FUNCTIONAL STUDIES ON VIBRISSAL SLOWLY-ADAPTING MECHANORECEPTORS

By

SENOK, Silas Solomon

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Division of Basic Medical Sciences
Department of Physiology, Faculty of Medicine,
The Chinese University of Hong Kong

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ABSTRACT

Most cells possess some degree of mechanosensitivity but some have become specialised detectors of mechanical perturbation. These, the mechanoreceptors, subserve functions from touch perception to hearing and maintenance of posture. The importance and ubiquity of mechanosensitivity notwithstanding, it remains a poorly understood physiological process. The present study examined the process of mechanosensitivity in functioning, isolated mammalian slowly adapting touch receptors. The particular receptor of interest is unique among the cutaneous mechanoreceptors in that it possesses a specialised cell (Merkel cell) to which the afferent nerve terminal is attached. The morphological features of the association are suggestive that the Merkel cells are involved in the mechanoelectric transduction process. However, this hypothesis is still controversial. The basic question that still persists is whether the Merkel cell is the mechano-sensory transducer or the apposed nerve.

To study the physiology of the mechanoreceptors, rat vibrissae (whiskers) were chosen as a model because they are versatile, highly differentiated vibrotactile sense organs which have a large number of Merkel cell receptors. To this end, an isolated preparation was developed using single vibrissal follicles which enabled electrophysiological recording of responses from single mechanoreceptors upon application of controlled mechanical displacements. Using computer programs developed in-house to control the stimulation as well as the acquisition, display and partial analysis of the data on-line, two major types of slowly adapting mechanoreceptors were found and characterised on the basis of their response characteristics.

The sinus hair type I (St I) is the Merkel cell receptor while the type II (St II) is considered to be the lanceolate endings as originally described by Gottschaldt and co-workers in cats. This is the first instance of a successful characterisation of the vibrissal slowly adapting mechanoreceptors in an isolated preparation.

Using pharmacological studies, this study reports the first findings of qualitative differences between the Merkel cell receptors and another slowly adapting mechanoreceptor (St II). Chloroquine inhibited Merkel cell receptor function while being excitatory to the St II receptors. Caffeine had a strong excitatory effect on Merkel cell receptors while inhibiting the St II. Ultrastructurally, chloroquine was shown to specifically affect the Merkel cells while sparing other nerve terminals and surrounding keratinocytes. Furthermore, the present studies provide for the first time functional evidence for a calcium induced calcium release (CICR) process in Merkel cell receptors - a process which could not be found in the St II. A modification of the current model of mechanoelectric transduction in Merkel cell receptors is thus suggested to accommodate this new step in the proposed sequence of events (Iggo & Findlater, 1984; Yamashita, *et al*, 1992).

It is concluded therefore that the process of mechanoelectric transduction in Merkel cell receptors is fundamentally different from that of another receptor (St II) which does not possess a specialised cellular attachment. The ultrastructural evidence from the chloroquine experiments suggest that the Merkel cells are responsible for the observed functional differences. These findings therefore strongly suggest that Merkel cells are the mechanoelectric transducers in this touch receptor.

Chapter 1

<u>INTRODUCTION AND OBJECTIVES</u>

Mechano-sensitivity - the ability to detect and react to mechanical stimulation - appears to be present in all living organisms. It is involved in such diverse processes as sound perception, cell volume regulation and fertilization. While certain cell types have become specialized detectors of mechanical stimulation (e.g. the cochlear hair cells of the inner ear), for many, mechanosensitivity is a house-keeping tool (e.g. osmotic swelling in red blood cells activates stretch-sensitive membrane K^+ and Cl^- channels (Hamill, 1983).

Mechanoreceptors are specialised groups of cells with the specific function of detecting and responding to a mechanical stimulus. In mammals, mechanoreceptors are either primary, where the transducer is a modified nerve ending, or secondary, where a non-nervous cell serves as the transducer. In the latter case, the afferent axon is subsequently activated synaptically as is the case in the cochlear hair cells.

In the skin and its appendages the mechanoreceptors subserve the very important function of touch-pressure (pressure is sustained touch) sensation. Most of the receptors are specialized nerve endings with or without accessory structures. Some of these nerve terminals (e.g. Pacinian corpuscles) are rapidly adapting, responding for only a short time at the onset (and often during removal) of a mechanical stimulus. They thus serve

mainly as velocity detectors and are especially adapted to respond to vibratory stimuli. Slowly adapting mechanoreceptors (e.g. Merkel nerve endings and Ruffini corpuscles), on the other hand continue to respond when an applied mechanical stimulus persists. They adapt only gradually, the extent and rate of adaptation varying between different types of receptors.

These basic functional differences were clear from the earliest electrophysiological recordings of single unit activity by Adrian and Zotterman (1926). Significant progress has been made especially in the second half of this century, with specific nerve endings being directly associated with specific functional characteristics (e.g. Adrian and Umrath, (1929) for Pacinian corpuscles; Iggo and Muir (1969) for Merkel endings (SA I) in hairy skin; and Chambers et al (1972) for Ruffini corpuscles in the skin (SA II)).

Mechano-electric transduction - the process that couples and hence converts mechanical energy into transmissible electrical nerve impulses - has however remained poorly understood. Even in the best studied, and perhaps best understood, of these receptors - Pacinian corpuscles - the precise mechanism of transduction and location of transducer sites are still being unravelled (see Bell et al., 1994 and Bolanowski et al., 1994 for review).

The ubiquity of mechanosensitivity in so many cellular processes notwithstanding, the mechanosensitive (MS) channels are among the least understood of membrane channels. Until fairly recently, standardised

non-destructive procedures for controlled application of mechanical stimuli to cell membranes have been in short supply (see (McBride & Hamill, 1992)). The problem is compounded by the lack of specific ligands that bind to mechanosensitive channels. Amiloride and its analogues, better known for inhibition of epithelial membrane Na^+ channels and the Na^+/H^+ antiporter, have been found to block MS channels in sensory (cochlear hair cells, Jørgensen and Ohmori, 1988; Rusch et al., 1994) and non-sensory (xenopus oocytes, Hamill *et al.* 1992) cells at relatively high concentrations. Unfortunately, amiloride, at such high concentrations, can affect so many other cellular processes that its use in functioning sensory preparations is limited since the data are often difficult to interpret. The precise mechanism of gating of the mechanosensitive channels has therefore remained within the realms of conjecture. The recent cloning of a bacterial mechanosensitive channel (Sukharev et al., 1994) would certainly appear to be a step in the right direction.

The situation is even less clear in the slowly adapting mechanoreceptors (the focus of the present study), especially in the Merkel cell terminal (called SA I in the skin, Iggo and Muir, 1969; St I in the sinus hair, Gottschaldt et al., 1973; and Ft I in frog skin, Yamashita et al., 1986), where there is enduring controversy regarding the function of the specialised cell with which the afferent terminal makes contact. All other sensory mechanoreceptors in mammalian skin and appendages consist basically of a specialised nerve terminal which serves as the mechano-electric transducer, with or without accessory structures which

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may modify the impinging mechanical stimulus (e.g. the onion-like lamellae of Pacinian corpuscles serve as 'high pass' mechanical filters).

While a lot is known about the morphology of the Merkel cell receptors, direct evidence for their possible mechanotransductive role in the receptor has not been as forthcoming. Among other reasons, two major difficulties may be identified to account for this tardiness in establishing a specific functional role for Merkel cells.

◆ a).   The receptors are small and inaccessible. Merkel cells are located in the epidermo-dermal border of the skin, with the layers of the epidermis above and the dermis below making them virtually inaccessible to direct investigation by conventional electrophysiological techniques in their native location; a microelectrode tip would be broken by such tough intervening tissues. Merkel cells are also small, measuring about 7 - 10µm in length and are transparent, further compounding the problem of accessibility since they cannot be easily visualized.

◆ b).   It would appear that the shape of the cells - and possibly their intercellular connections as well - are important for normal mechanosensitivity. Merkel cells in the skin send cytoplasmic extensions into the layer of keratinocytes with which they establish desmosomal junctions, while in sinus hairs Merkel cells in the external root sheath send cytoplasmic extensions into the glassy membrane and also form desmosomal junctions with the overlying keratinocytes.

Although Merkel cells have been isolated as single cells (see Nurse and Cooper, 1988; Yamashita et al., 1992) using enzymatic digestion and mechanical dissociation. Isolated Merkel cells fail to retain their natural ovoid shape but become spherical (see Yamashita et al., 1992). The inability to demonstrate mechanosensitivity in these isolated Merkel cells, apart from the question of their viability, has been attributed largely to the shape change (Yamashita et al., 1992).

Sinus hairs contain an abundance of Merkel cells. As many as 2000 cells may be found in one sinus hair follicle, depending on animal species and size of hair follicle (Halata, 1990). The sinus hair thus affords a good model for studying the function of Merkel cells.

OBJECTIVES OF STUDY

The principal objectives of the present study are therefore as outlined below:

◆ a)   To develop a viable isolated vibrissal preparation to enable functional studies on slowly adapting mechanoreceptors. This has the advantage that the receptors are still *in situ* and at the same time eliminating the numerous confounding factors and limitations of *in vivo* experiments. Furthermore, it was considered, based on the location of the receptors in vibrissae (see full description of structure in the next

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chapter), diffusion of superfused test substances was likely to be superior to previous isolated preparations using the skin-nerve preparation (Reeh, 1986), where the receptors are also relatively sparse.

◆ **b)** To further functionally characterise the slowly adapting (SA) vibrissal mechanoreceptors, enabling the receptor types to be functionally distinguished one from the other. This is important for two reasons.

Firstly, there is disagreement in the literature regarding vibrissal slowly adapting mechanoreceptors. While most workers accede to the functional distinction of rapidly and slowly adapting terminals in vibrissae (Fitzgerald, 1940; Zucker & Welker, 1969; Hahn, 1971; Gottschaldt *et al.* 1973; Dykes, 1975; Gibson & Welker, 1983a,b; Lichtenstein *et al.* 1990; Waite & Jacquin, 1992), few have been able to functionally sub-characterise the slowly adapting mechanoreceptors into two main classes as done by Gottschaldt et al. (1973). Either the characterisation was not attempted (e.g. Lichtenstein et al., 1990; Waite and Jacquin, 1992) or attempts failed . For example Gibson and Welker (1983b) concluded that the slowly adapting mechanoreceptors could not be subcategorised on the basis of discharge regularity and even found that adaptation properties in vibrissal afferents formed a continuum from the two extremes of rapid to almost no adaptation. There is clearly a need to clarify these

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discrepancies and possibly explain why other workers have experienced these difficulties.

Secondly, and perhaps of greater importance, one needs to be able to say categorically, based on the response characteristics of the receptors under examination, what receptor is being recorded from. Failing this, the proposed vibrissal preparation will not be suitable for functionally studying the vibrissal mechanoreceptors, since there is as yet no other established procedure (e.g. pharmacological) for differentiating between the receptor types.

◆ c)   Having established (a) and (b), to use the preparation to explore the transduction mechanism in the Merkel cell receptor (named St I in vibrissae, Gottschaldt et al., 1973), with a view to shedding some light on the mechanotransductive function (or the lack thereof) of Merkel cells in St I afferents. The identification and characterisation of any other slowly adapting mechanoreceptor(s) as described by Gottschaldt et al. (1973) will be a convenient 'control' in this regard, since only St I endings have cellular attachments by way of Merkel cells.

Chapter 2

<u>LITERATURE REVIEW</u>

2.1 CLASSIFICATION OF CUTANEOUS MECHANORECEPTORS

Several attempts have been made to classify the cutaneous mechanoreceptive terminals (e.g. Iggo, 1974; Halata, 1975; Iggo & Gottschaldt, 1974; Bannister, 1976; Malinovsky, 1986). This underscores the complex morpho-functional properties of the cutaneous mechanoreceptors. It is easy to understand the difficulties encountered by early investigators like Krause and Merkel in the last century (as reviewed by Bannister (1976)) who were restricted to using the light microscope. Since the first published electron microscopical study of a cutaneous mechanoreceptor (the Pacinian corpuscle) by Pease and Quilliam (1957), most of the receptors have been described ultrastructurally.

There is however, no universally accepted method of classifying the receptors. The presence of a large array of transitional forms of the receptors, coupled with the fact that functional characterisation lagged behind morphology have made objective classification on the basis of structure and function rather difficult.

2.1.1 Morphological classification

On the basis of electron microscopical studies, Halata (1975) classified the cutaneous mechanoreceptors into three main groups, called type I, II and III respectively (see figure 2.1):

Type I: These are the epidermal nerve endings. They are characterised by the absence of Schwann cells. Receptors in this group include the free nerve endings which are found in the granular layer of the epidermis and Merkel endings which consist of an expanded nerve terminal making contact with a Merkel cell located in the basal layer of the epidermis and glandular ridges.

Type II: This group is made up of the dermally located 'bulboid nerve endings'. Generally, these receptors consist of a nerve terminal invested by lamellae of Schwann cells with no distinct perineural capsule and are located just below the basal layer of the epidermis. Receptors in this group include the palisade (lanceolate) endings around hairs, the lanceolate terminals of sinus hairs and the Meissner corpuscles in the digital skin of the monkey.

Type III: The third group of receptors, which are also dermally located differ from the second group by being encapsulated corpuscles with an inner core formed by Schwann cell lamellae. The capsule is a direct continuation of the perineurium of the afferent fibre. This group

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consists mainly of the Pacinian corpuscles and their variations including the Golgi-Mazzoni (or simple lamellated) corpuscles and Krause end bulbs.

This purely morphological classification does not take into account the functional properties of the receptors.

Endings	Structure	Localisation	Synonyms
Free nerve endings		Epidermis: stratum granulosum	
----Type I----			
Merkel nerve endings		Epidermis: stratum basale	Brown bodies Iggo corpuscles Merkel cell-neurit complex
Simple bulboid nerve endings		Dermis stratum papillare	Papillary nerve endings Free nerve endings of the dermis
----Type II----			
Dentritic bulboid nerve endings		Hair, sinus hair: mesenchymal sheath. ridged skin: stratum papillare	Hair: palisade NE SH: lanciform NE RS : Meissner's corpuscles Ruffini end-bulbs Dogiel end-bulbs Genital corpuscles
Simple encapsulated corpuscle with inner core		Dermis: below the epidermal cone	Krause end-bulbus Golgi-Mazzoni corpuscles Mucocutaneous end-organs Paciniform corpusles Innominate corpuscles
----Type III----			
Pacinian corpuscles		Deep layers of the dermis	Vater-Pacini corpuscles Rauber's end-organs

Figure 2.1 Morphological classification of mammalian cutaneous mechanoreceptors. From Halata, (1975).

2.1.2 Morpho-functional classification

Partly based on earlier suggestions by Iggo and Gottschaldt (1974) and Andres (1974), Malinovsky (1986) proposed this classification. The nerve endings were classified on the basis of their locational relation to cells of ecto-, meso-, or endodermal origin and their functional characteristics (mainly their rates of adaptation). There are also 3 groups of endings according to this classification.

Class I: Terminals are related to cells of the mesodermal origin. This group includes free subepidermal endings, arborrized nerve endings in subcutaneous tissue and Ruffini endings. Receptors of this group are functionally considered to be slowly adapting displacement detectors.

Class II: Terminals are related to cells of endodermal origin. These terminals have inhomogenous adaptation characteristics and are thus considered to be intermediate. The group is supposed to be best exemplified by the vibrissal mechanoreceptors where both slowly and rapidly adapting responses have been described (*vide infra*).

Class III: This group is made up of the Pacinian corpuscles and Herbst corpuscles which are related to cells of ectodermal origin (Schwann cells). They are rapidly adapting velocity detectors which are highly sensitive to vibration.

2.1.3 Functional classification

A detailed functional classification was put forward by Iggo (1974). The receptors are divided into two major groups of rapidly adapting and slowly adapting mechanoreceptors, with further subdivisions within.

i.   Rapidly adapting mechanoreceptors:

These are the velocity or acceleration detectors. They are further subclassified as follows:

Pacinian corpuscle: This is the most prominent member of the group which is specialised to detect high frequency mechanical disturbances and thus responds best to vibratory stimuli. (Described in greater detail below).

'Other' high frequency detectors: These include the Golgi-Mazzoni corpuscles in sinus hairs (Andres, 1966) and periosteal tissue of the jaw (Sakada & Aida, 1971). Generally, these receptors possess a tuning curve with maximum sensitivity to mechanical stimulation at frequencies of 400 - 800 Hz. It is worth noting however, these 'other' detectors are considered by other workers to be variants of Pacinian corpuscles (Andres, 1966; Stephens *et al.* 1973; Halata, 1975; Halata & Munger, 1980) and it would appear that this type of response is characteristic of lamellated corpuscles.

Hair follicle receptors: These are considered as a special subgroup of rapidly adapting receptors (Iggo & Gottschaldt, 1974). They are excited only by moving stimuli, being phasic or velocity detectors. The sinus hair receptors are not included in this category, but are considered as a special group. Brown and Iggo (1967), describe distinctive groups of receptor responses in down, guard and tylotrich hair follicles which they called **D**, **G** and **T types** respectively. The differences were based on the size and type of receptor field, mechanical sensitivity of the afferent unit and on the diameter of the afferent fibres as estimated by their conduction velocities.

Type G fibres had receptive fields of 0.5 - 6.0 cm² in the cat, are myelinated with mean conduction velocity of 54m/s (cat) and 28.9m/s (rabbit). Brown and Iggo, (1967) described two subtype of the G fibres while Burgess et al. (1968) found a G receptor with intermediate properties between the G_1 and G_2 of Iggo and Brown. T units are much fewer with receptive fields ranging between 0.25 and 6.00 cm² with larger axons than the G units (conduction velocities of 68.2m/s and 35.6m/s in cat and rat respectively). D units associated with the down hairs are the most numerous and are said to be highly sensitive and may even discharge in synchrony with the arterial pulse (Brown & Iggo, 1967). They have smaller diameter myelinated axons (conduction velocities of 19.9m/s and 9.0m/s in cat and rat respectively). It is estimated that these fibres make up the majority (up to 80%) of δ fibres in the saphenous nerve (Brown & Iggo, 1967).

Movement of hairs on the body is thus an effective stimulus for activation of cutaneous afferent fibres. Under controlled conditions, impulses are generated only during movement of a single hair or group of hairs (Brown & Iggo, 1967). By measuring the interspike intervals, Brown and Iggo (1967) showed that responses were uniform for any given velocity over limited angles of deflection and concluded that discharge frequency in these hair receptors provided unambiguous information to the brain about velocity of movement.

ii.   Slowly adapting mechanoreceptors:

These detect the amplitude of displacement rather than velocity and constitute the second major group of mammalian cutaneous mechanoreceptors. Characteristically, they discharge responses during both dynamic and static phases of mechanical displacement of skin (see figure 2.2). Two types of SAs have been identified in the skin and directly correlated with morphologic entities. These have been called SA I and SA II respectively (Iggo & Muir, 1969; Chambers *et al.* 1972).

Mech. displacement

Pacinian corpuscle

Hair follicle afferent unit

Slowly adapting Type I

Slowly adapting Type II

-200 -100 0 100 200 300 400 4 5 6

msec

Time

sec
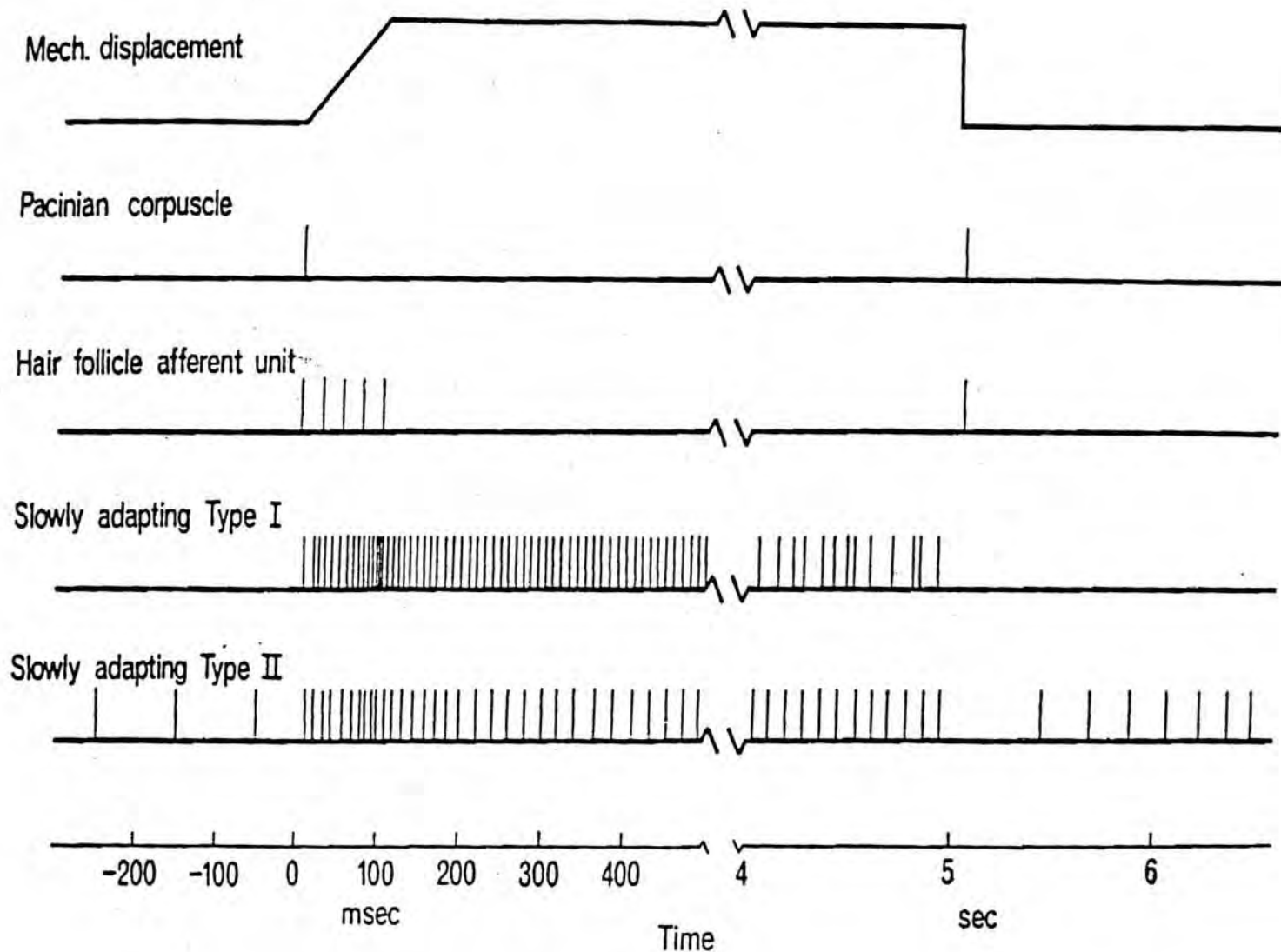
Figure 2.2

Diagramatic representation of the responses of the 4 major myelinated cutaneous mechanoreceptors in response to a sustained mechanical stimulus (uppermost trace). Pacinian corpuscles adapt very rapidly while SA II (Ruffini) endings are very slowly adapting. The rapidly adapting receptors exhibit an 'off response' when the stimulus is withdrawn while the SA I and SA II do not. Note also the spontaneous firing by the SA II (From Iggo and Gottschaldt, 1974).

