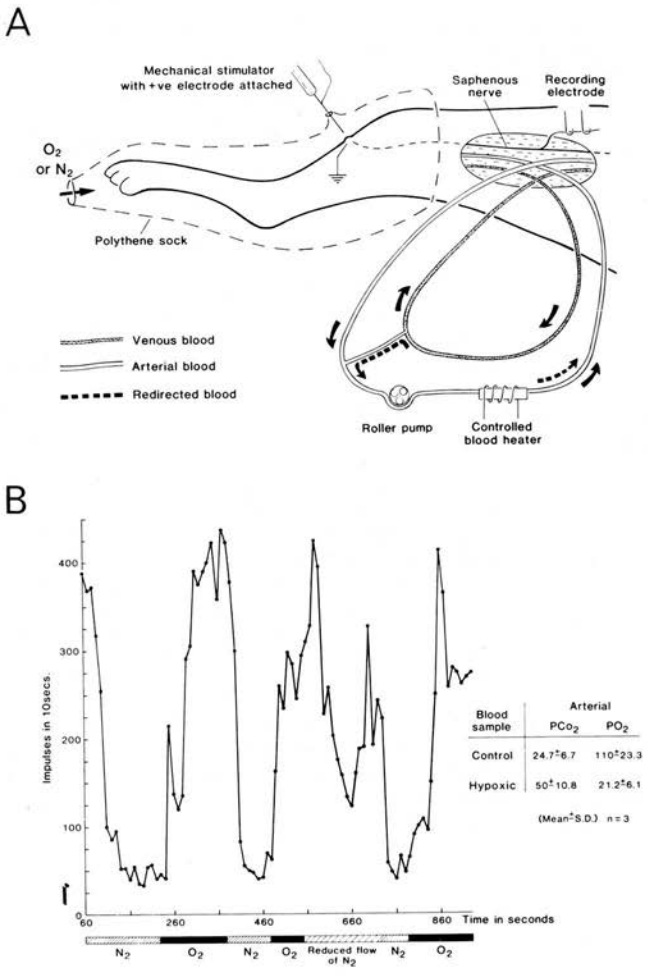

FIGURE 4. (A), Schematic diagram of the preparation used to make the limb hypoxic. Venous blood is redirected into the arterial circulation perfusing the limb while N_2 is made to pass over the limb within the polythene sock. Also shown is the arrangement used to stimulate the receptor both mechanically and electrically. (B), Graph of the response to continuous mechanical stimulation against the time after venous blood enters the arterial system. The duration of each application of N_2 and O_2 into the polythene sock around the limb is shown below

the abscissa. The table gives arterial pO_2 and pCO_2 levels in control and hypoxic conditions.

which the limb and its associated SAI mechanoreceptors can be made reversibly hypoxic. This is achieved by occluding the femoral artery and redirecting the venous femoral blood to the femoral artery below the occlusion point. Blood pO_2 and pCO_2 levels in the closed loop circuit are measured periodically throughout the procedure to determine the degree of hypoxia present. N_2 or O_2 is then made to flow through a polythene sock encasing the limb. Reversal of the hypoxic condition is obtained by redirecting arterial blood back down to the limb. Using this method, similar results to those of Anand et al. (unpublished) have been obtained. Under hypoxic conditions we have also induced, by electrical stimulation, repetitive propagated potentials in the afferent fibre through the probe of the mechanical stimulator as described by Lindbolm and Tapper (51). Under these conditions, although the receptor becomes exhausted and fails to respond to mechanical stimulation, the nerve fibre can still (20) be excited electrically and can conduct propagated action potential at high frequency. Thresholds for electrical stimulation and conduction velocities are unaltered between the control or 'normal' conditons and the hypoxic conditions. This result, when interpreted in conjunction with the loss of granules from the stimulated Merkel cells suggests that hypoxia has its greatest and most rapid effect on the Merkel cells. This possibly indicates that the transduction in the Merkel cell-neurite complex is an O_2-sensitive process. Even though arterial pO_2 levels are very low (12mm Hg), when O_2 is applied to skin containing the receptor, sufficient O_2 diffuses through the skin to maintain the ability of the Merkel cell to respond to mechanical stimulation. This could be expected in a sensory receptor cell in which there must be a rapid uptake of neurotransmitter precursors, synthesis of the transmitter substance, its subsequent storage and eventual release. In slowly adapting receptors such as Merkel cell-neurite complexes, this process has to be maintained for some time, hence their sensitivity to O_2.