Slowly adapting type I (SA I) mechanoreceptor: The characteristic responses of this receptor has been identified on little dome-like elevations of hairy skin for some time, but became fully established by Iggo (1963), hence they are often called Iggo's domes (see Iggo & Gottschaldt, 1974 for review). The small dome-like elevations are innervated by large myelinated afferent fibres. When a mechanical probe is applied to the dome, a stream of action potentials can be recorded from the afferent fibre. Typically, the adjacent skin is unresponsive nor is the receptor activated by stretching the surrounding skin.

The SA I has been directly identified as the Merkel nerve ending (Iggo & Muir, 1969). An SA I unit consists of a cluster of some 50 -100 Merkel cells in the epidermo-dermal border of the skin and its innervation. The small dome-like elevations referred to above are associated with the position of this cluster of Merkel cells. A single cluster is supplied by one large-calibre myelinated primary afferent axon which gives off terminal branches to each of the Merkel cells (see figure 2.3).

The typical response properties of the SA I include the very low threshold (as low as 1μm of displacement in the touch dome (Iggo & Gottschaldt, 1974)) of activation and the characteristically irregular discharge pattern as evinced by the variable interspike intervals (ISI) which are described as having a *Poisson* distribution (see fig 2.3). The SA I rarely has a resting discharge. Iggo and Muir (1969) attributed the irregular discharge pattern, at least in part, to the fact that with so many

Merkel cells per unit, there should be multiple spike generation sites (Horch *et al.*, 1974).

The static discharge is related to the amplitude of mechanical stimulation by a power function of the form:

$$R=kS^n \text{ (Tapper, 1965; Werner \& Mountcastle, 1965),}$$

while the discharge during dynamic stimulation is related both to amplitude and velocity of displacement (Iggo & Muir, 1969).

Slowly adapting type II (SA II) mechanoreceptor: The responses of this mechanoreceptor have been well described (Iggo, 1966; Chambers *et al.* 1972). The receptor is also excited from a single identifiable spot on the skin but it also responds to stimulation of the adjacent skin and to stretch. There are no distinct surface markings on the skin for the location of the receptor. It has been identified as the Ruffini ending (q.v.).

The SA II receptor tends to have spontaneous discharge but the most notable feature is the highly regular discharge pattern. The ISIs are regularly spaced with a normal (*Gaussian*) distribution (see figure 2.3). The stimulus-response relationship can be equally well described by a power or exponential function.
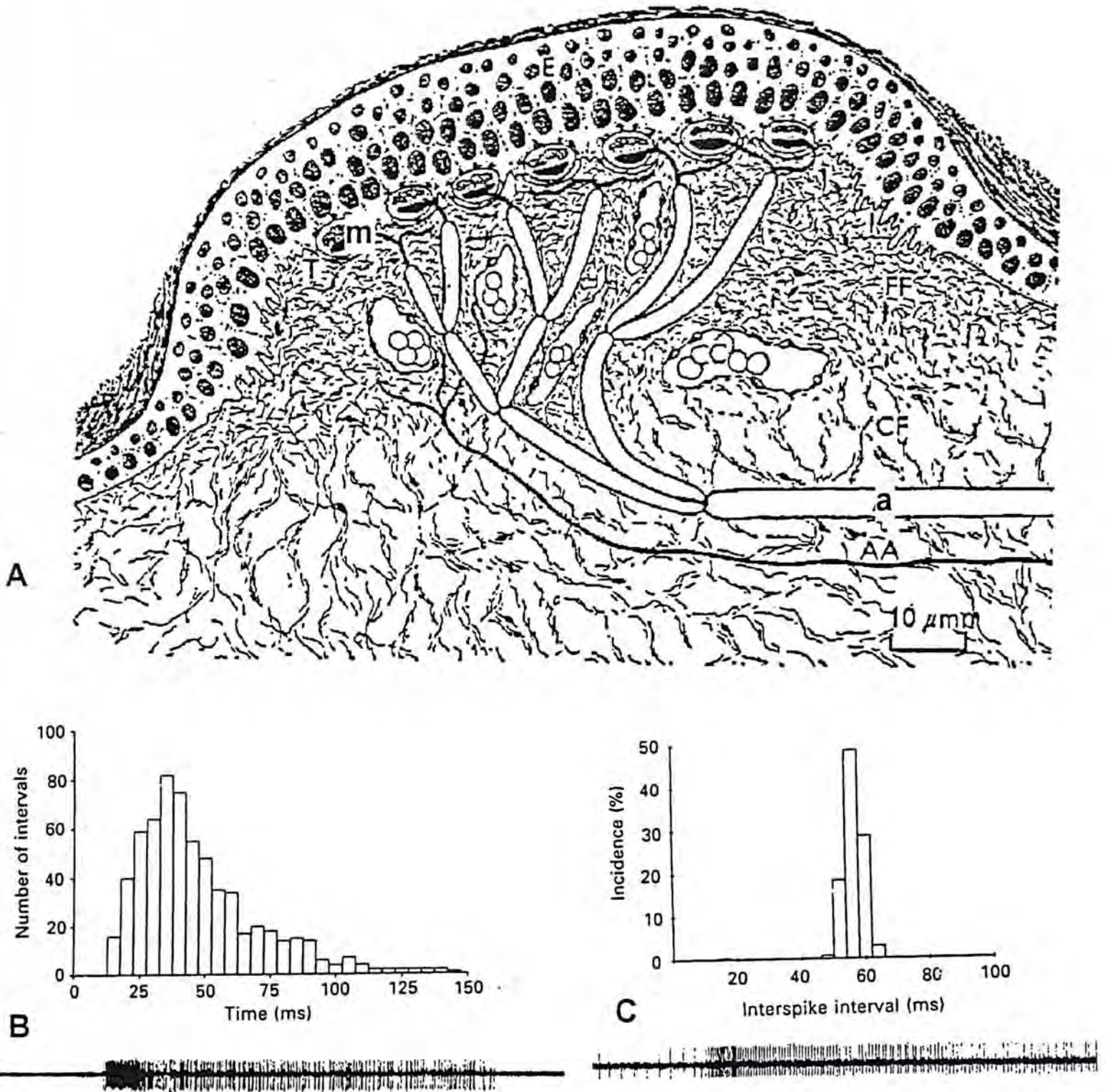
Figure 2.3

A. Diagramatic representation of a touch dome with the nerve endings and Merkel cells that constitute a single SA I unit (**a** myelinated axon; **m** Merkel cell; *from Iggo and Muir, 1969*).

B,C. SA I and SA II response characteristics compared. The discharge patterns (bottom traces) show the irregular nature of SA I (**B**) firing, with a skewed (*'Poisson'*) *interspike* interval distribution while the SA II (**C**) receptor has a regular pattern wih a normal distribution of interspike intervals (*Modified from Baumann et al., 1990*).

C-mechanoreceptors: These are cutaneous mechanoreceptors with non-myelinated afferent fibres. Up to 50% of cutaneous non-myelinated afferents supplying hairy skin end as sensitive mechanoreceptors (Iggo, 1960; Bessou *et al.* 1971). Bessou et al., (1971) found that C-mechanoreceptors had small receptive fields and required relatively prolonged contact with the skin surface before responses were elicited. Responses were slowly adapting and declined to zero within 20 - 30 seconds of steady maintenance of the stimulus (CF SA I and SA II receptors which can continue to discharge for several minutes or even hours).

C-mechanoreceptors are markedly affected by repeated stimulation and may become totally unresponsive following stroking of the skin for say, 20 seconds, requiring up to 8 minutes before further responses can be elicited (Iggo, 1960). Thus, to obtain constant responses, intervals of 3 - 4 minutes are required between stimuli (Iggo, 1960; Iggo & Kornhuber, 1968; Bessou *et al.* 1971; Hahn, 1971). Few C-mechanoreceptors have been described in glabrous skin.

There are recent suggestions that the C-mechanoreceptors may form part of a pool of 'silent' nociceptors which may be recruited in conditions of hyperalgesia (Schmelz *et al.* 1994; Schmidt *et al.* 1995).

The foregoing examples of attempts at classifying cutaneous mechanoreceptors highlight the existing confusion. The same receptors are to be found in different groups in the first two classifications (e.g.

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Ruffini endings are classified with free nerve endings in Class I (Malinovsky, 1986) but are to be found with Meissner and lanceolate endings in Halata's Type II. Though a morpho-functional classification should be ideal, Malinovsky's classification has shortcomings. By lumping the sinus hair terminals into one 'intermediate' group, this classification fails to recognise the complex, distinct sinus hair terminal (see later) which represent a unique microcosm of most of the mechanoreceptive terminals found elsewhere in the skin and its appendages, of which the sinus hairs form a part. Iggo's classification is detailed and uses function as the principal criterion; it however seems to have difficulties fitting the sinus hair receptors into any group. This was probably due to the uncertainties regarding the response properties of sinus hair mechanoreceptors.

The simplest and possibly least controversial classification would be one that looked at the mechanoreceptors on the basis of their rates of adaptation (i.e. they can be either slowly or rapidly adapting receptors) with their thresholds possibly forming a basis for sub-grouping within the two broad classes. This is what Iggo's classification has done to a great extent. Even this is limited by the fact that it may be found to hold true only for mammals since the supposed avian equivalents of Merkel cell endings called Grandry cells (Gottschaldt & Lausmann, 1974) and Merkel endings in salamander skin (Diamond et al. 1986) are rapidly adapting. It would also appear that lanceolate terminals in sinus hairs may be slowly adapting unlike their counterparts in other types of hair follicles (q.v.).

2.2 CHARACTERISTICS OF SOME MAMMALIAN CUTANEOUS MECHANORECEPTORS

The specific characteristics of some of the mechanoreceptive afferents in mammalian skin are summarised below.

2.2.1 Pacinian corpuscles

Also called Vater-Pacini, these receptors are large (may be up to 2.5 mm in diameter). Their size and accessibility makes them the most studied and possibly best understood of the receptors. Data from them have been valuable in understanding the function of receptors in general. Found in the subcutaneous layers of all types of skin, mesentery and other connective tissue, they are ovoid, cylindrical or spheroidal in shape. A corpuscle is made up of an afferent neurite surrounded by an accessory capsule formed by concentric layers of lamellae, typically described as resembling a cocktail onion (Figure 2.4). The layers of lamellae are separated by fluid-filled spaces which serve as a 'high pass' mechanical filter to impinging stimuli.

Pacinian corpuscles are rapidly adapting mechanoreceptors which respond particularly well to rapid vibratory stimuli. Bolanowski (1988) suggested that there were two types of Pacinian corpuscles, one responding best to compression and the other to decompression stimuli. Recently, two types of extensions (filopodia) have been demonstrated in

the neurite ending (Bolanowski *et al.* 1994). These were suggested to be the structural basis for the bi-directional sensitivity of Pacinian corpuscles (see Bell *et al.* 1994 for recent review).
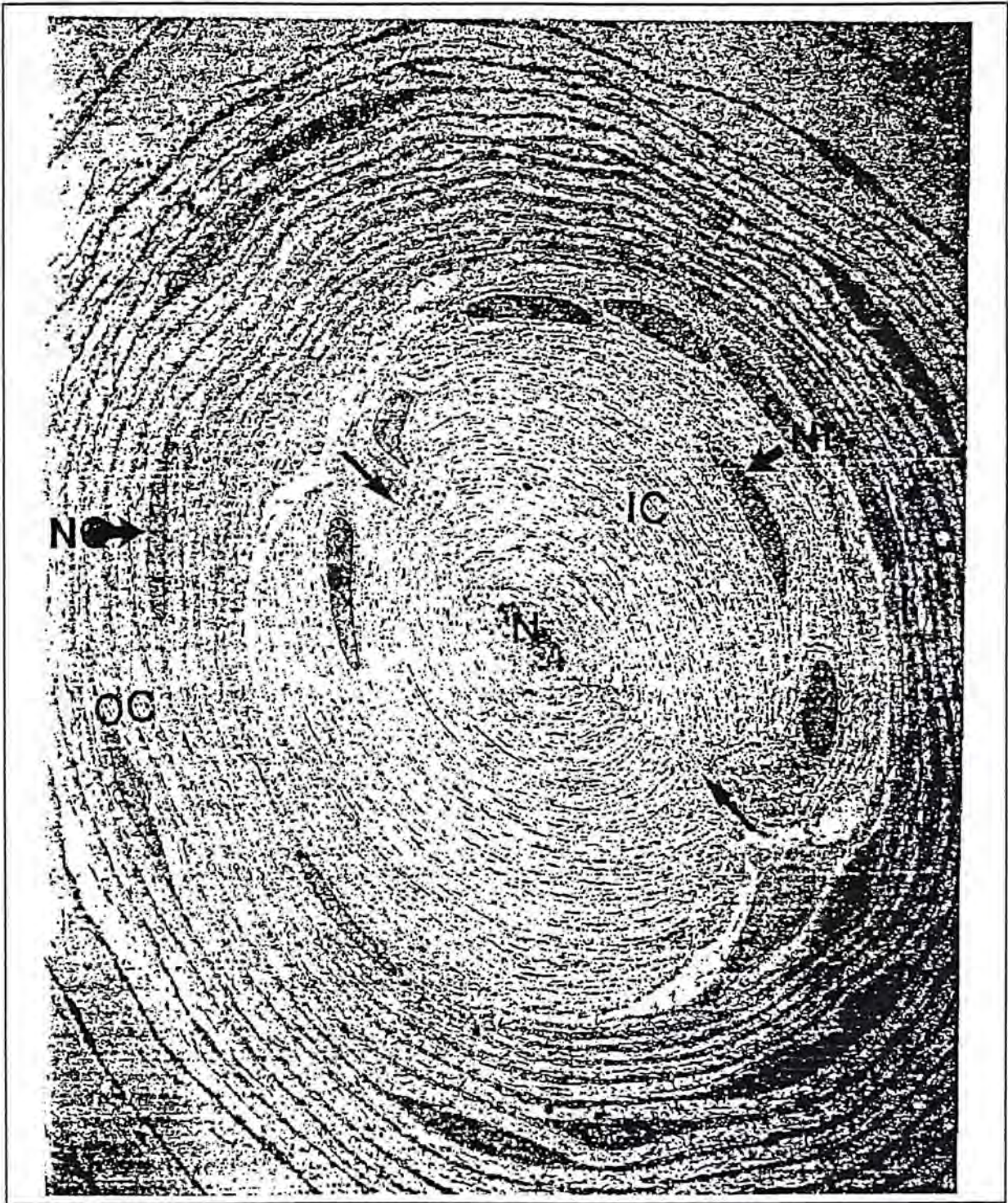

Figure 2.4

Electron micrograph of a Pacinian corpuscle showing inner and outer layers of lamellae formed by Schwann cells. The outer layers of lamellae (OC) are continuous while the inner layer (IC) forms two hemilamellae with the eliptically shaped terminal neurite (N) lying in the extracellular clefts (arrows) formed by the hemilamellae. From Bolanowski et al., (1994).

2.2.2 Ruffini 'spray' endings

This spindle shaped ending, first described in human glabrous skin by Ruffini in 1893 (Bannister, 1976) is also found in hairy skin. Biemesderfer et al., (1978) described a Ruffini-like terminal which was associated with hair follicles, below the sebaceous gland, the 'pilo-Ruffini complex'. Structurally, the receptor, which is located in the dermis, ranges in length from 0.5 -2 mm and has a distinct outer capsule formed by 3 - 5 lamellae, surrounding a fluid-filled and compartmentalised capsule space. The inner core of myelinated axon breaks up to form a dense meshwork of finer branches and terminals in the inner core. The fine terminals are covered only by basal lamina and may make contact with collagen fibrils which run within the inner core .

Ruffini endings were identified as the origin of the slowly adapting type II (SA II) responses by Chambers and co-workers (1972) in the cat. Figure 2.5 shows the structural similarities with the Golgi tendon organ.

2.2.3 Merkel cell nerve endings

The structure and function of these receptors are described in other sections - **2.1.3 (ii), 2.3.2** and **2.4.**
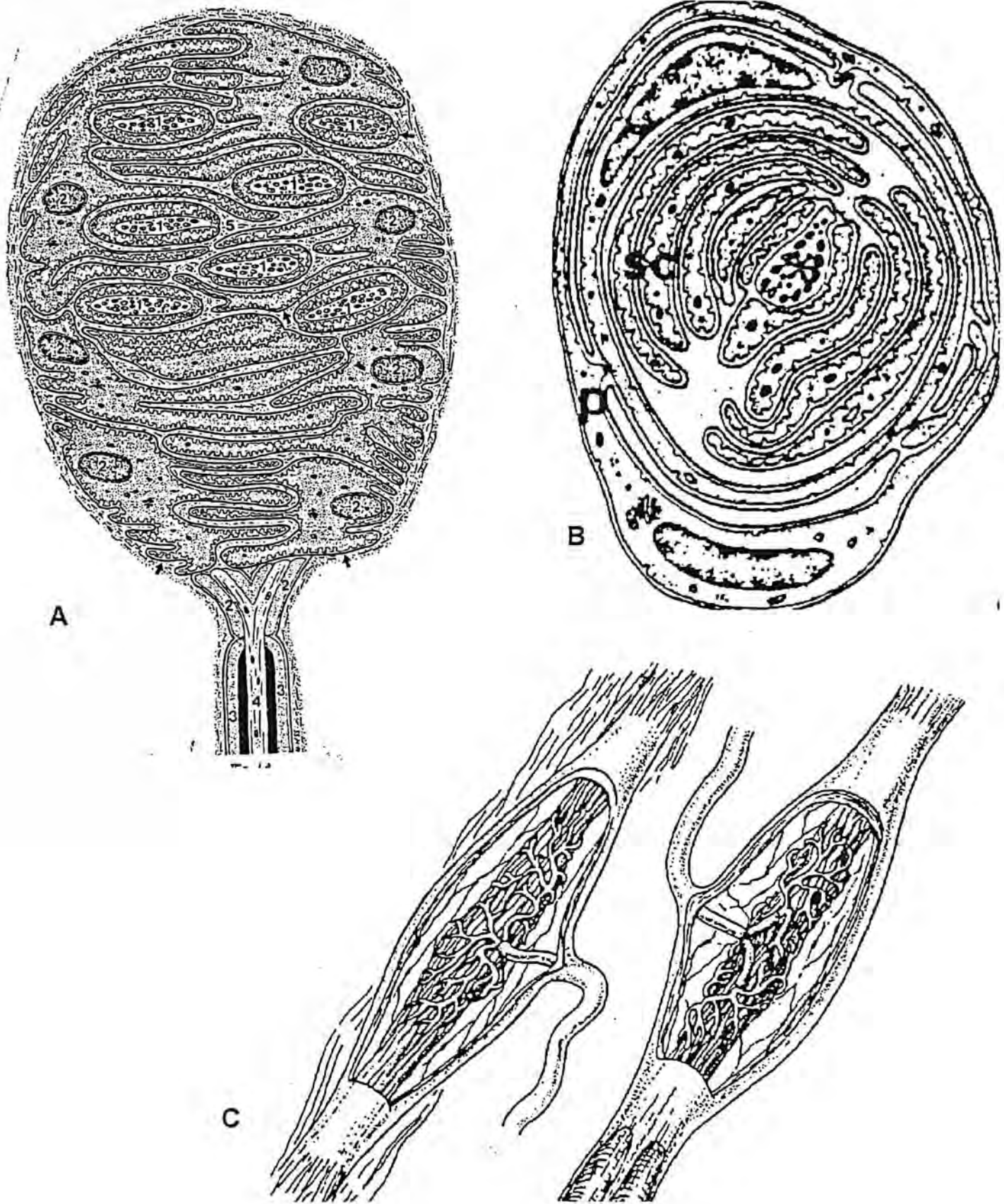
Figure 2.5

Meissner corpuscle (**A**), a lamellated (Golgi-Mazzoni) corpuscle (**B**) and **C** a Ruffini 'spray' ending (left) beside the structurally similar Golgi tendon organ. See text for description. Note the striking similarity of the lamellated corpuscle to a Pacinian corpuscle. (*From Halata, 1975 (**A**), 1993 (**B**) and Bannister, 1976 (**C**)*)

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2.2.4 Lanceolate terminals

Lanceolate terminals are associated with all types of hair (see Halata, 1993) and appear to be peculiar to the innervation of hairs (Andres, 1966). In guard hairs and down hairs, they are arranged as palisades immediately below the sebaceous gland (Cauna, 1969; Halata, 1993). The receptor terminals are fine, radially flattened endings of myelinated fibres. The unmyelinated terminal portion of the fibre is sandwiched between 2 sleeves of Schwann cell cytoplasm with cytoplasmic extensions protruding through occasional gaps (Figure 2.6)

In sinus hairs, the straight lanceolate endings project many finger-like processes into the glassy membrane surrounding the hair shaft. The terminals in the sinus hair are also longer with a more complex internal structure, ending (usually) one-axon:one-terminal basis (Andres & von Düring, 1973).

Lanceolate terminals have been considered as rapidly adapting receptors because only rapidly adapting responses are usually associated with the down hairs and guard hairs (Iggo, 1966). In sinus hairs however, the lanceolate terminals are considered to be slowly-adapting (Gottschaldt et al. 1973). Andres and von Düring (1973), suggested that the structural similarities of non-sinus hair lanceolate endings with lamellated corpuscles could account for their rapidly adapting nature.
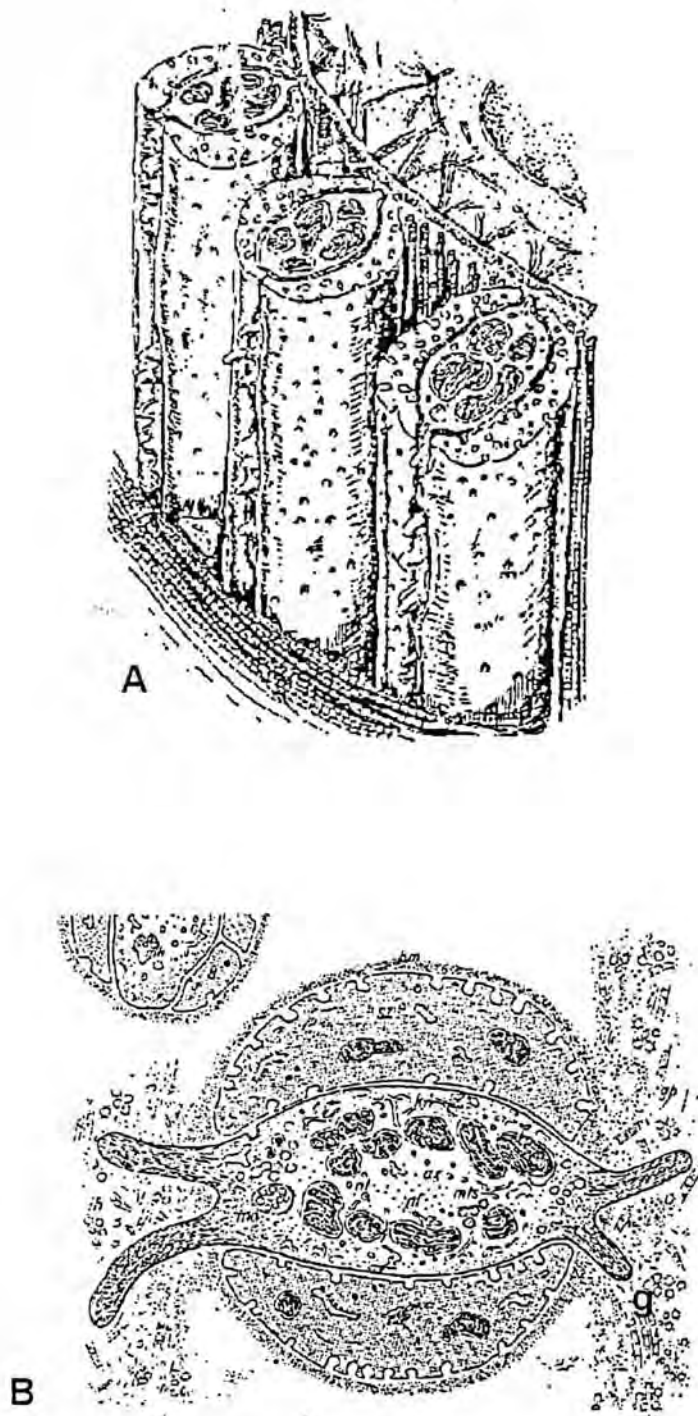
Figure 2.6

Schematic representation of lanceolate terminals showing palisade arrangement (**A**). The cross-section (**B**) shows the nerve terminal sandwiched between two sleeves of Schwann cell cytoplasm with long cytoplasmic extensions into the glassy membrane (**g**) in vibrissae. (*From Bannister, 1976 (**A**) and Andres and von Düring, 1973 (**B**)*)

2.2.5 Golgi-Mazzoni corpuscles

These corpuscles are found in the dermis below the epidermal ridges in glabrous skin. The receptor has a perineural capsule of several layers, an indistinct capsular space and inner core containing a centrally situated single nerve terminal. The receptor has a diameter of about 150 - 250µm. First described in muscle and tendon by Golgi and Mazzoni (see Iggo & Andres, 1982) and in subcutaneous tissue by Ruffini in 1893. They are generally considered along with the simple lamellated corpuscles of sinus hairs (Andres, 1966; Stephens *et al.* 1973; Halata, 1975; Halata & Munger, 1980), to be variants of Pacinian corpuscles which are much bigger; and like Pacinian corpuscles they are considered to be rapidly adapting mechanoreceptors.

2.2.5 Meissner corpuscles

Meissner corpuscles are found in the dermal papillae of glabrous skin. They are numerous in the fingertips and other sites with a high tactile sensitivity. This prompted von Frey (1906) to consider them touch endings. They were first described by Meissner in 1853. In the mature primate, Meissner corpuscles are oval in longitudinal section and measure about 200 by 80µm, orientated at right angles to the skin surface. Their precise shape varies with age (Cauna, 1965), being longer and vermiform in later years. The receptor consists of a group of myelinated fibres which penetrate the base of the corpuscle; up to 9 fibres may penetrate one capsule. Once in the capsule, the fibres lose their

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myelin sheaths and form spirals which branch among the flattened processes of the capsule cells. (see Cauna & Ross, 1960; Cauna, 1965; Cauna, 1966).

Functionally, Meissner corpuscles are considered to be rapidly adapting receptors (Jänig *et al.* 1968; Lynn, 1969; Jänig, 1971).
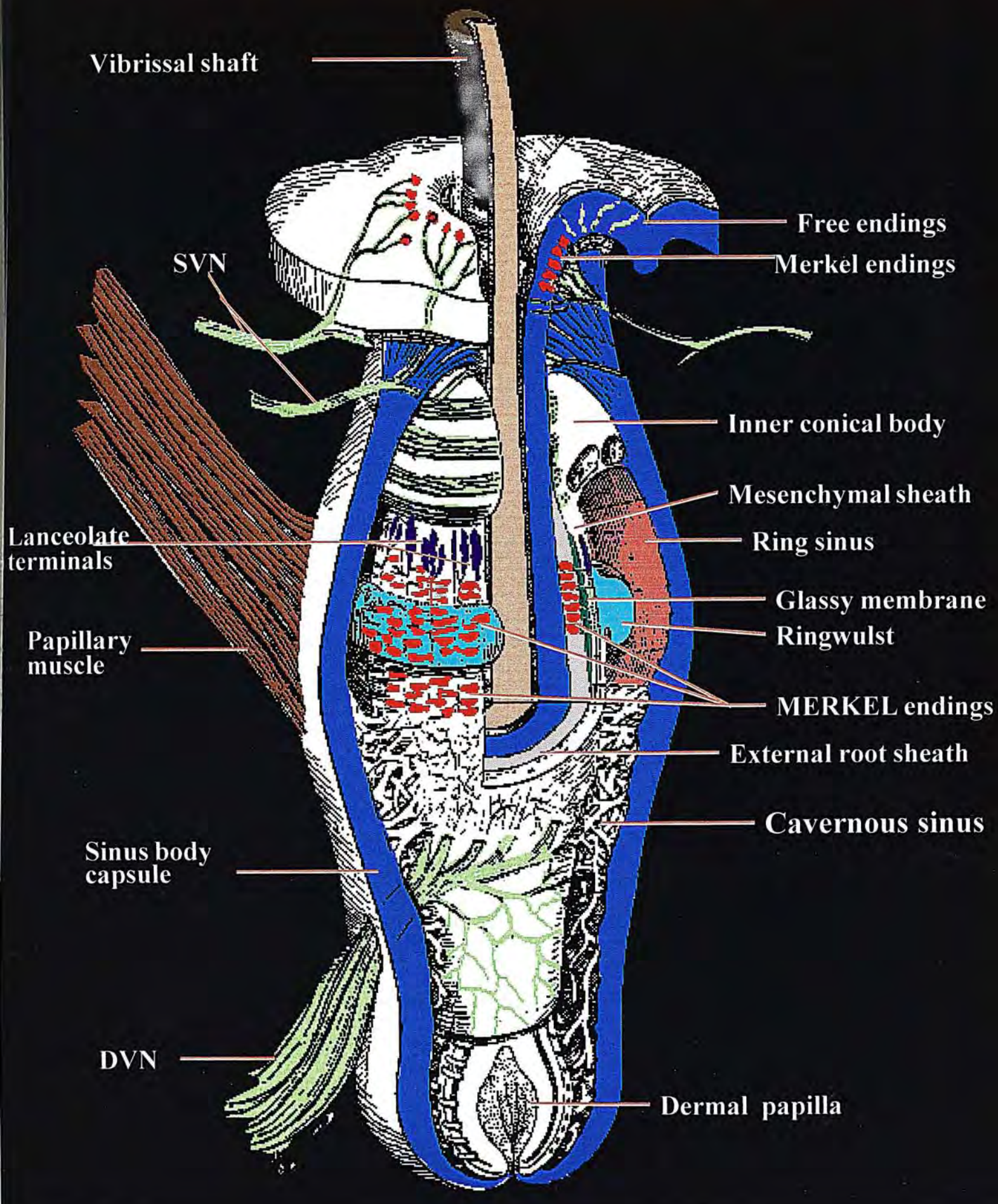
Figure 2.7

Schematic representation of the innervation of a sinus hair follicle. (*Modified from Rice et. al. 1993 J. Comp. Neurol. 337:366*)

2.3 VIBRISSAE

Vibrissae are long large diameter hairs found on the face of virtually all mammals other than man (Stephens *et al.* 1973; Dykes, 1975; Halata, 1990, 1993). They are a subgroup of sinus hairs present in the perioral facial regions, on the cheeks and around the eyes of many mammals e.g. the rows of vibrissae on the upper lips of cats, dogs and rats. In the upper lip or mystacial pad of these animals, the vibrissae are also called whiskers. Less complex sinus hairs may be present on other parts of the body e.g. the inner aspect of the lower foreleg of the cat (Gottschaldt *et al.* 1973).

They are highly differentiated vibrotactile sense organs in rodents and other mammals (Halata, 1990). For example, Yohro's (1977b) study of the big-clawed shrew, a nocturnal insectivore, found that the elongated snout of this animal has as many as 540 vibrissae with an extremely thick trigeminal nerve forming up to three quarters of the widest diameter of the medulla oblongata and a third of the widest diameter of the brain. This contrasts with its thin, comparatively rudimentary, optic nerve. The sinus hair is therefore considered to be one of the most important sensory organs in this animal (Yohro, 1977b). Other reports that demonstrate the important sensory functions of sinus hairs have shown that without sinus hairs, rats orientate poorly and when forced to swim, drown within 8 minutes as compared to a duration of up to 60 hours in normal controls (Riesenfeld, 1979). Thus vibrissae are not just obstacle detectors but

serve as a tracking system (Hahn, 1971). Yohro (1977b) also suggests that vibrissae may complement the ear in the low frequency range since the capability for echo-location demonstrated in the shrew (Gould *et al.* 1973) may decrease the ability of the ear to detect low frequency sounds to which mammals like the shrew are known to be quite sensitive (Abe, 1968). A possible role in sonar perception has also been suggested for the vibrissae in the California sea lion (Stephens *et al.* 1973).

Sinus hairs have been the subject of considerable study for more than a century. The structure of vibrissae has been well documented in several species including: rat, rabbit and cat (Andres, 1966); mole and dwarf pig (Halata, 1975); monkey (van Horn, 1970; Halata & Munger, 1980); big-clawed shrew (Yohro, 1977b); opossum and tree shrew (Montagna *et al.* 1975); whale (Nakai and Shida, 1948); sea lion (Stephens *et al.* 1973). In their comparative light microscopic studies, Rice et al. (1986) found only minor interspecies variations in the structure of vibrissae. More recently, the high density of afferent terminals in vibrissae has been further elucidated in the rat using immunofluorescence and tracer techniques (Rice, 1993; Rice *et al.* 1993; Fundin *et al.* 1994).

2.3.1 Structure of Vibrissae

The most distinguishing structural hallmark of sinus hairs is the presence of a distinct blood sinus within a thick capsule which almost completely encloses the hair follicle (Andres, 1966; Stephens *et al.* 1973; Halata, 1975).

i. *The capsule* of the blood sinus (sinus body capsule) is made up of dense connective tissue. Superficially, the capsule, which is dermally derived is continuous with the superficial dermis of the overlying skin, forming the **outer conical body** (Halata & Munger, 1980). The deep portion of the capsule forms the bulbous base of the blood sinus which the capsule encloses (see Figure 2.7). Vibrissal movements are under some degree of voluntary movement; the muscles are attached to the external surface of the sinus body capsule. These whisking movements help the animal to feel its way around (see Yohro, 1977a; Carvell & Simons, 1988).

ii. *The blood sinus* enclosed by the sinus body capsule is cavernous distally, being criss-crossed by trabecullae of mesenchymal tissue connecting the hair bulb to the capsule, especially in the upper segments of the **cavernous sinus** (Fundin *et al.* 1994). These anastomotic sinuses are lined by squamous epithelium (Stephens *et al.* 1973). Proximally, the blood sinus forms one continuous ring-like cavity which completely surrounds the middle /upper segments of the hair follicle. This part of the blood sinus is called the **ring sinus**.

Functionally, the blood sinus is thought to 'protect' the hair follicle receptors from the surrounding tissues and the prominent blood sinus apparently shields the receptors from arterial pulsations as well, thus filtering out the 'mechanical noise'. Indeed it was found that vibrissal mechanoreceptors were only activated by movement of the appropriate

hair and not the surrounding skin or structures (Gottschaldt *et al.* 1973; Iggo, 1974).

iii. *The Ringwulst* is a characteristic structural adaptation at the level of the ring sinus which is present in most species. It has also been variously referred to as the *haarwulst,* pulvinus, kissen or bourellet annular, and is absent in the horse, cow and swine, animals that appear to have a minimal requirement for vibrissae (see Stephens *et al.* 1973). It is made up of a well defined connective tissue ring in the rat (Andres, 1966), but consists of fine collagenous fibrils in the sea lion (Stephens *et al.* 1973). The position and shape of this ring-like structure has been taken to mean that it plays some as yet poorly understood function in the mechanisms within the sinus hair. Suggested functions have included 'balancer' structure (Melaragno & Montagna, 1953), vascular regulation (Ling, 1966), while Stephens et al., (1973) suggest that the ringwulst may play an important role in transmitting vibration from the shaft to the ring sinus which could then be propagated through the cavernous sinus to activate the lamellated corpuscles (q.v.) which are so intimately related to the trabecullae of the cavernous sinus.

iv. *Glassy membrane and mesenchymal sheath:* The glassy membrane separates the epidermally derived external root sheath from the dermally derived components within the blood sinus. It is made up of fine collagenous fibrils intermeshed at random angles. It begins above the

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base of the hair follicle bulb and extends to about the lower demarcation of the inner conical body; thus it is absent in the upper portion of the sinus hair (Halata & Munger, 1980). It is thickest beneath the ringwulst where its external surface is irregular. The fine collagen fibrils of the ringwulst insert as tufts on the glassy membrane, which becomes thin above the ringwulst (Stephens *et al.* 1973).

The glassy membrane invests the outermost layer of the external root sheath formed by a monolayer of Merkel cells which have been shown to send cytoplasmic extensions into it (Stephens *et al.* 1973; Halata, 1975; Halata & Munger, 1980). This investiture is thought to be important for mechanical stimulation of Merkel nerve endings and might, in part, explain their directional sensitivity. It is conceivable that bending the hair, even by very small angles of deflection will be effective in the presence of this type of arrangement; that is indeed the case (see Gottschaldt *et al.* 1973; Lichtenstein *et al.* 1990). The **mesenchymal sheath** forms the inner lining of the blood sinus, lying on the outside of the glassy membrane. It is present at both the cavernous and ring sinus levels (see Figure 2.7).

v.   *Inner conical body and rete ridge collar:* The inner conical body is dermally derived and surrounds the hair follicle at the apex of the blood sinus. The **rete ridge collar** envelopes the uppermost reaches of the hair follicle as the external root sheath becomes continuous with the epidermis

(Halata & Munger, 1980). The rete ridge collar is separated from the inner conical body by the outer conical body.

2.3.2 Innervation of Vibrissae

In consonance with the important sensory function of vibrissae alluded to above, vibrissae have an impressive array of afferent mechanosensory terminals (Figure 2.7). Consequently, studies of the innervation of vibrissal follicles abound. To mention just a few: Merkel, 1880; Retzius, 1892; Tretjakoff, 1911; Vincent, 1913 (as reviewed in Halata & Munger, 1980); Melaragno & Montagna, 1953; Andres, 1966; Patrizi & Munger, 1966; van Horn, 1970; Halata, 1975; Rice *et al.* 1993; Fundin *et al.* 1994. Each vibrissal follicle is innervated by a large deep vibrissal nerve (DVN), and a small superficial vibrissal nerve (SVN).

i.   *The Deep Vibrissal Nerve (DVN)* arises as direct branch of the infra orbital nerve from the maxillary division of the trigeminal nerve. It penetrates the sinus body capsule at the level of the cavernous sinus. In the rat, this penetration is on the side of the capsule around the lower third of the follicle, the DVN being almost always single. In the sea lion, the nerve enters through the base, at the distal tip of the capsule (Stephens *et al.* 1973) while in the monkey, two or more DVNs penetrate the capsule on the sides (Halata & Munger, 1980).

On penetrating the sinus body capsule, the nerve splits into separate bundles and hug the distal hair follicle at the level of the

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cavernous sinus like and open hand, sending 'fingers' of fascicles around to completely surround the hair follicle. Some myelinated axons are distributed within the mesenchymal sheath and trabecullae of the cavernous sinus extending both downwards and upwards, terminating as the **lamellated corpuscles**. These corpuscles were described as Golgi-Mazzoni corpuscles (Andres, 1966), and **simple lamellated corpuscles** or **paciniform corpuscles** (Stephens *et al.* 1973; Halata, 1975; Halata & Munger, 1980).

Rice et al. (1993), using immunofluorescence marking describe a dense network of large calibre **'reticular-like' endings** in the uppermost region of the cavernous sinus in close proximity to the glassy membrane as well as other small-calibre profiles that were distributed throughout the cavernous sinus. They contend that these 'reticular-like' endings were the same as those previously described as **Ruffini nerve endings** and **branched lanceolate endings** by Andres (1966), and **circumferential lanceolate endings** in the shrew by Yohro (1977b). The lamellated corpuscles are considered as rapidly adapting like their counterparts in the skin and elsewhere (Gottschaldt *et al.* 1973).

Ruffini endings or **pilo-Ruffini endings** were described in non sinus hair by Biermesderfer et al., (1978) as being slowly adapting. Their response pattern in the sinus hairs is yet to be established. Although Rice et al. (1993) contend that Ruffini endings are absent in rat vibrissae, as described by Andres (1966) and Halata (1975; 1980), EM studies from rat vibrissal follicles done as part of this study, do show the presence of

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the pilo-Ruffini endings (e.g. Figure 4.15).

Nerves that do not terminate in the cavernous sinus continue on in small bundles along the shaft of the vibrissa, outside the glassy membrane, run through the basal part of the ringwulst, still in small bundles. These give rise to 2 major types of large-calibre afferents with prominent myelin sheaths.

One group terminates outside the glassy membrane as longitudinally oriented **lanceolate terminals** which are arranged in a palisade fashion, parallel to each other and completely surrounding the hair follicle. They lie within the mesenchymal sheath adjacent to the glassy membrane. Though the nerve terminals are ultrastructurally the same as those of lanceolate terminals elsewhere in other hair types, the sinus hair lanceolates tend to be longer, ending on a 'one-axon-one-terminal' basis (Halata & Munger, 1980). There are also reports of 2 - 3 lanceolate terminals arising from one myelinated axon (Rice *et al.* 1993; Fundin *et al.* 1994).

The other group of myelinated nerve endings penetrate the glassy membrane. Upon emerging on the internal surface of the membrane, the nerve axon branches and continues parallel to the glassy membrane for some distance making contact with **Merkel cells** in the outer root sheath. Unlike in the skin where the nerve terminal approaches the Merkel cell from the dermal surface, the vibrissal Merkel cells are approached and contacted by their neurites from the epidermal side of the external root sheath, leaving the Merkel cells free to send their cytoplasmic protrusions

into the glassy membrane, which separates them from the dermal mesenchymal sheath and the blood sinus. It is estimated that a sinus hair might contain as many as 2000 Merkel cells and these might be supplied by some 50 - 60 axons (Halata & Munger, 1980; Halata, 1993). Thus a single unit might be made up of anything from 10 - 100 Merkel cells.

Merkel cells and their associated axons are found in the epidermis and cutaneous appendages of all mammals and some submammalian vertebrates including lampreys (Whitear & Lane, 1981), fish (Fox *et al.* 1980), salamanders (Fox & Whitear, 1978; Tweedle, 1978; Scott *et al.* 1981), frogs (Tachibana, 1978), and lizards (Landmann & Halata, 1980), where they may be associated with rapidly adapting responses. The avian equivalent of Merkel cells referred to as Grandry cells (Andres & von Düring, 1973; Halata, 1975), are part of rapidly adapting receptors (Gottschaldt & Lausmann, 1974).

The Merkel nerve ending is unlike any of the other nerve endings described above by virtue of the fact that the nerve terminal makes synaptiform contact with a specialised cell - Merkel cell. Though Merkel (1880) called the cell a *Tastzelle* or touch cell, based on light microscopic studies he, of course had no way of showing this to be the case functionally. Although it has been demonstrated clearly that this receptor gives rise to the characteristic SA I type of responses (Iggo & Muir, 1969), it is yet to be proven conclusively that the Merkel cell is the site of mechano-electric transduction in this receptor.

ii. *Superficial Vibrissal Nerve (SVN):* The one or two twigs of innervation that enter the sinus hair above the level of the blood sinus has been called the superficial vibrissal nerve (Rice *et al.* 1986), but was previously called the conus nerve (e.g. (Andres & von Düring, 1973)). The SVN supplies the rete ridge collar and the inner conical body (Figure 2.7).