If Merkel cells do secrete a neurotransmitter in response to mechanical stimulation, in what way might this process be initiated and what might be its function? Although the following is speculative, it is based on what is known about the morphology and physiology of the Merkel cell-neurite complex and by comparison of this receptor system with other well studied systems. Morphologically, certain comparisons can be made between the Merkel cell-neurite complex and the hair cell arrangement in the mammalian auditory system. The superficial half of the Merkel cell is attached to the overlying epidermal cells by desmosomes. Rod-like cylindrical cytoplasmic process from the Merkel cell fill corresponding indentations in adjacent epidermal cells and are distributed randomly over the superficial half of the Merkel cell. These processes are not attached by desmosomal contact to the epidermal cells. An analogy can be made between the processes of the Merkel cell and the sensory hairs or stereocilia of the mammalian auditory receptor

cells. These sensory hairs are tubular projections of the cell membrane arising from the cell surface. Bending of the sensory hairs is brought about by displacement of auxiliary sensory structures to which the tips of the hairs are attached. The displacement of the sensory hairs is the first step in the excitation process in the receptor cell. It is now known that the fine filaments running down the length of each stereocilium are composed of actin (52). Similar filaments, found within the Merkel cell processes, project up into the epidermis. Deformation applied to the skin overlying the Merkel cell-neurite complex could be expected to cause these processes to bend in a similar manner to that of the stereocilia of the auditory receptor cells. The simplest transduction process to visualize is one in which bending of the Merkel cell processes causes stretching or compression of the cell membrane. Assuming Ca^{2+} channels to be present in the cell membrane, alterations in the tension across the cell membrane may alter the structural configuration of these channels allowing the entry of Ca^{2+} into the cell. This in turn would cause the release of neurotransmitter from the granules of the Merkel cells producing a graded depolarization of the adjacent nerve terminal. With sufficient stimulation, transmitter release would be such that an action potential would be initiated in the afferent fibre. It is our intention to test out various aspects of this hypothesis using the preparation described above. Various substances can be introduced to the local circulation of the limb and their effects on normal and exhausted receptors tested. One obvious substance is VIP which, as stated earlier, has been shown to be present in Merkel cells (Hartschuh et al., in press). The role of Ca^{2+} in the transduction process may also be determined by the infusion of Ca^{2+} channel blockers or chelators into the limb circulation to see if the response to mechanical stimulation is modified in any way.

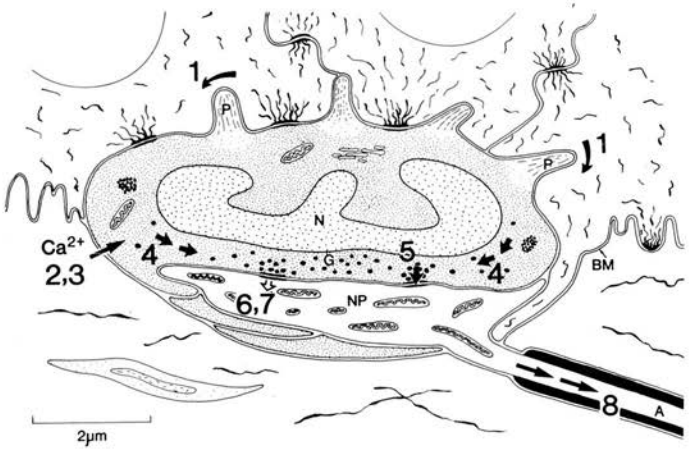

FIGURE 5. Merkel cell-neurite complex showing the suggested sequence of events in the proposed transduction process. See text for details.

P-cytoplasmic processes; N-Merkel cell nucleus; G-granular vesicles; BM-basement membrane; A-myelinated axon.

In conclusion, the following sequence of events during natural stimulation of an SAI receptor is postulated and summarized in figure 5.

1) Mechanical distortion of filamentous rods of Merkel cells, 2) alteration of membrane permeability of epidermal surface of Merkel cell leading to, 3) entry of Ca^{2+} ions into Merkel cell, 4) mobilization of osmophillic granules, 5) release of granule contents at synapse-like junction between the Merkel cell and the nerve plates, 6) alteration in permeability of nerve plate membrane leading to, 7) development of generator potential with the consequent, 8) initiation of an impulse in the myelinated afferent fibre.

The search for a functional role for the Merkel cells is sustained by the knowledge that they are a necessary (30), if not sufficient, element in the normal transduction process in the SAI afferent unit.