In the rete ridge collar, large- and medium-calibre myelinated fibres terminate in contact with Merkel cells which form a kind of neck collar for the sinus hair entry. It is thought that these receptors detect the degree of bending or displacement of the sinus hair relative to the surrounding skin. Other fine radially disposed profiles were recently described in this region using immunofluorescence labelling (Rice *et al.* 1993).

At the inner conical body, SVN fibres terminate as thin lanceolate endings, Aδ axons and clusters of varicose endings from bundles of C fibres (Mosconi *et al.* 1993).

Terminals of the SVN are not of great importance in the present work because recordings were made solely from the DVN.

2.4 MECHANOSENSORY TRANSDUCTION

Bannister (1976) has outlined four stages in the process of sensory perception: (1) stimulus accession, the process by which the impinging stimulus gains access to the appropriate receptor; (2) stimulus transduction, whence the adequate stimulus (mechanical in this case) gets converted to electrical energy; (3) action potential generation; and (4) neural processing.

i. ***Stimulus accession:*** Since the receptors are not in direct contact with the environment, the stimulus has to pass through the intervening layers of cells and connective tissue. Some receptors have additional accessory structures that may further modify the stimulus that finally gets to the nerve terminal. The Pacinian corpuscle is a good example - its fluid-filled lamellae absorb the energy of all but very rapidly changing deformation by the displacement of fluid between the concentric layers of flattened cells. The accessory structure thus serving as a 'high pass' filter for the mechanical stimuli (Loewenstein, 1971).

ii. ***Stimulus transduction:*** In virtually all receptors, the impinging stimulus results in a partial or complete depolarisation of the resting potential, the magnitude of depolarisation depending on the strength and duration of the applied stimulus. This graded response has been called the generator or receptor potential (Katz, 1950). The receptor potential

consists of a dynamic phase of rapid depolarisation and a static phase of reduced polarisation following the quickly adapting dynamic phase (Katz, 1950; Grundfest, 1971).

The receptor potential is the result of sudden changes in membrane conductance to small inorganic ions. In the Pacinian corpuscle, the generator potential is Na^+-dependent (Diamond et al. 1958). The same appears to be the case in other mechanoreceptors studied e.g. crayfish stretch receptor (Brown et al. 1978). Unfortunately, most mammalian cutaneous endings are too small for direct electrophysiological investigation.

The molecular mechanisms underlying the conductance changes in the receptor have remained poorly understood. Recent work on mechano-gated ion channels in sensory (cochlear hair cells, (Jørgensen & Ohmori, 1988; Rusch et al. 1994)), and non-sensory (Xenopus oocytes, (Hamill & McBride, 1992)) cells suggest that mechanosensory processes occur through activation of specific mechanically gated ion channels.

Exploration of the mechano-gated channels has however been hampered by the lack of specific ligands. Amiloride and some of its analogues have been shown to bind to the mechano-gated channel (Jørgensen & Ohmori, 1988; Rusch et al. 1994). Based on these observations, antibodies against the amiloride-sensitive epithelial Na^+ channel was used to immunolocalize putative mechanotranducer sites on the ciliary processes of the cochlear hair cells (Hackney et al. 1992). Furthermore, a bacterial mechano-gated channel protein has recently

been cloned, sequenced and expressed (Sukharev *et al.* 1994). When functionally reconstituted in liposomes, mechanosensitivity could be elicited, albeit at higher thresholds, suggesting the involvement of cellular cytoskeletal structures in normal mechanosensitivity (Hamill & McBride, 1994).

The emergent properties of the mechano-gated channel include: (1) relatively rapid adaptation in the presence of sustained application of the mechanical stimulus (Hamill & McBride, 1992); (2) they are only weakly selective among cations in hair cells and *Xenopus* oocytes, allowing Na^+, K^+ and Ca^{2+} to permeate while excluding anions (Howard *et al.* 1988; Sachs, 1988), Ca2+ being the most permeable through the channel (Jørgensen & Kroese, 1994; Ohmori, 1992); and (3) a surprising voltage sensitivity (Sukharev *et al.* 1993; Hamill & McBride, 1992).

It would appear therefore that mechanical perturbation of a receptor terminal directly activates the opening of mechano-gated channels which process initiates the observed conductances associated with the receptor potential. The precise coupling of mechanical stimulation to mechano-gated channel opening however remains unknown.

iii.   *Action potential initiation:* The receptor potential electrotonically invades the adjacent unmyelinated segment of the receptor terminal where action potentials are triggered by opening of voltage-gated ion channels. The number of action potentials generated is directly related to

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the amplitude of the receptor potential. Thus there is a frequency coding at the level of the receptor. Adaptation may also occur at this level (see French, 1992 for review).

In secondary receptor cells, the receptor potential and subsequent events are transmitted to the afferent terminal through release of chemical neurotransmitters; in which case the afferent terminals' action potential generating process is activated via opening of ligand-gated channels in the membrane of the afferent fibre.

iv.   *Neural processing* of impulses from the periphery are complex and may occur at various levels of the CNS. On the whole, analysis of peripheral input by the CNS would appear to depend largely on sampling impulse frequency (see Bannister, 1976). It is therefore expected that regular trains of impulses would be more efficiently sampled than irregular ones (Burgess & Perl, 1973) as is the case in some receptors including the irregularly firing SA I mechanoreceptor (Iggo & Muir, 1969) and the carotid body chemoreceptor (Biscoe & Taylor, 1963).

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2.5 THE MERKEL CELL CONTROVERSY

As noted earlier, there is still uncertainty regarding the precise role of the Merkel cell in the SA I (or St I in vibrissae) receptor. Basically, the argument centres around a mechano-electric transduction role (or the lack thereof) for Merkel cells. One group considers the Merkel cell to be the transducer proper and propose that transduction involves a mechanism of synaptic transmission between the Merkel cell and the opposed nerve terminal (see the reviews by Iggo & Findlater, 1984; Hamman, 1992). The other group consider the nerve terminal to be the transducer proper and seek to ascribe other non transducer functions to the Merkel cell (e.g. Gottschaldt & Vahle-Hinz, 1982; Mills & Diamond, 1995). Both groups agree however that Merkel cells are not mere 'passive abutments for the nerve endings' (Gottschaldt & Vahle-Hinz, 1981).

2.5.1 Evidence for a transducer function for Merkel cells

i.   *Structure of Merkel cells:* Certain structural features of the receptor make a hypothesis of synaptic transmission between the Merkel cell and nerve terminal appealing (see Iggo & Muir, 1969; Iggo & Findlater, 1984). The Merkel cell has a characteristic elongated shape with a highly lobulated nucleus. It sends cytoplasmic protrusions between adjacent keratinocytes in the skin or glassy membrane in sinus hairs. These look like adaptations for mechanical responsiveness as seen in

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other mechanosensitive nerve terminals, e.g. the filopodia in Pacinian corpuscles or the protrusions in lanceolate terminals. These protrusions have indeed been likened to the cilia in auditory hair cells (Iggo and Findlater, 1984).

Secondly, Merkel cells have dense cored granules in the cytoplasm that resemble secretory granules. These granules are polarised towards the side of the Merkel cell where the nerve terminal makes contact. In this respect the Merkel cells bear some similarity to the type I cells of the carotid body, where the afferent fibres have an irregular firing pattern (Biscoe & Taylor, 1963; Biscoe *et al.* 1970). Though the contact lacks some features of typical synaptic clefts, symmetrical thickenings in the membrane of the afferent terminal and the Merkel cell plasma membrane are well recognised (Iggo & Muir, 1969; Toyoshima & Shimamura, 1991). Diamond et al. (1986), describe reciprocal synapses in salamander skin where the afferent terminals also show clear vesicles.

Chen et al., (1973) reported fusion of dense cored granules with the membrane at the synaptic cleft. In a study of Merkel cells in the tongue of the finch *Lonchura striata*, Toyoshima and Shimamura (1991) demonstrated unequivocal evidence of exocytotic discharge of Merkel cell granules at the plasma membrane facing both the nerve terminal and the basal lamina. Toyoshima (1989) had shown in earlier preliminary experiments that 'massive' exocytotic release of granules was evident when the finch lingual tissue was fixed shortly after mechanical stimulation.

Immunohistochemical methods have been used in an attempt to identify the possible neurotransmitter substance(s) in Merkel cells. This has led to the identification of several peptides and putative neurotransmitters in Merkel cells, often shown to be localised in the dense cored granules (Hartschuh *et al.* 1989; Chew & Leung, 1991; English *et al.* 1992).

The structural features of the receptor make the hypothesis of synaptic transmission compelling.

ii.   *Functional evidence:* Several experimental approaches have been used. Since calcium influx is known to be involved in chemical synaptic transmission (Llinas, 1977), Ca^{2+} blockers have been used in attempts to interfere with synaptic transmission. Yamashita et al. (1986) demonstrated a differential effect of the inorganic Ca^{2+} blockers Mg^{2+} and Mn^{2+} on frog types I and II units, the type I being inhibited to a much greater extent than the type II. This finding suggested that an additional process of higher calcium sensitivity e.g. chemical synaptic transmission, was involved (Yamashita *et al.* 1986). Other studies using rats have also demonstrated that calcium influx is required for normal mechano-electric transduction in the SA I (Cooksey *et al.* 1984; Pacitti & Findlater, 1988; Tsu & Baumann, 1992).

Based on observed similarities between Merkel cell receptor and cochlear hair cells, Baumann et al. (1986; 1990) tested the effect of the aminoglycoside neomycin on cat and rat SA I receptors. Acute application

of aminoglycosides in cochlear hair cells had been shown to cause a nonspecific decrease in permeability to cations which was counteracted by an increase in extracellular Ca^{2+} concentration (Kroese & van den Bercken, 1982; Hudspeth & Kroese, 1983). These studies, which compared the effect of neomycin on SA I and SA II receptors found that stimulus evoked activity in SA I was suppressed to a greater degree than in SA II following close arterial infusion (Baumann et al. 1990).

Attempts have been made to destroy Merkel cells by photobleaching them after quinacrine loading. Merkel cells take up the fluorescent dye quinacrine which is also used as a fluorescent marker for them (Crowe & Whitear, 1978). It has been shown that acute photobleaching of Merkel cells resulted in cessation of responses to mechanical stimulation, often with thresholds to electrical stimulation remaining unchanged (Baumann & Senok, 1993; Ikeda et al. 1994). Using a much criticised technique (see Hamman, 1992 for review), Diamond et al. (1988) contend that photobleaching of quinacrine labelled Merkel cells in Salamander skin failed to eliminate mechanical responsiveness. Evidence has now emerged however that photobleaching of quinacrine labelled Merkel cells is neither specific nor complete. This study (Senok et al., in press), which used smaller doses of UV irradiation in skin and sinus hair receptors, showed ultrastructural evidence of nerve terminal abnormalities even while receptors were still responsive. Figure 2.8

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summarises a proposed mechanism of mechano-electric transduction in
Merkel cells (Iggo & Findlater, 1984).
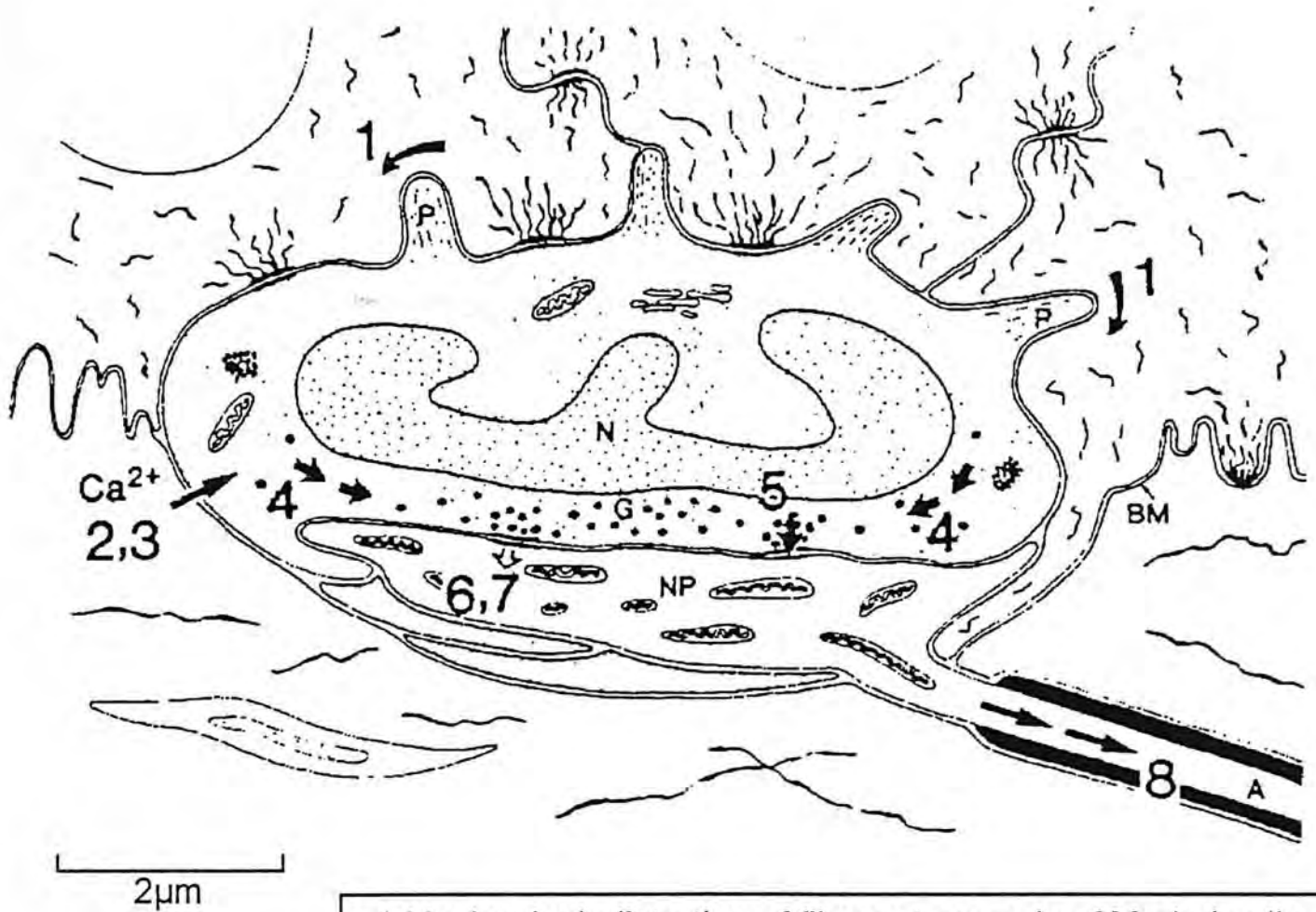
Figure 2.8

Proposed mechano-electric transduction process in the cutaneous
Merkel cell receptor by Iggo and Findlater (1984)

2.5.2 Evidence against Merkel cell transducer function

Arguments against Merkel cell transducer function have arisen more because of the shortage of direct evidence in support of Merkel cell transducer function than anything else. While some have sought alternative functions for Merkel cells, others have taken the paucity of direct evidence for a transducer function to infer that Merkel cells are merely mechanical abutments on the nerve endings with a possible function of enhancing the effectiveness of mechanical stimulation (Gottschaldt & Vahle-Hinz, 1981).

i.   _Non-sensory functions for Merkel cells._ There is evidence to support neurotrophic functions for Merkel cells. Munger's group concluded from their studies on rat and human material that Merkel cells have neurotrophic functions, serving as targets of regenerating nerves (Renehan & Munger, 1986). Based on their staining properties with quinacrine (Crowe & Whitear, 1978), the presence of CGA and multiple peptides in mammalian Merkel cells (Hartschuh et al. 1989; English et al. 1992), the suggestion that Merkel cells are paraneurons (Fujita, 1977) has been strengthened. Merkel cells in culture have also been shown to produce NGF (Vos et al. 1991), and dermal Merkel cells (during development) have been shown to be NGF-positive (Narisawa et al. 1992). Kim and Hobrook (1995), from embryological studies suggest that Merkel cells may have some trophic roles in the development of sweat glands and hair follicles. None of these however preclude Merkel cells

from being mechano-electric transducers.

ii. ***No neurotransmitter, no neurotransmission:*** It has not been possible so far to functionally identify the neurotransmitter involved in the proposed synaptic transmission between Merkel cells and their nerve terminals. Met-encephalin, in particular generated a lot of interest, being one of the first to be immunohistochemically identified in the Merkel cell. Opiod receptor blockers have been unable to block responses of the receptors in a cat vibrissa (Gottschaldt & Vahle-Hinz, 1982), and rat skin-nerve preparation (Baumann & Chan, 1992). It has been shown however that met-encephalin immunoreactivity was restricted to certain species, being consistently found only in mice and dogs (Cheng Chew & Leung, 1992).

Earlier experiments involving topical testing of several substances on identified touch domes by Smith and Creech (1967) found that neither classical cholinergic nor adrenergic processes were involved in SA I function.

In other experiments, Gottschaldt and Vahle-Hinz (1981) considered the ability of the vibrissal mechanoreceptors to follow vibration at frequencies in excess of 1500Hz and the short difference in latencies found (about 0.2 - 0.3 ms) between topical electrical and mechanical stimulation of cat vibrissal presumptive Merkel cell mechanoreceptors, to be evidence against synaptic transmission. First, it has been shown (Llinas, 1977), that there are synapses with synaptic delay of about 0.2

ms, which is of the same order of magnitude as the figures reported by Gottschaldt and Vahle-Hinz. Such a high frequency of discharge is therefore not incompatible with chemical synaptic transmission. Secondly, in the light of present understanding of the nature and complexities of vibrissal afferents, there is some doubt regarding the actual identity of the receptors being recorded from.

iii.   *No mechanosensitivity, no mechanotransduction:* Merkel cells have been isolated from vibrissal hair follicles and from rat glabrous skin (Nurse & Cooper, 1988; Yamashita *et al.* 1992 respectively). Nurse and Cooper (1988) reported the presence of only one type of ionic channel which was non-selective for Na^+ and K^+. The technically superior work by Yamashita et al. (1992), however found Merkel cells to have the electrical properties expected of an excitable cell. They also found a high threshold (L-type) Ca^{2+} channel and K^+ channels. Both groups of workers were however unable to find currents that were mechanically activated.

As has been noted earlier, the principal problem with isolated Merkel cells, as was clearly demonstrated by Yamashita et al. (1992), is the fact that the cells lose their normal oblong structure and become spherical. The shape of Merkel cells and their cytoplasmic extensions are considered important for their mechanosensitivity (see Iggo & Findlater, 1984). It is therefore not surprising that these workers were unable to elicit mechanosensitivity in their isolated Merkel cells.

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Thus there is evidence for and against Merkel cell transducer function. The weight of evidence so far, though mostly circumstantial, will appear to favour a mechano-electric transducer function for Merkel cells.

2.6 INTRACELLULAR CALCIUM MOBILISATION

Calcium is involved in diverse cellular processes and increases in cytoplasmic calcium concentration ($[Ca^{2+}]_i$) triggers many cellular events. In many cellular systems, Ca^{2+} influx from the extracellular space does not get to the intracellular target fast enough (or at all) by diffusion alone because the presence of a large number of almost immobile Ca^{2+} binding sites (Allbritton et al. 1992) in the cells makes Ca^{2+} diffusion in the cytosol much slower than that of any other second messenger (see review by Pozzan et al. 1994). This becomes progressively important as the size of the cell gets bigger. Thus cells do not rely on the large, virtually limitless extracellular Ca^{2+} pool. Instead intracellular Ca^{2+} stores are ubiquitous in cells. The nature and function of the pools are diverse; in some (e.g. the SR or ER) the Ca^{2+} is in very rapid dynamic equilibrium with the cytosol - a few tens of miliseconds - (Lytton & MacLennan, 1992), while in some (e.g. secretory granules) the pool is virtually frozen (Fasolato et al. 1991).

To rapidly release Ca^{2+} from intracellular stores, there are Ca^{2+} release channels within the membranes of the stores. Two major types of Ca^{2+} release channels have been identified which are controlled by specific second messengers to release Ca^{2+} from apparently distinct pools within the stores - i.e. there appears to be compartmentalisation within the SR/ER (see reviews by Henzi & MacDermott, 1992; Pozzan et al. 1994). These are the Inositol 1,4,5-trisphosphate receptor (IP_3R) and the Ryanodine receptor (RyR).

2.6.1 Inositol 1,4,5-trisphosphate receptor (IP$_3$R)

This receptor was named after its endogenous agonist, inositol 1,4,5-trisphosphate (IP$_3$). IP$_3$ is generated by the enzymatic breakdown of phosphatidyl inositol 4,5-bisphosphate after activation by protein kinase C (see Berridge, 1993). Production of IP$_3$ in the cell releases Ca^{2+} from a distinct intracellular pool. IP$_3$'s ability to activate the channel is highly sensitive to Ca^{2+} leading to suggestions that Ca^{2+} may be the actual activating messenger for the IP$_3$R (Lechleiter et al. 1991). IP$_3$ however has a greater sphere of activity within the cell because of the intracellular Ca^{2+} buffering referred to above (Allbritton et al. 1992).

Apart from IP$_3$, there are few known agonists of the IP$_3$R. Ca^{2+} and ATP are coagonists of the receptor but will not activate the channel in the absence of IP$_3$ (Bezprozvanny et al. 1991; Bezprozvanny & Ehrlich, 1993). A specific antagonist of the receptor is heparin, which, along with other polyanions competitively inhibits IP$_3$ binding to the IP$_3$R (Ghosh et al. 1988; Taylor & Richardson, 1991). Caffeine, used as an activator of the RyR (see below) was found to inhibit IP$_3$-induced Ca^{2+} release in Xenopus oocytes (Parker & Ivvora, 1991), cerebellar microsomes (Brown et al. 1992), and permeabilized smooth muscle cells (Hirose et al. 1993). Caffeine decreases channel opening without altering IP$_3$ binding (Brown et al. 1992; Bezprozvanny et al. 1994); near complete inhibition was achieved at 10mM caffeine (Bezprozvanny et al. 1994).

2.6.2 The Ryanodine receptor (RyR)

Named after the plant alkaloid, the specific binding of which helped purify the receptor, the ryanodine receptor mediates the process that has come to be known as calcium-induced calcium release (CICR); small amounts of Ca^{2+} influx have been shown to induce Ca^{2+} release from the stores (see recent review by Meissner, 1994). Though most cells have both types of Ca^{2+} release channel, skeletal and cardiac muscle Ca^{2+} stores are predominantly of the ryanodine type, being largely IP_3-insensitive.

There is a direct mechanical coupling of the RyR and the plasma membrane dihydropyridine (DHP) receptor in skeletal muscle (McPherson & Campbell, 1993), such that Ca^{2+} influx directly activates the RyR. Recent reports suggest that the new second messenger cyclic ADP ribose (cADPR) is one of the physiological agonists of the RyR (Galione et al. 1991; Lee et al. 1994).

Ryanodine activates Ca^{2+} release from the receptor by binding irreversibly to it and locking it in the open state (see Ehrlich and Bezprozvanny, 1994). Caffeine is however considered the 'gold standard' (Ehrlich & Bezprozvanny, 1994) for demonstrating activation of CICR in intact and semi-intact systems; e.g. SR vesicles (Meissner et al. 1986), direct activation of RyR channel in native membranes (Ehrlich & Watras, 1988), purified reconstituted RyR (Sorrentino & Volpe, 1993), and cloned, expressed RyR (Brillantes et al. 1994). As stated above, the same concentrations of caffeine that activate CICR were also found to inhibit

Ca^{2+} release from the IP$_3$R, thus in experiments where caffeine is used to induce Ca^{2+} release, the Ca^{2+} release must have come through the RyR (Ehrlich & Bezprozvanny, 1994).

Specific inhibitors of the RyR include among others, ryanodine (>10µM), Dantrolene, Ruthenium red and procaine (see Galione *et al.* 1991; Henzi & MacDermott, 1992; Khoyi *et al.* 1993). Heparin, the specific inhibitor of the IP$_3$R has also been shown to activate the RyR (Ritov *et al.* 1985; Bezprozvanny *et al.* 1993).

Figure 2.9 shows the schematic representation of the structures of the two receptors and their activation in neurons while table 2.1 summarises their functional properties and modulation.
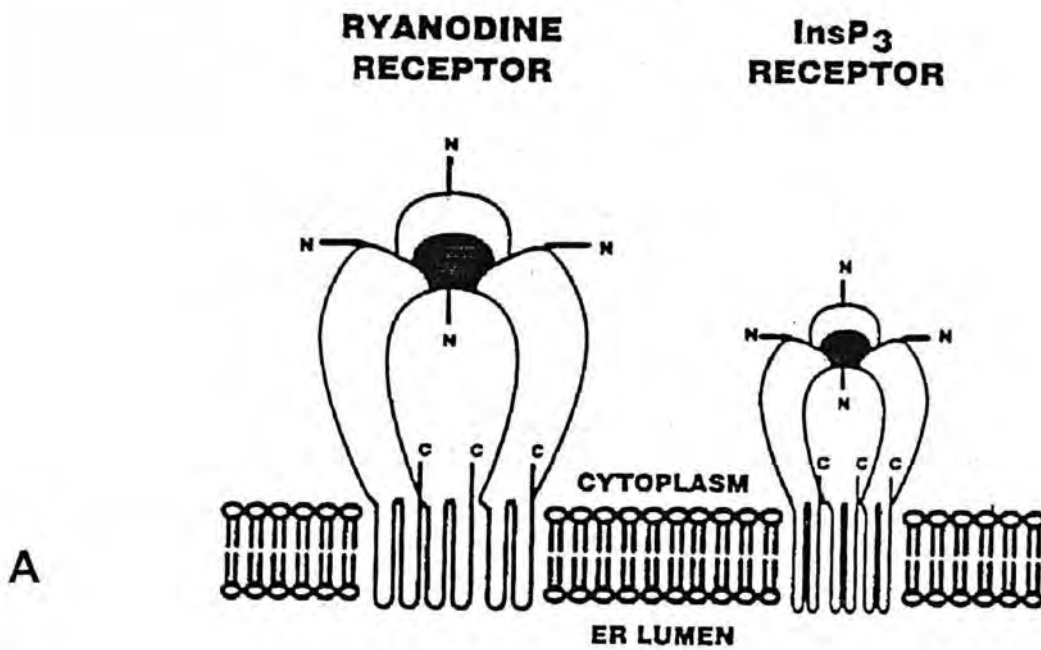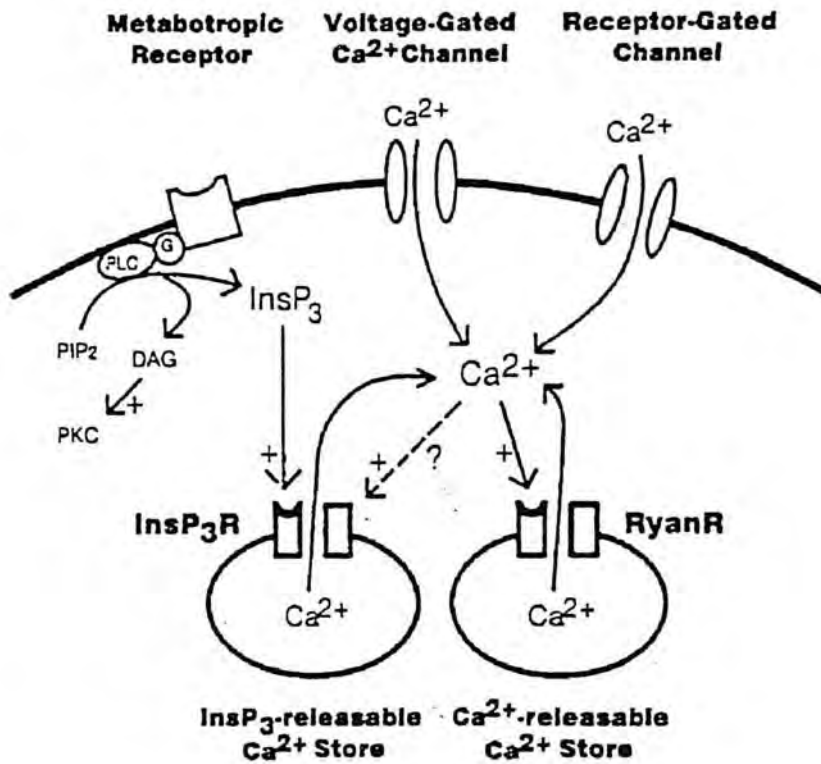
Figure 2.9

A. Schematic representation of ryanodine and IP_3 receptors showing their relative sizes and orientation with respect to the endoplasmic reticulum (ER) membrane. Both receptors have a homotetrameric structure.

B. Diagram showing activation of the two intracellular calcium release channels in neurons (*From Henzi and MacDermott, 1992*).

Table 2.1. Summary of the functional properties and modulation of intracellular calcium release channels

	IP_3 receptor	Ryanodine receptor
Molecular weight	313 kDa (x4)	565 kDa (x4)
Physiological activators	Inositol 1,4,5-trisphosphate (IP_3)	Ca cADP ribose mechanical coupling (skeletal muscle)
Modulation by Ca	< 0.3µM Ca ↑ activity > 0.3µM Ca ↓ activity	< 100µM Ca ↑ activity > 1mM Ca ↓ activity
Modulation by ATP	< 2mM ATP ↑ activity > 2mM ATP ↓ activity	ATP ↑ activity
Pharmacological activators	?	caffeine heparin doxorubicin ryanodine (< 10µM)
Pharmacological inhibitors	heparin caffeine	ruthenium red ryanodine (> 10µM)

Source: Ehrlich and Bezprozvanny, 1994

Chapter 3

METHODS

3.1 THE ISOLATED VIBRISSAL PREPARATION

3.1.1 Animals

Experiments were carried out on male Sprague-Dawley rats weighing about 300g with the approval of the Animal Research Ethics Committee of the Chinese University of Hong Kong (CUHK). All animals used were obtained from the CUHK Animal House, being requested for and used on a *per diem* basis.

Animals were first anaethetised with urethane (20% w/v 6ml kg^{-1} I.P.) and subsequently killed by an intracardiac injection of a similar amount of urethane. In some early experiments, animals were killed by cervical dislocation following a stunning blow to the head, to exclude the (unlikely) possibility of a urethane effect on the preparation. Killing the animal was necessary to afford a bloodless dissection field in an otherwise highly vascular location.

3.1.2 Dissection: removal of vibrissae

One of the whisker pads (usually the left) was rapidly excised from the killed animal by first making a cut in the junction between the

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glabrous and hairy part of the upper lip, then cutting rostrally, keeping as near to the maxilla as possible. A sharp but sturdy pair of scissors was found to be most effective for this procedure. Cutting to the bone ensured that the infraorbital nerve was cut at its exit from the infraorbital foramen, leaving a longer nerve stump on the vibrissa for later use.

The excised whisker pad was immersed in a pool of carbogen (95% O_2 + 5% CO_2)-bubbled synthetic interstitial fluid (henceforth referred to as SIF). The solution (see below and table 3.1) is basically a modified Krebs solution (Bretag, 1969). Whiskers to be dissected were then selected.

Selection of vibrissae was based mainly on the length and hence sturdiness of a hair; thinner hairs buckled more easily and were therefore more difficult to use for sustained mechanical stimulation. Single hairs were also preferred for basically the same reason (two hairs may be seen emerging from the same vibrissal follicle as part of the replacement process, but this growth and replacement of hairs takes place within the outer root sheath without affecting the innervation). The most suitable hairs were found to be the most rostral and dorsal hairs: A1, A2, B1 and B2 and the stradllers α and β (see fig 3.1). These hairs were almost always among the 3 - 5 vibrissae dissected each time.

Further dissection was carried out under stereo microscopic view (Stemi SV11 Zeiss, Germany). To remove a selected vibrissa, the intervibrissal skin was incised with fine scissors and then cut circumferentially. Number 3 forceps were used to hold the skin edge still

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attached to the vibrissa and gently pulled, lifting it up and exposing the muscular attachments to the external surface of the sinus body capsule.
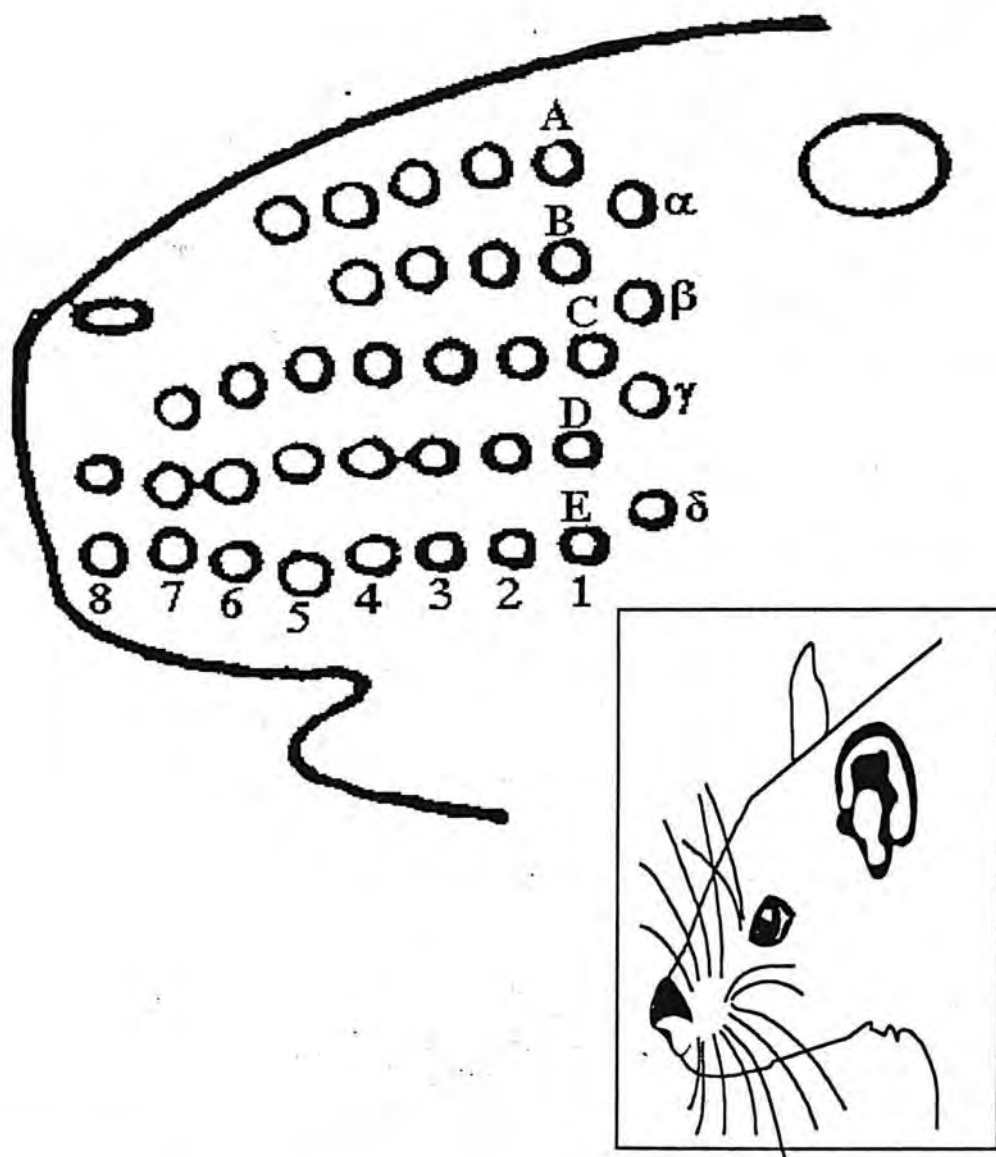

Figure 3.1

Schematic representation of the whisker pad showing the nomenclature of the vibrissae. The 5 rows of vibrissae are labelled **A - E** from dorsal to ventral and numbered from caudal to rostral while the hairs straddling the rows are labelled α - δ as shown (see Zucker & Welker, 1969; Woolsey & van der Loos, 1970). The most frequently dissected hairs were the caudal and dorsal hairs **A1, A2, B1, B2** and the stradllers α and β. Inset rat face with whiskers for orientation.

The deep vibrissal nerve (DVN) is visible at this point as it penetrates the side of the sinus body capsule. The point of entry was found to be always on the caudal aspect of the follicle. The muscular and other attachments to the sides and the base of the sinus body capsule can thus be cut, while sparing the nerve. A subsequent gentle tug on the skin edge with the forceps neatly pulls out the nerve stump from its loose attachments, thus freeing the vibrissa and attached nerve stump (DVN) of about 1 - 2cm. The required number of hairs were removed in like fashion.

Removed vibrissa were further dissected. A vibrissa was immersed in a fresh pool of synthetic interstitial fluid (SIF) on a silgard (Sylgard 184, Dow Corning, USA) base. If required, the follicle was restrained on the silgard with fine insect pins. A sharp blade was then used to make a longitudinal incision in the capsule of the blood sinus, taking care to avoid cutting into the underlying mesenchymal tissue and nerve. A single straight longitudinal cut was best. This released the blood from both cavernous and ring sinuses. An interesting observation at this point was that the blood within the capsule did not clot as long as the capsule remained intact. Unslit hairs left in SIF could be slit hours later. On opening the capsule however, the blood clotted soon afterward.

The blood was cleared away with SIF directed at the opened capsule using a Pasteur pipette. The position of the longitudinal slit appears to be important and best results especially for drug applications seemed to be obtained when the cut was made on the side, close to the penetration of the DVN, rather than on the opposite surface of the

capsule. The reason for this is uncertain but it is likely that the circumferential arrangement of the receptors around the hair follicle is not uniform, with a possible bias towards the side of DVN entry. Variations in the dissection procedure included cutting off the part of the cavernous sinus below the entry level of the DVN and completely removing the sinus body capsule.

Removing the capsule completely involved more delicate manouvering and a new sharp blade was imperative. The capsule was slit on two or three sides and then, with the aid of two fine pairs of scissors, holding the edges, the cut ends were gently peeled apart from top (skin surface) to bottom. The part of the capsule attached to the penetrating nerve was peeled last by holding on to the hair shaft. The epineurial sheath of the DVN is continuous with the sinus body capsule, so gentle intermittent tugs can remove the last bit of the capsule clean along with the epineurial covering of the DVN (the one occasion when a shorter nerve stump was a helpful tradeoff!). Often the DVN was pulled out as well (success rate about 2 out of 3 vibrissae tried). The result was a "desheathed" hair with the hair follicle and DVN devoid of their usual coverings.

Dissected hairs were kept in SIF (continuously bubbled) at room temperature until they were required for electrophysiological recordings.

Table 3.1 Materials for preparation of 5L of SIF

	Material	Supplier	Weight/5 Litres
1	NaCl	Merck	31.50g
2	KCl	Merck	1.30g
3	$MgSO_4 .5H_2O$	Merck	0.85g
4	$NaHCO_3$	Merck	11.00g
5	D-Gluconic acid	Sigma	10.50g
6	$NaH_2PO_4.2H_2O$	Merck	1.30g
7	D-Glucose monohydrate	Merck	5.00g
8	Sucrose	BDH	13.00g
9	$CaCl_2$*	Merck	1.125g

Not added until the solution has been prepared and bubbled with carbogen for some minutes to prevent precipitation.

3.1.3 Synthetic Interstitial Fluid (SIF) preparation

For a 'normal' day of experimentation, 5 litres of solution was prepared. All materials used were at least of analytical grade. For 5 litres of solution, the materials were weighed as shown in Table 3.1.

The final composition of the solution is shown in Table 3.2 with the pH being maintained at 7.4.

Table 3.2 Composition of SIF

	Ions	Concentration (mM)
1	Na^+	145
2	K^+	3.5
3	Ca^{2+}	1.5
4	Mg^{2+}	0.69
5	Cl^-	114
6	HCO_3^-	26.2
7	PO_4^{2-}	1.7
8	SO_4^{2-}	0.69
9	Gluconate	9.6
10	Glucose	5.55
11	Sucrose	7.6

pH = 7.4

Osmolality = 290 mosm

Buffered with 95% O_2 + 5% CO_2

3.1.4   The organ bath

The organ bath used was the same as the one used for the *in vitro* skin-saphenous nerve preparation (Reeh, 1986; Tsu & Baumann, 1992), with some modifications. The bath consists of two main compartments, an outer (larger) stimulation chamber and an inner tissue chamber. The modifications made in the organ bath were (a) reduction in the volume of the outer chamber by about two thirds. This was achieved by filling up the redundant rectangular space (see fig. 3.2a) with silgard. (b) The inflow of SIF was in the smaller (tissue) chamber where the entire hair follicle and nerve were placed. The inflow was arranged to achieve turbulent flow within the chamber. A rectangular silgard platform, about 1cm x 0.5cm x 0.2cm, was fixed to the middle of the tissue chamber to serve as a stage on which to fix the vibrissal follicle.

Essentially, the organ bath is as shown in Figure 3.2. It was made of perspex and weighed about 250g including the silgard additions described above. The tissue chamber was separated from the outer chamber (henceforth referred to as the stimulation chamber), by a removable perspex 'separator' which had a hole in the middle (diameter of about 2mm). This was the only connection between the two chambers.

The flow of solution within the bath was as shown by the curly arrows in Figure 3.2. Continuous superfusion was maintained using a peristaltic pump (Ismatec MS-Reglo, Switzerland). SIF was pumped at controlled rates (1.5 - 2 ml/min) into the tissue chamber and flowed out into the stimulation chamber through the separator hole from whence it

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was actively pumped out by the peristaltic pump. The total SIF volume in the tissue chamber was maintained at about 2ml and the temperature at 33 - 35°C, by pre-heating the SIF in the inflow tube using a temperature controller.