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[*From the Proceedings of the Physiological Society*, 20–21 *July* 1984
Journal of Physiology, **357**, 30*P*]

The responses to mechanical stimulation of SA I receptors of cats and rats in the presence of calcium antagonists

By Elizabeth J. Cooksey, G. S. Findlater and A. Iggo. *Department of Veterinary Physiology, University of Edinburgh, Edinburgh EH9 1QH*

Merkel cells of slowly adapting type I (SA I) cutaneous mechanoreceptors contain dense-cored granules in the cytoplasm adjacent to the expanded nerve ending. This has led some investigators to postulate that Merkel cells function as primary receptor cells responding to mechanical deformation by the secretion of a chemical transmitter (Iggo & Muir, 1969; Horch, Whitehorn & Burgess, 1974), or that they are abutments making mechanical stimuli efficient in deforming the mechanosensitive nerve ending (Gottschaldt & Vahle-Hinz, 1981).

As calcium is implicated in secretory processes we have used the preparation previously demonstrated to the Society (Cooksey, Findlater & Iggo, 1984) to infuse into the localized circulation of the hind limb of the chloralose-anaesthetized cat (70 mg/kg) the calcium channel antagonists, Co^{2+} or verapamil. Co^{2+} serum levels, P_{O_2} and P_{CO_2} levels of the limb circulation were periodically measured throughout the experiments. In other experiments, with normal limb circulation, Co^{2+} or verapamil were injected subcutaneously under SA I receptors in urethane-anaesthetized rats (175 mg/100 g) and chloralose-anaesthetized cats. In most experiments fine wire electrodes were inserted through the skin close to each side of the receptor for electrical stimulation of the afferent fibre innervating the receptor. At the end of each experiment the receptor was removed for histological examination.

Slow injection of Co^{2+} to give serum levels of 0·1–1·2 mM caused, within 12 min, failure of the SA I receptor to respond to mechanical stimulation. At the same time the afferent fibre still responded to electrical stimulation. Return of the general circulation to the limb led to recovery of the receptor. Subcutaneous injection of a solution containing 5 mM-Co^{2+} produced the same effects, but more quickly, and there was no recovery. Infusion of 100 μM-verapamil into the closed loop circulation reduced the response to mechanical stimulation. When injected subcutaneously with verapamil the receptor ceased to respond to mechanical stimulation but not to electrical stimulation. Ultrastructural examination of the receptors which had been stimulated in the presence of Co^{2+} or verapamil showed Merkel cells with depleted numbers of granules.

These results suggest that calcium is a requirement for the normal function of the SA I receptor. We suggest that the granules are required for normal transduction.

G. S. Findlater is a MRC scholar. Supported by the SERC.

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[*From the Proceedings of the Physiological Society*, 23–24 *September* 1983
Journal of Physiology, **346**, 14*P*]

A limb-perfusion preparation allowing manipulation of the internal and external environment of cutaneous receptors of the cat

By Elizabeth J. Cooksey, G. S. Findlater* and A. Iggo. *Department of Veterinary Physiology, University of Edinburgh, Edinburgh EH9 1QH*

Anand, Iggo & Paintal (1979) described a method of demonstrating the lability of granular vesicles in the Merkel cells of type I slowly adapting cutaneous receptors under extreme hypoxia. This has been modified to produce a reversible local limb hypoxia and to permit the perfusion of fixative while still recording from the afferent fibre of the receptor. The preparation has been used to examine the possible transduction process between Merkel cell and nerve terminal by testing the effects of extreme hypoxia on the responses of type I S.A. receptors to mechanical stimuli and by correlating this with the number and distribution of Merkel cell vesicles.

The saphenous nerve in cats anaesthetized with chloralose was dissected out in a paraffin pool and the responses of the receptor to known fixed mechanical stimuli recorded under normal and hypoxic conditions. Local limb hypoxia was obtained by occluding the femoral artery and redirecting the venous femoral blood to the femoral artery below the occlusion point. Blood P_{O_2} and P_{CO_2} levels in the closed-loop circuit were measured to determine the degree of hypoxia. Simultaneously nitrogen at 4 l/min was made to flow through a polythene sock encasing the limb to prevent oxygen diffusion through the skin to the receptor. Under these conditions repetitive propagated potentials were induced in the afferent fibre through the probe of the mechanical stimulator as described by Lindblom & Tapper (1967). The effect of oxygen on the receptor was determined by replacement of nitrogen with oxygen within the polythene sock. Results show that under extreme hypoxia the receptor becomes exhausted, while the nerve fibre is still conducting, and this correlates with a reduction in the number of Merkel cell vesicles. Replacement of nitrogen with oxygen around the limb under hypoxic conditions resulted in a return of response to mechanical stimulation. Using this preparation chemical substances could be delivered to the skin and their effect on normal and exhausted receptors tested.

Support by a grant from the S.E.R.C.

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