Silver wire electrodes, consisting of recording, reference and earth components, were used for recording. The recording electrode was electrically separated from the other electrodes by immersing it in a layer of Fluorinert FC-40 (Sigma Chemical Co.), with which the tissue chamber was filled up to just below the level of the silgard dissecting platform. Fluorinert is a liquid mixture of completely fluorinated aliphatic compounds. Fluorinert FC-40, used in these experiments has a density of 1.85g/ml, thus the turbulent SIF flow could be maintained above while the active electrode was isolated below (see Figure 3.2a). The choice of Fluorinert was fortuitous. Having tried to use paraffin oil as a layer above the SIF as in the rat skin-nerve preparation (see Tsu & Baumann, 1992), and in the salamander skin-nerve preparation (Diamond *et al.*, 1986), it was quickly discovered that the oil was carried away with the SIF into the outer chamber and the electrical isolation was soon no more. A heavier grade of paraffin oil was tried (Heavy white oil from Sigma), but that too was washed out after 20 - 30 minutes. It was only then that it was discovered that Fluorinert FC-40 had in fact been in use in the laboratory in the *in vivo* setup.
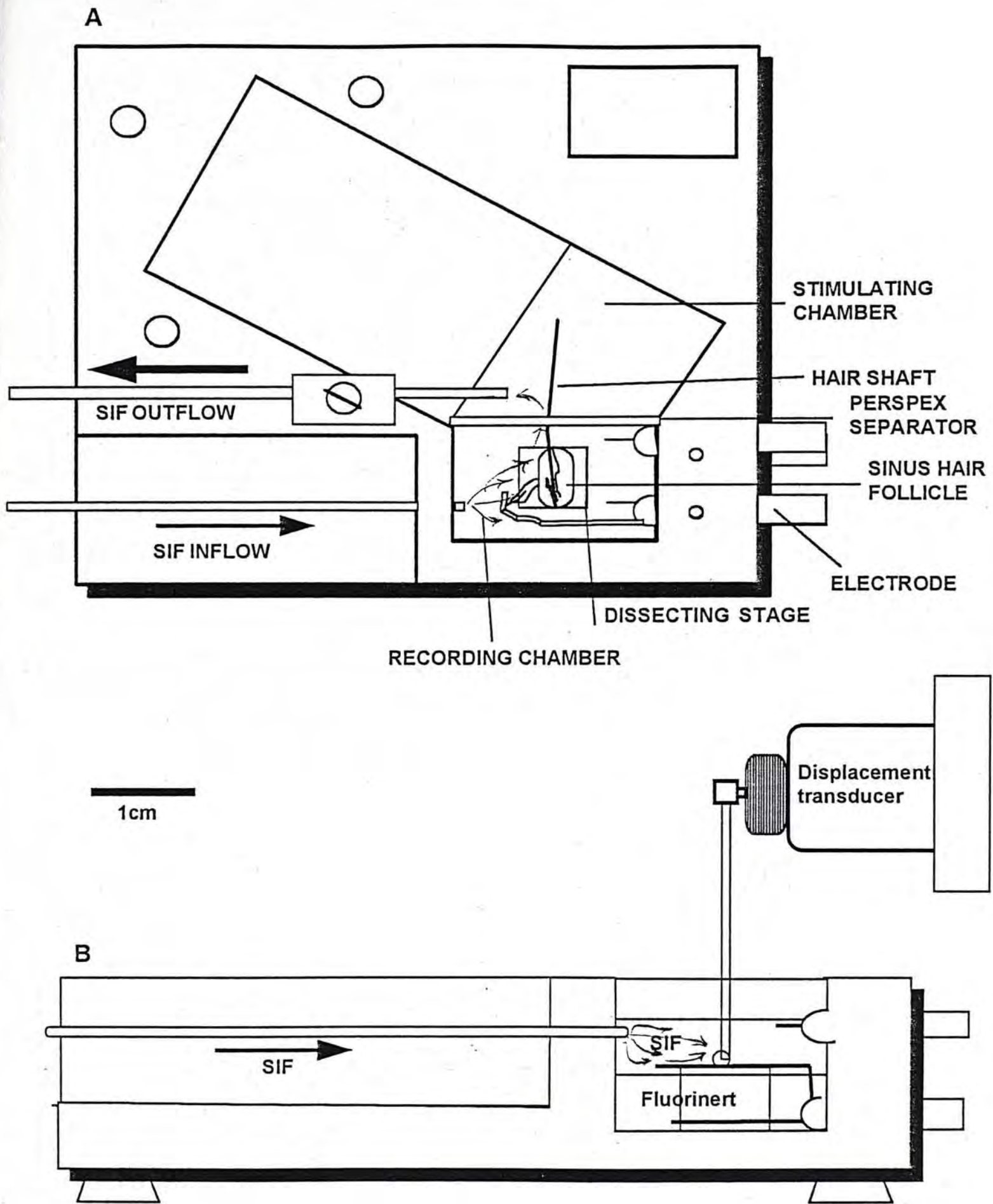
Figure 3.2

Drawing of the modified organ bath used for the isolated vibrissal preparation.

A top view. **B**. side view. See text for description.

3.1.5 Recording procedure

a). Mounting:

To make recordings the dissected hair was mounted on the silgard platform in the tissue chamber using fine insect pins. This was after the perfusion had been stabilised both in terms of flow and temperature, and electrodes put in place. Three to four pins were required; one on either side at the rete ridge collar (where there may still be a bit of intervibrissal skin), and one at the base of the follicle, at the distal tip of the cavernous sinus. Two pins may be used at this level, to attain a 'wide open' configuration of the slit capsule and hence improve diffusion, especially when the part of the cavernous sinus below the level of entry of the DVN was cut off as described earlier. Care was taken to avoid pinning the DVN directly or applying too much tension on it.

This stage was the most difficult part when dealing with 'desheathed' hairs, with the capsule completely removed. Apart from the distal tip of the follicle, there was hardly any tissue to pin the the hair to the platform, making it difficult to achieve a steady controlled stimulation when recording from such hairs. The other end of the hair was thus fixed indirectly by inserting two pins on either side of the hair shaft, just distal to the level of the inner conical body (the rete ridge collar having been removed with the capsule), thus restraining the upper part. This was often still unsatisfactory since the capsule tended to rotate when the hair shaft was displaced as part of the stimulation process (vide infra). Thus the mounting process further limited the success rate when using desheathed

hairs. It was well worth the trouble though because, diffusion was excellent and the onset of drug effects were usually manifested must faster.

b).   **Dissecting the DVN:**

Having properly mounted the hair follicle, the DVN was further dissected using fine No. 5 stainless steel forceps. The epineurial sheath was dissected off the tip of the nerve stump and then gently eased away towards the capsule where it may be left or partially cut off, depending on the length of nerve stump still available. It often helped to pin this bit of the epineurium to the silgard platform as well. This was not necessary in 'desheathed' preparations since the capsule and the epineurial sheath would have been removed already.

The nerve was then gently teased and split into smaller bundles. Each bundle was placed on the recording electrode in the Fluorinert layer below and tested for the presence of mechanosensitive fibres by pushing the hair shaft in the recording chamber with a hand-held probe while monitoring the generated signals (the voltage difference between the recording and reference electrodes) on an oscilloscope. The teasing process was continued until single unit responses were obtained, with the spikes being of uniform amplitude and shape (Figure 3.3 for sample oscilloscope screens).

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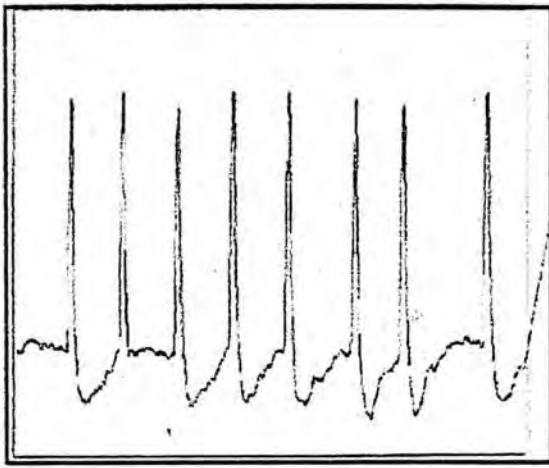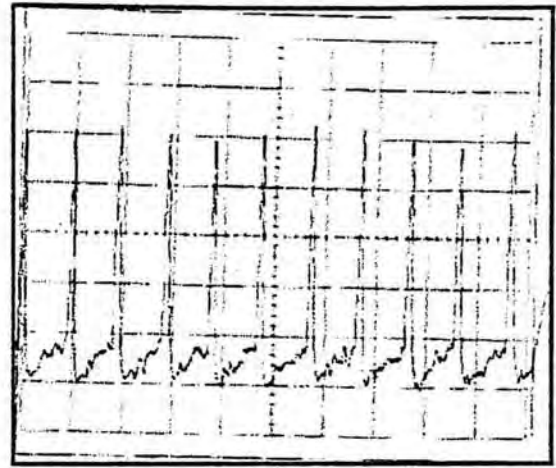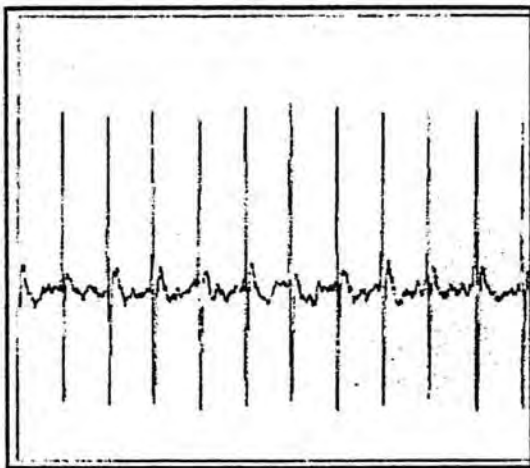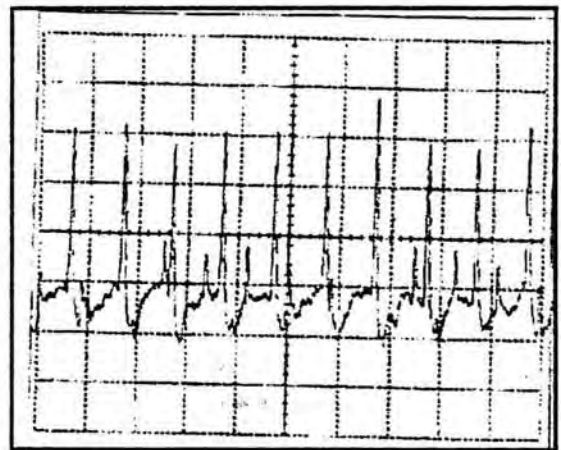
Figure 3.3

Sample oscillograms. Single units (**A,B**) with uniform spike shapes and amplitudes may be biphasic (**C**). **D.** Multiunit window with sufficient differences in spike amplitudes to enable recording of either unit by cutting off the undesired one using the 'window' discriminator.

c). Mechanical stimulation:

The hair shaft was firmly attached to the probe of a mechanical transducer (Figure 3.2b) about 5 to 10mm from the hair bulb. Mechanical stimulation was applied as feedback controlled displacements using an electro-mechanical transducer (Brad & Uper 4810). Displacements were applied in the most sensitive direction which was usually either a pull (-ve) or push (+ve) in the horizontal plane. Displacement amplitudes used were between 0.5 and 1.5mm (push or pull). The stimuli (mechanical displacement) were applied for 5sec (500ms ramps and 4000ms plateau) every 30sec (or 60sec in some early experiments when it was thought that 30sec cycles may lead to early exhaustion of the receptors).

d). Signal processing:

The signals were preamplified with an a.c. preamplifier (NL 104) and band filtered (NL 125, 70 - 5000 Hz), using the Neurolog modular system (Digitimer Ltd, England). Signals were subsequently passed through a spike processor (Digitimer D130), for window discrimination and counting. The spikes were then displayed on a digital storage oscilloscope (Tektronix, 2430A), and recorded on a chart recorder (Astro-Med MT 95000) as cumulative spikes. The whole system was controlled by an 80486-based microcomputer through a CED1401+ laboratory interface (Cambridge Electronic Design, UK) using a program written in our laboratory using Borland's TurboPascal. The number of spikes during the dynamic and static phases of each stimulus period

including their interspike interval histograms were computed and displayed online (see Figure 3.4 for a sample screen), and stored on hard disk for offline analysis. From the stored data, the interspike intervals could be computed for any period during the applied stimulus with a bin width of 1ms. The number of spikes during the stimulus intervals were recorded, displayed online and stored on disk in like manner. The main parameter used as an indicator of responses was the number of action potentials for the 5-second duration of the applied stimulus. The stored data was however available for use in examining other parameters like the interspike intervals, rate of adaptation, e.t.c.
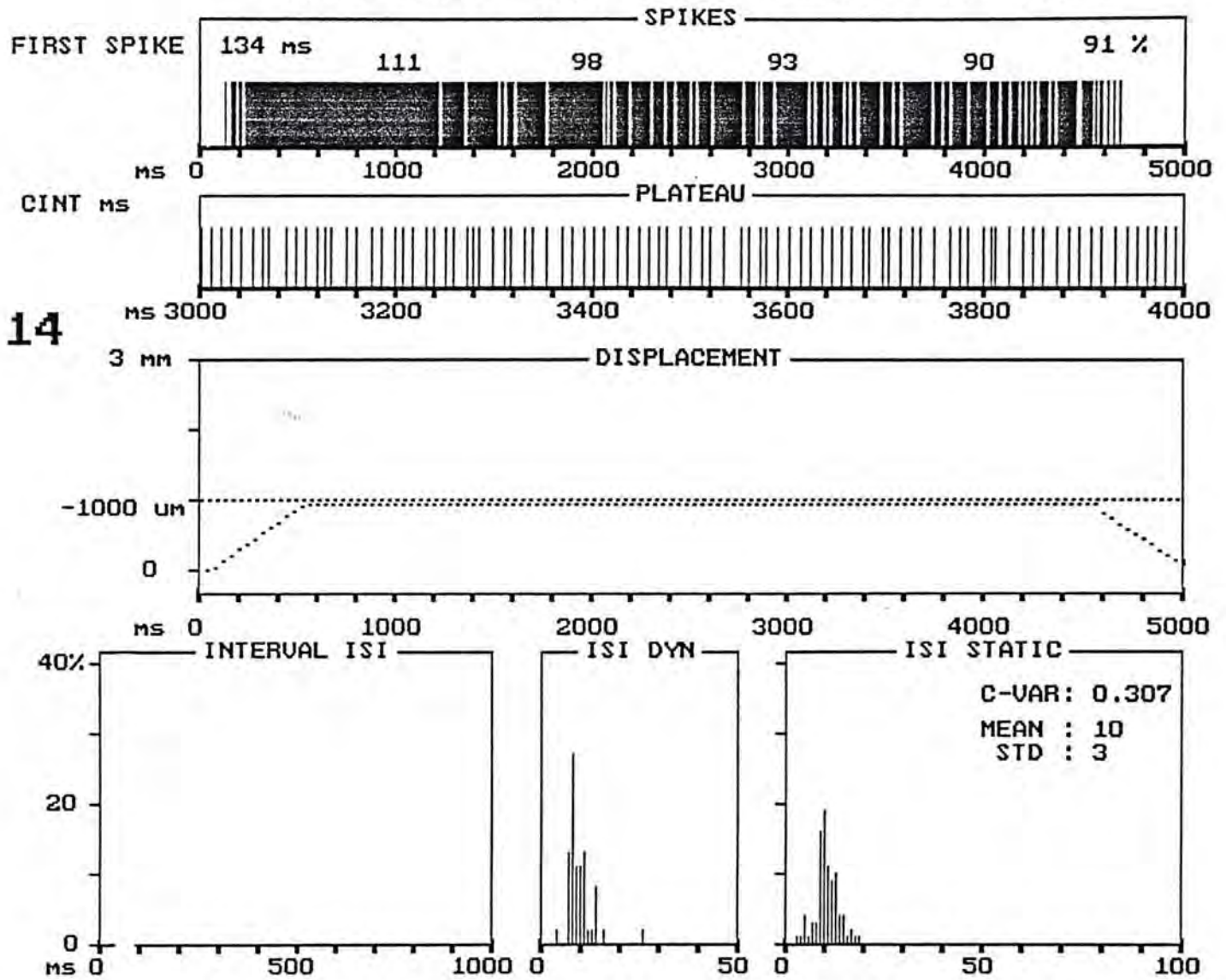
Figure 3.4

Sample computer screen showing the data acquired and computed online during each 30sec cycle. The upper line of text shows the stimulus number, diplacement (µm), spike counts during the 2nd (C1) and 3rd (C2) 1000ms of the plateau phase, the coefficient of variance (CVAR x1000) and the spike counts during the dynamic (CDYN), plateau (CPLAT), total period (CSTIM) and static (CSTAT) phases respectively. A code may also be entered through the keyboard (as an event marker) as the experiment progresses. The "spikes" and "plateau" boxes show the spike trains. The 'plateau' box displays a faster time scale of the period from 3000 - 4000ms and better shows the pattern of discharge. As a rough index of adaptation, the number of spikes during the static period from 3500 - 4500ms is divided by the number of spikes during the period from 1000 - 2000ms (91% in this case). Interspike interval (ISI) histograms during the interval (spontaneous counts), dynamic phase and static phase are shown in the lower three boxes. The shape of the displayed ISIs also aids in identification of the type of unit online. This unit is 'silent', hence the empty 'interval ISI' box. *CDYN=dynamic counts; CSTAT=static counts; CPLAT=plateau counts (CSTIM-CDYN); CSTIM=stimulus counts (CDYN+CPLAT).*

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e). Pharmacological testing:

When a pharmacological agent was to be tested on the preparation, the required concentration was prepared in SIF and switched for normal SIF using the same inflow system. Normal SIF superfusion was then restored at the end of application of the test substance.

f). Identification of receptor types:

To identify the type of receptor being recorded from, the discharge pattern of a receptor in response to mechanical stimulation was analysed online. Parameters considered included, sensitivity of the receptor to mechanical stimulation (see later) and the regularity of discharge during the static phase of stimulus application. The latter was the most readily evident distinguishing hallmark, since the two receptors of interest were both slowly adapting. With experience, the audio output from the spike processor was often diagnostic. From the stored data, the characteristic discharge pattern of the receptors could be further confirmed offline.

3.2   EXPERIMENTAL PROTOCOL

3.2.1 To test suitability and stability of the preparation

To ascertain the suitability of the preparation, prolonged recordings (5s every 30s) were carried out over periods of 5 hours or more on identified single units.

Viability was further tested by storing dissected hairs in normal SIF in the refrigerator for 24 - 48hrs. Stored hairs were retrieved, allowed to gradually return to room temperature and then used for recording as described above.

The ultrastructure of the vibrissal receptors were examined before and after prolonged stimulation as well as after storage to ensure that the receptors retained their structural integrity. Some 'fresh', '24-hour' and '48-hour' hairs were thus fixed in 6% glutaraldehyde for 20 minutes and further processed for electron microscopy.

3.2.2 Mechano-electric transduction mechanisms

Pharmacological agents were tested on identified single units as part of experiments aimed at elucidating the mechanism(s) of mechano-electric transduction in the receptors. In this regard, the Merkel nerve endings were of particular interest. The agent to be tested was first prepared in SIF at the appropriate concentration from a stock solution (where applicable), and superfused through the inflow system. Thus the

same temperature and flow conditions as for normal superfusion were maintained. Perfusion tubes were regularly washed out with H_2O_2 (after experiments) to prevent accumulation of agents in the walls of the tubes.

In some experiments, the effect of the applied pharmacological agent on ultrastructure of the receptors was examined. Such hairs were fixed at the onset of recognised drug effects, during maximal effect and after recovery.

Table 3.3 List of compounds used.

	COMPOUND	SOURCE	Cat. no.
1	3-methyladenine	Sigma Chemical Co.	M9281
2	Caffeine	Rein	Art. 2584
3	Chloroquine diphosphate	Sigma Chemical Co	C6628
4	Heparin Na salt Grade II from porcine intestinal mucosa	Sigma Chemical Co	H7005
5	Magnesium Cl	Sigma Chemical Co	M5005
6	Methylamine HCl	Sigma Chemical Co	M0505
7	Pentosan polysulphate (Av. Mol. Wt. 3000)	Sigma Chemical Co	P8275
8	Procaine HCl	Sigma Chemical Co	P9754
9	Quinacrine dihydrochloride	Sigma Chemical Co	Q3251
10	Ryanodine	RBI	R100
11	Verapamil HCl	Sigma Chemical Co	V4629

3.2.3 Electron microscopy

Specimens for electron microscopy were fixed in 6% glutaraldehyde in 0.1M sodium phosphate buffer for 20 minutes at 4°C and post-fixed in 1% OsO_4 in 0.1 M phosphate buffer with 1% sucrose. The specimens were stored in 0.1M sodium phosphate buffer for several weeks, cut in ca. 1mm thick blocks and dehydrated in alcohol. Embedding in Epon 812 was done according to the method described by Luft (1961). Serial semithin sections (1µM thick) were cut on OmU4 ultramicrotome (Reichert and Porter Blum) and stained with toluidine blue and pyronin red according to Laczko and Levai (1975). Selected semithin sections were re-embedded for electron microscopy and thin sections were stained according to Reynolds (1963) with lead citrate. Sections were examined with a Philips 200 electron microscope in Prof. Z. Halata's Department of Functional Anatomy at the University of Hamburg.

3.3 DATA ANALYSIS

3.3.1 Online

Online, all data were digitized using the CED1401+ general laboratory interface (Cambridge Electronic Designs, UK) and a 80486-based microcomputer. Online analysis was restricted mainly to examination of the computer screens which were refreshed during each

30sec cycle with the main aim of ascertaining the receptor type being recorded from.

3.3.2 Offline

Offline data analysis was carried out using an in-house program written in Borland's TurboPascal. With this program, relevant data of choice could be extracted for use from the original storage files on hard disk. The most common extraction procedure was to concentrate on the number of spikes during the dynamic and static phases of stimulation, the total number of counts as well as the number of spikes during the intervals. Most of the graphs plotted utilized data from this extraction. Interspike interval (ISI) data used to plot the ISI histograms were also extracted as required using the same program.

To characterise the slowly adapting receptor types, the following parameters were compared:

i. **Dynamic sensitivity:**

As an index of the sensitivity of the receptor, the number of spikes during the 500ms dynamic phase of stimulation was compared to the counts during the last 1000ms of the static phase. This was computed as the **Dyn:Stat ratio.**

ii. **Adaptation rate:**

This was computed as a percentage by dividing the spike count

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during the last 1000ms of the static phase by the counts during the second 1000ms of the static phase.

iii. Discharge regularity:

The coefficient of variance (CVAR) of the interspike intervals were computed and used as an index of regularity of the spikes.

iv. Spontaneous activity:

The presence and magnitude of spontaneous firing as well as the regularity of such firing was also examined and compared for the two types of receptors.

Graphs were plotted for individual and grouped data using the commercial software SigmaPlot for Windows (Jandel Scientific).

Mathematical transformations and statistical analyses were carried out using Sigmastat for Windows (Jandel Scientific). Individual experiments were normalised by taking an early period of 10 stimuli as 100%, to eliminate inter-unit variations. For testing of drugs, the response to mechanical stimulation at the end of drug superfusion was compared to the period immediately before drug application. Thus each unit served as its own control. Unless otherwise stated, results were expressed as mean±SEM. Student's t-test was used for statistical analysis. A p value of ≤ 0.05 was considered significant.

Chapter 4
<u>RESULTS</u>

4.1 CHARACTERISATION OF THE RECEPTORS

Both slowly- and rapidly adapting mechanoreceptors were identifiable in the preparation. The rapidly adapting receptors were not examined any further in the present study. Two major types of slowly adapting mechanoreceptors were identified. A possible third type of slowly adapting unit was also recognised. Because the characteristics of the receptors found closely resembled those descbribed by Gottschaldt *et al.* (1973) in whole animal experiments using cats, their nomenclature has been adopted i.e. Sinus hair type I, II, etc (St I, St II etc), with any subclassifications being denoted by lower case letters, e.g. St IIa).. All types of receptors could be found regardless of the particular variant of the dissection procedure used, including the 'desheathed' hairs.

4.1.1 Sinus type I (St I) mechanoreceptor

This receptor was found to be highly sensitive to bending of the hair. When the hair shaft was affixed to the stimulating probe, even slight tapping of the of the heavy iron table on which the setup was rigged, elicited responses, in tune with the tapping. As a rough index of this sensitivity, the dynamic-static ratio was computed by dividing

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the number of counts during the 500ms dynamic phase of stimulation by the number of spikes during the last 1000ms of the static phase of stimulation. This ratio was found to be consistently >50% in this type of receptor and was significantly higher than the second receptor ($p<0.0001$, see Figure 4.3).

The St I had an irregular discharge pattern (Figures 4.1 and 4.4), with the interspike interval histograms having a wide distribution with a coefficient of variance (CVAR) >0.1 (mean 0.505 ± 0.190 (mean $\pm$ sd), range 0.180 - 0.861 in 14 consecutive St I units, (see Figure 4.4)). There was a low tendency to spontaneous discharge. When present, this was found to be of an irregular pattern (e.g. see Figure 4.1 'Interval ISI' box).

The approximate location of the receptor was determined by gently probing the follicle for the point/area of maximum sensitivity; the St I receptors were found to be always located within the upper 1/2 - 1/3 of the hair follicle, at the level of the ring sinus, usually above the ringwulst.

4.1.2 Sinus Type II (St II) mechanoreceptor

The most notable characteristic of the second receptor was its highly regular discharge pattern (Figure 4.2), with CVAR <0.1 (0.067 ± 0.016, mean $\pm$ sd; range 0.044 - 0.096, in 15 consecutive St II receptors, Fig. 4.4). There was a higher tendency to discharge spontaneously; e.g. 50% of 57 consecutive St II units examined

exhibited spontaneous discharge while the figure for St I units was about 35%. The spontaneous discharge was found to be highly regular (Figure 4.2 'interval ISI' box). The approximate location of the receptor was usually within the middle third of the follicle, also at the level of the ring sinus but mostly at or slightly below the ringwulst.

4.1.3 Sinus type Ib or Sinus type III?

A third, infrequent type of unit was found which had distinctly different response characteristics from the two described above. A total of 13 such unit were recorded from. Typically, the spikes occurred in groups as couplets or triplets but may have as many as 6 - 8 spikes in a group. The spikes within a group were separated by short intervals while the individual groups were separated by longer intervals (see Figure 4.5). Thus there were 2 peaks in the ISI histogram. This feature was clearest during the static phase of stimulation. This distribution of ISIs also meant that the Coefficient of Variance was high, usually >0.5 and often >1. On the audio monitor, these units had a characteristic 'staccato' sound as the periods of short and long interspike intervals alternated.

Occasionally, the 'staccato' features became less prominent in the same unit, looking more like the St I but the CVAR of the ISIs remained relatively high (Figure 4.6). Thus the burst firing unit is likely to be a variant of the St I ending.

Figure 4.1

Sample computer screen of data acquired during a 5-sec simulus period from an St I unit. The 'plateau' box displays a faster time scale of the period from 3000 - 4000ms and better shows the irregular pattern of St I discharge. Interspike interval (ISI) histograms during the interval (spontaneous counts), dynamic phase and static phase are shown in the lower three boxes. The broad distribution (CF Fig. 4.2) is characteristic of St I receptors, with the CVAR being typically >0.1. Note that the ongoing activity (interval ISI) is also irregular in the St I. See Fig. 3.4 for a more detailed description of the sample screen.

STIM	DMIN	DMAX	DAMP	C 1	C 2	CVAR	CINT	CDYN	CPLAT	CSTIM	CSTAT	CODE
3	4	-1023	-1027	145	129	81	568	95	569	664	117	

DATE : 18/11/1994
UNIT 2   RUN 1   HAIR - 5 sec   STIMULI - CYCLE : 30 s
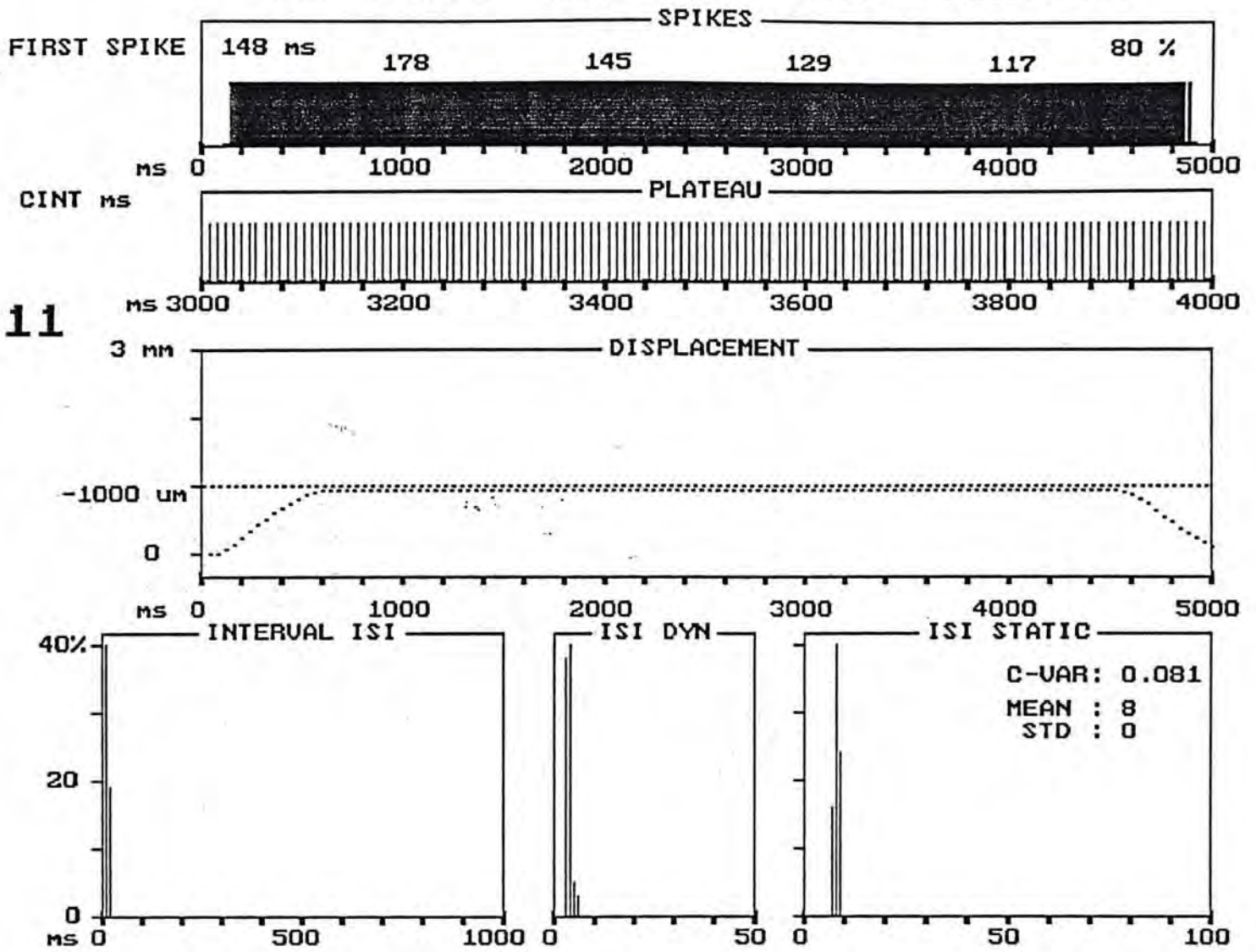
Figure 4.2

Sample computer screen of data acquisition during a 5 second simulus period showing the response characteristics of.a typical St II receptor with moderately high spontaneous firing. The 'plateau' box displays a faster time scale of the period from 3000 - 4000ms and better demonstrates the regularity of the discharge (CF Fig. 4.1). The interspike interval (ISI) histograms in the three lower boxes clearly demonstrate that ISIs in the St II are regular both in stimulated ('ISI STATIC' and 'ISI DYN' boxes) and spontaneous ('INTERVAL ISI' box) spikes; the histograms being tall and narrow (CF St I in Fig. 4.1). The CVAR of ISIs during the static phase is typically <0.1 as in this example. (see Fig. 3.4 for more detailed description of the sample screen).
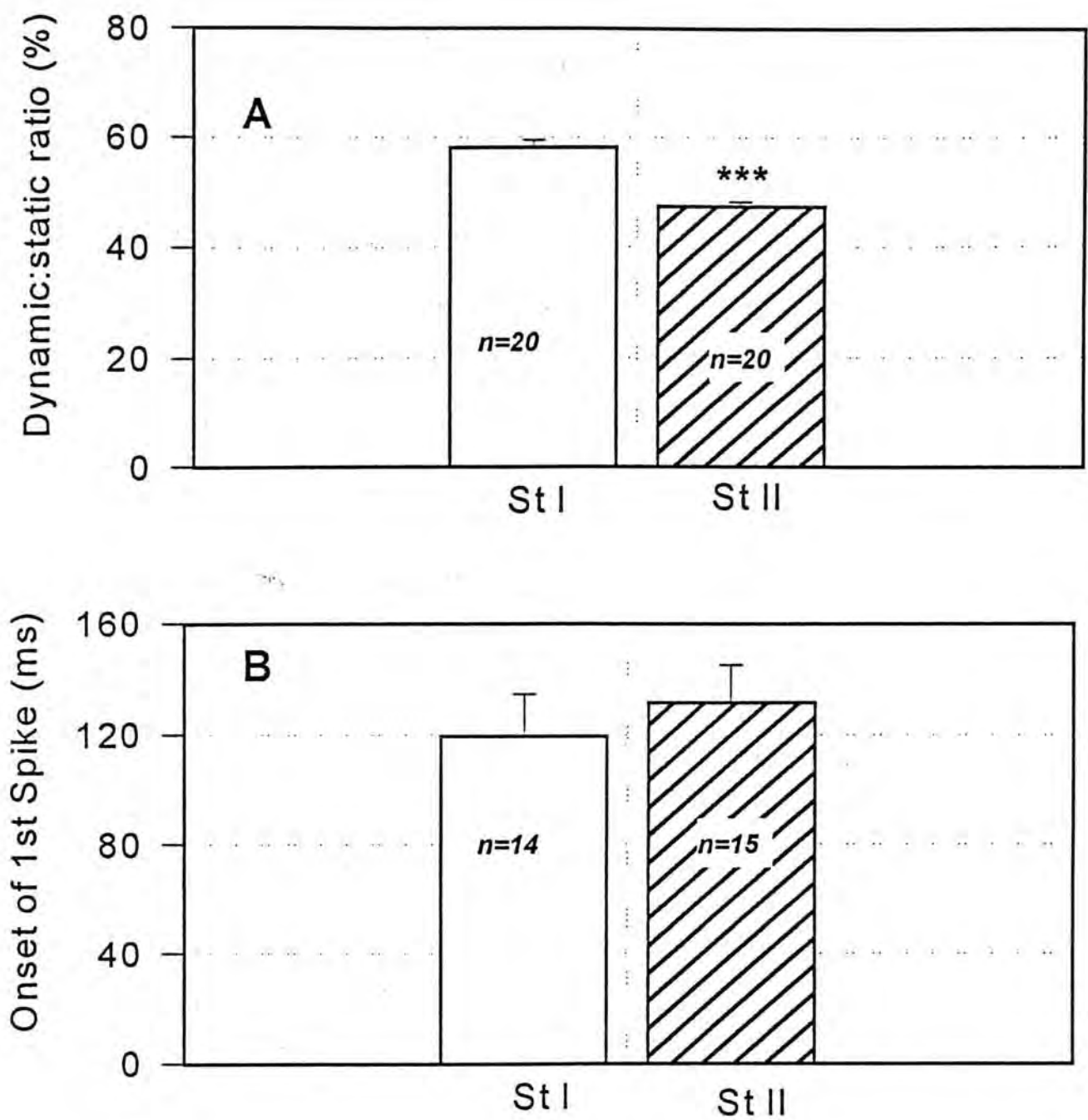
Figure 4.3

Relative sensitivity of St I and St II receptors compared in 20 consecutive St I and 20 consecutive St II receptors (**A**). The dynamic:static ratio was computed by dividing the number of spikes during the 500ms dynamic phase by the number during the last 1000ms of the static phase. St I receptors are more sensitive than St II receptors ($p<0.0001$). The onset of the first spike (**B**) tended to be earlier in the St I receptors (in keeping with the higher dynamic sensitivity shown in **A**), but the difference was not statistically significant. Units included in the '1st Spike' analysis were stimulated using the same (1mm) displacement amplitude, had similar (350 - 450 spikes per stimulus) response magnitudes, and were without spontaneous firing (at least during the segment chosen for analysis).
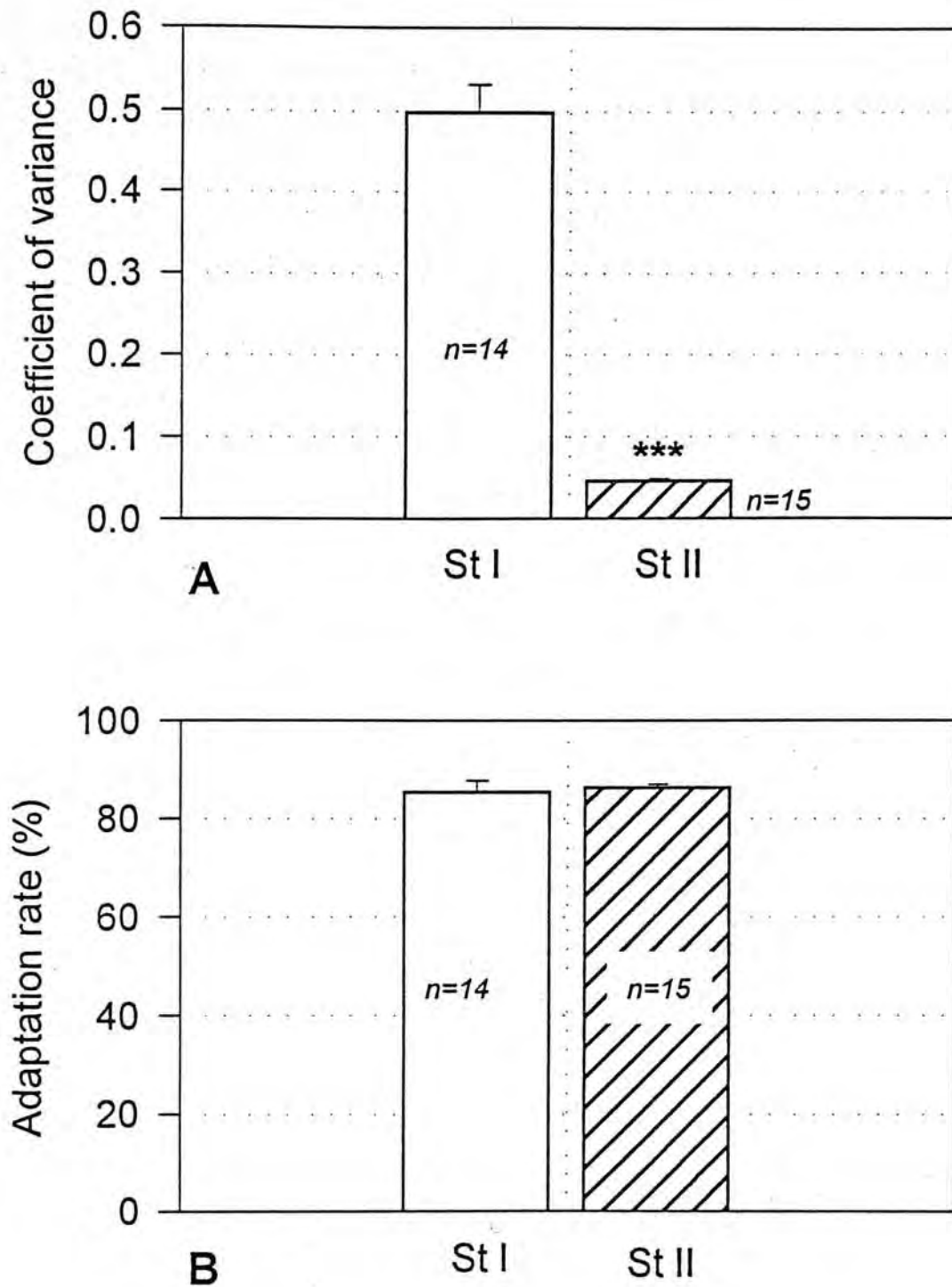
Figure 4.4

The coefficient of variance (A) and rates of adaptation (B) of 14 St I and 15 St II mechanoreceptors were compared. Within the 5s stimulus period used, there was no difference in the adaptation rates of the 2 receptor types. The coefficient of variance (CVAR) is an index of regularity of the interspike intervals and illustrates the differences between the highly regular St II spikes and the irregular St I spikes.
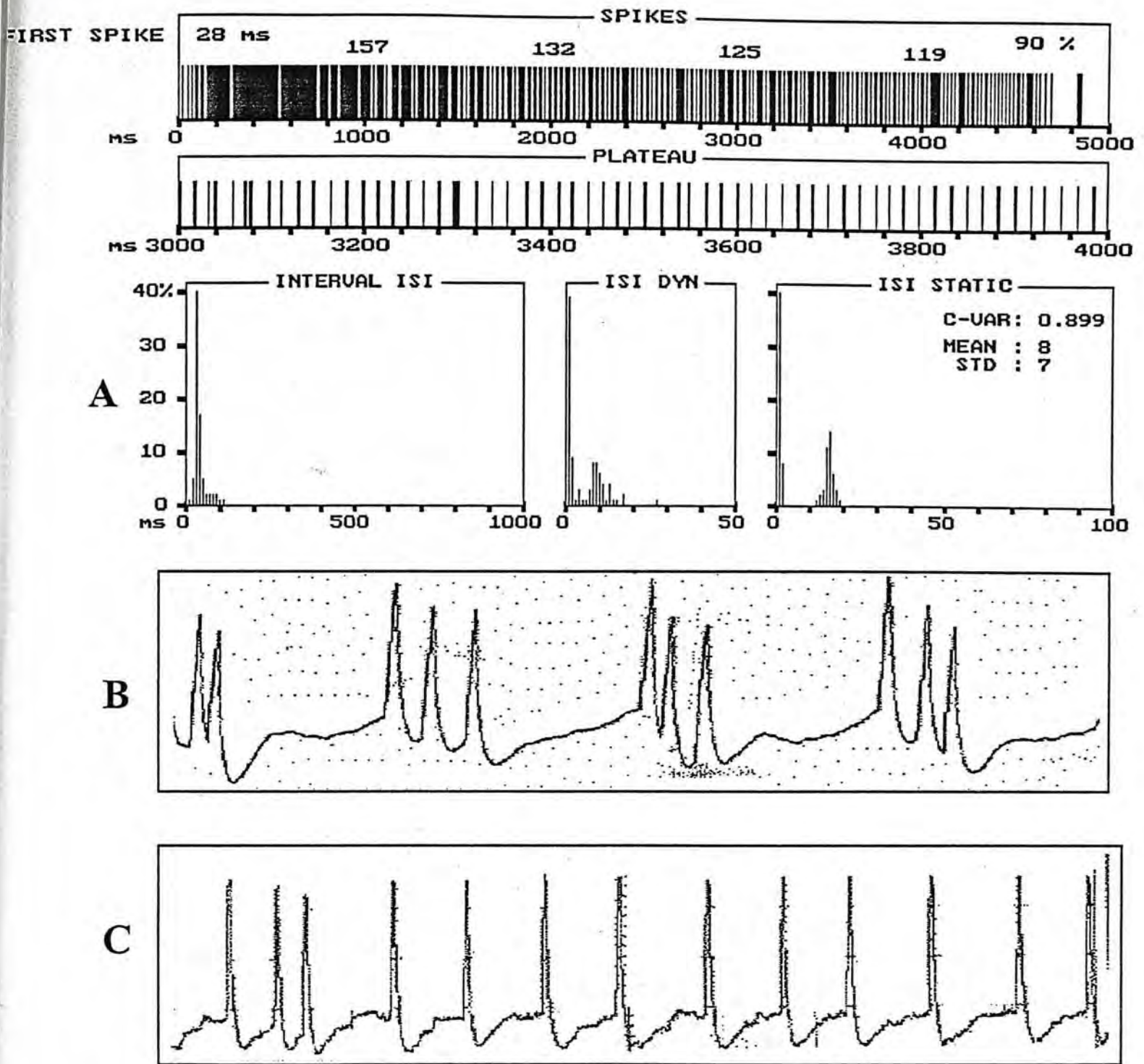
Figure 4.5

Response characteristics of the 'staccato' type of slowly adapting receptor. **A.** shows portions of a sample screen. The responses are irregular but appear to be regullarly so with groups of spikes separated by fairly regular intervals. The ISIs are typically double-peaked.Note the high CVAR of the of the interspike intervals. **B.** An oscillogram showing the action potentials occurring in triplets in this particlular example. **C.** Oscillogram from an St I for comparison.
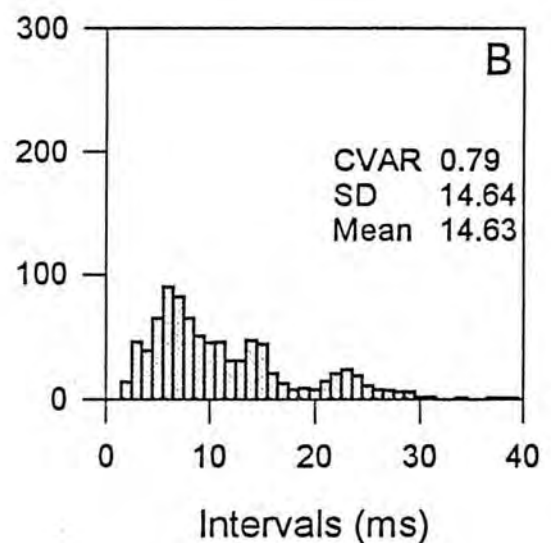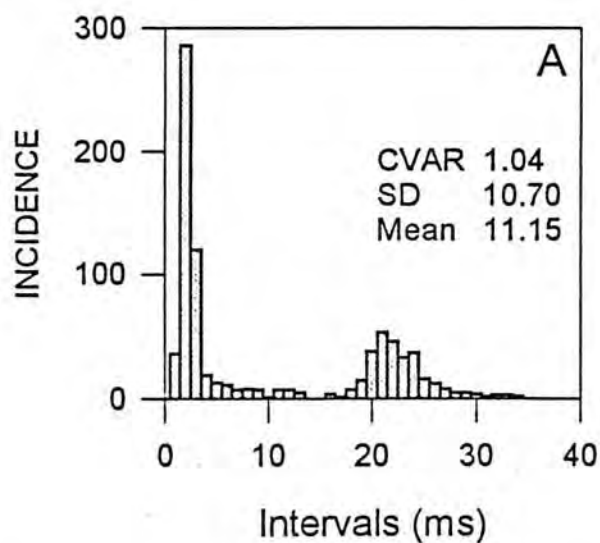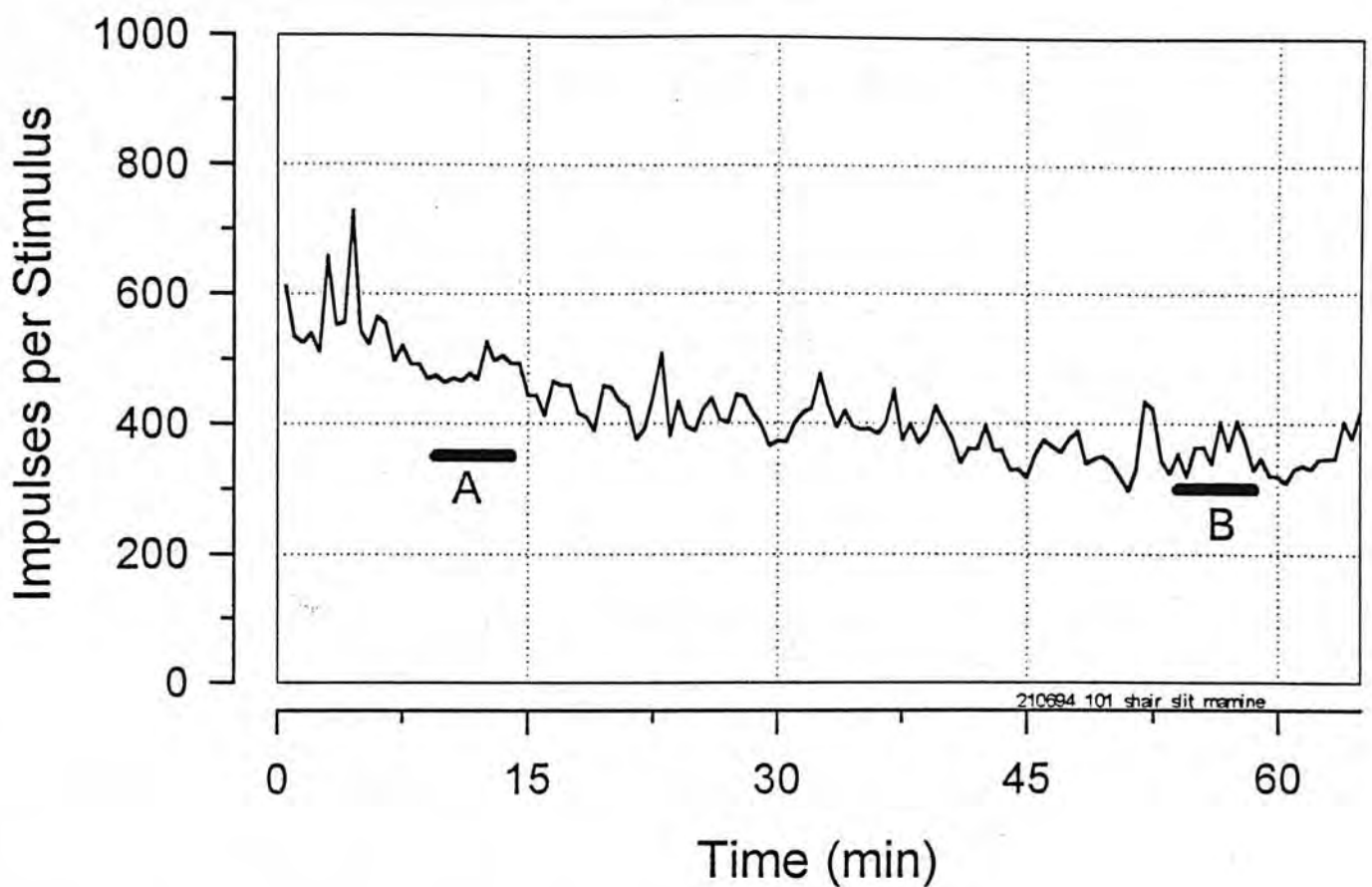
Figure 4.6

A 'staccato' type of slowly adapting vibrissal mechanoreceptor. The period of 10 stimuli marked A and B in the graph on the upper panel represent the times for which the interspike interval histograms A and B were plotted. A shows the typical pattern of ISI distribution as exemplified by the single sample screen in Figure 4.5. B however shows a pattern that looks more like that of the St I; the CVAR however remains relatively high (CF Figure 4.8).

4.1.4 Stimulus-response relationship

Within the limited range of displacement amplitudes used, the relationship between the amplitude of displacement and the responses (total number of spikes per stimulus) could be approximated by straight lines for both type I and II receptors (Figure 4.7). With higher displacement amplitudes one would expect a levelling off of the responses. The plot of the relationship also showed that St I receptors fired more spikes in response to the same amplitude of stimulation than did St II receptors ($p < 0.01$).

4.1.5 Relative frequency of the mechanoreceptors

Proportionately, the St II receptor was the most frequently encountered. For example, out of 345 slowly adapting mechanoreceptors recorded from, only about 40% were type I receptors in spite of the fact that the St I units were being actively sought out. When the number is broken down into time epochs, it can be seen that the likelihood of recording from St I units rose steadily with time (and experience). For example Table 4.1 shows that during three consecutive 6-month periods, the proportion of St I units recorded from went from 33% to 46% to 50%, appearing to improve with time and experience. The real proportion of St I receptors relative to St II is probably about 20%. Excising most of the cavernous sinus region below the level of entry of the DVN appeared to have made no difference on the relative ease with which type I or II units were found.
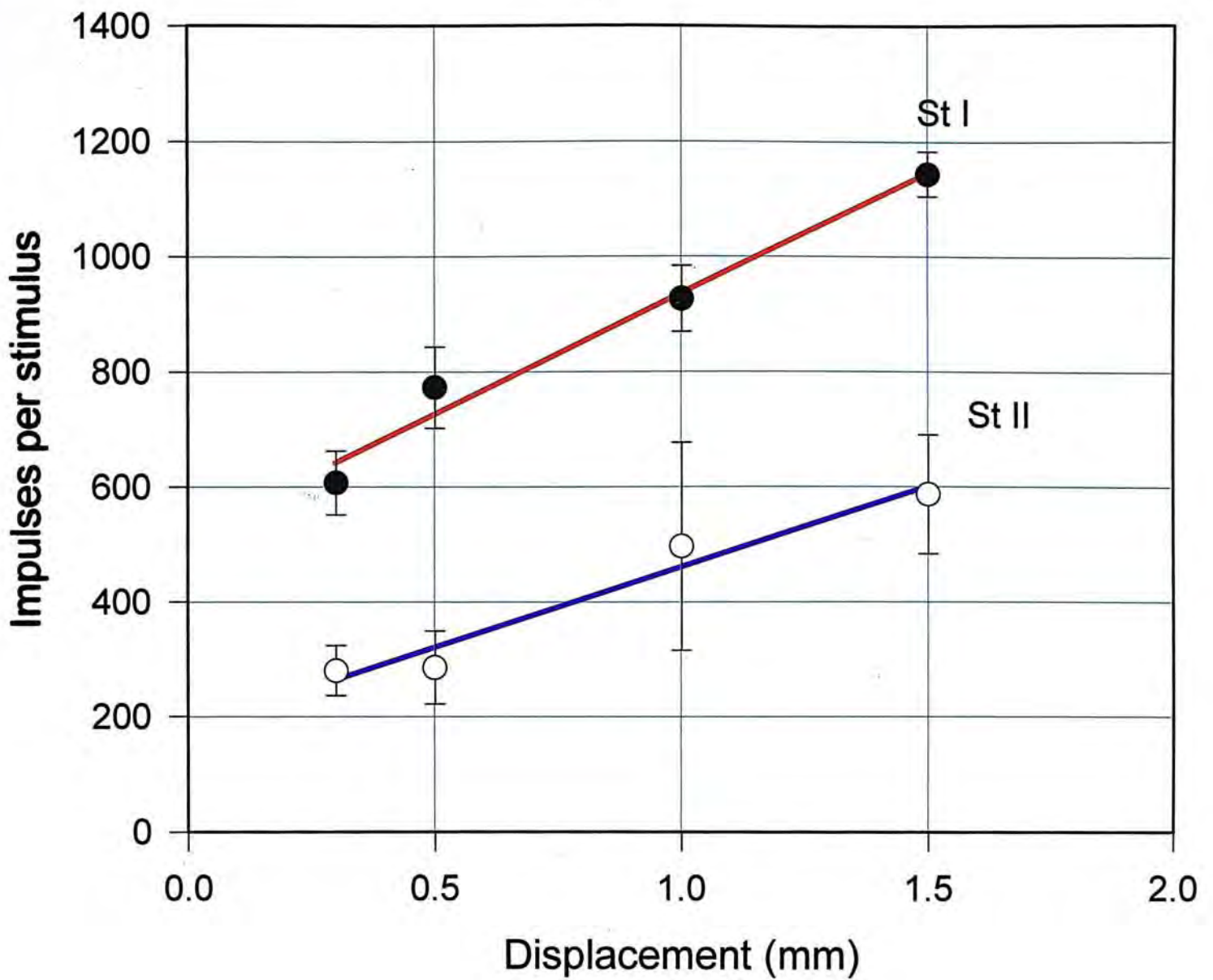
91

Figure 4.7

Stimulus-response profile for St I and St II receptors. Plotted points are means±SEM of 4 sets of observations. The relationship is well fitted by a 1st order linear regression line and would suggest a linear relationship between displacement and number of spikes in both St I and St II receptors. This is probably so because of the narrow range of displacements used as one would expect the number of spikes to level off as higher displacements are applied. For the same displacement amplitudes, St I receptors have higher counts than St II receptors ($p<0.001$, paired t-test).

Table 4.1 Number of St I and St II units recorded during 3 consecutive 6-month periods*.

6-mth. periods	St I		St II		Total	
	No. of units	% of total	No. of units	% of total	St I + St II	%
1	40	33	78	67	**118**	100
2	54	46	61	54	**115**	100
3	56	50	56	50	**112**	100
Total	**150**	**43.5**	**195**	**56.5**	**345**	**100**

This was in spite of the fact that St I units were being actively sought after. This table does not therefore represent the true relative frequency of the two types of receptor in the vibrissal follicle.

4.2   STABILITY AND VIABILITY OF THE PREPARATION

4.2.1 Stability during acute experiments

Steady responses could be recorded from both St I and St II receptors for at least 5 hours (Figures 4.8, 4.9) with only a slow decline of about 15 - 20% in responses observed at the end of 5 hours (Figure 4.10). Interspike interval histograms computed for 10 stimuli during the static phase of stimulus application from 3500 - 4500ms, remained well preserved during the 5 hour period (Figures 4.8, 4.9).

4.2.2 Viability of preparation

Recordings were made on vibrissae after 24 - 48 hours of storage at 4°C. After 24 hours of storage at 4°C sinus hairs were functionally indistinguishable from freshly dissected hairs (Figure 4.11); prolonged recordings of 5 hours could still be made on such hairs. Ultrastructually, the receptors were also well preserved. (Figures 4.14); as compared to the freshly dissected hair (Figure 4.13).

After 48 hours of storage however, responses to mechanical stimulation tended to be highly varied, stabilising only with difficulty and not lasting longer than about three hours (Figure 4.12). Ultrastructurally, Merkel cells in the outer root sheath still retained their characteristic features except for the apposing neurite terminal which was swollen. The keratinocytes also appeared shrunken after 48 hours.w
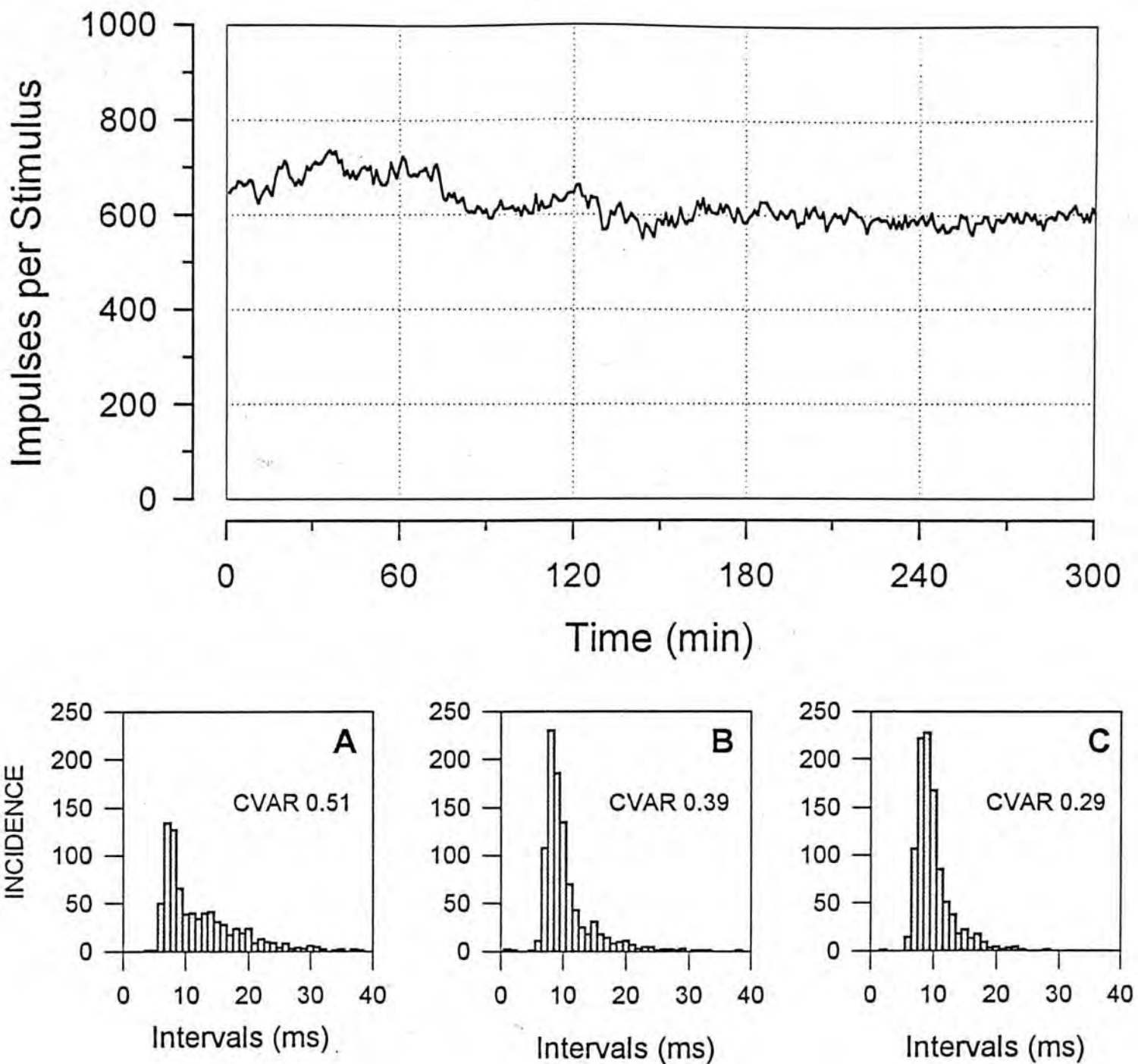
Figure 4.8

Responses from an St I receptor, showing stability over 5 hours of stimulation. The interspike interval histograms **A**, **B** and **C** are representative for responses to 10 consecutive stimuli during the 1st (minutes 21-25), 3rd (minutes 141-145) and 5th (minutes 261-265) hours of recording respectively. The shape of the ISI histograms are typically skewed in the St I (CF St II in Fig. 4.9).
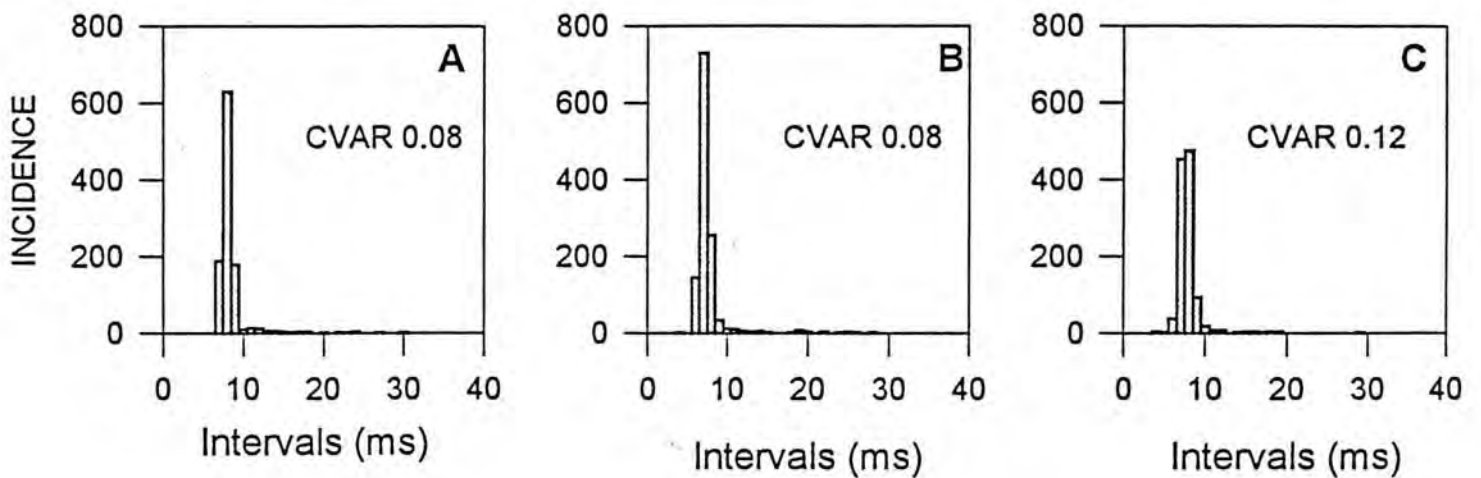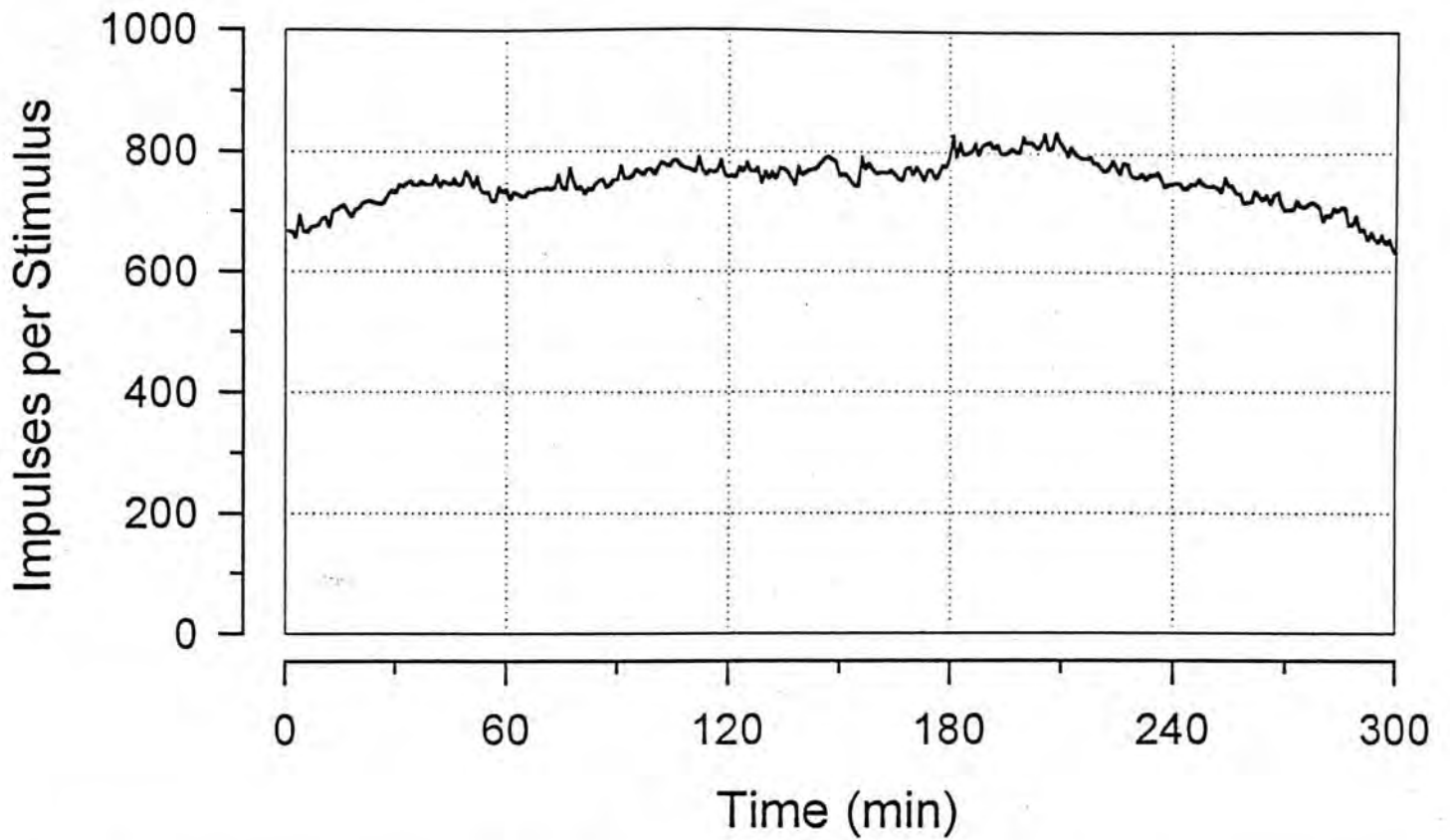
Figure 4.9

Stability of responses in an St II receptor over 5 hours of stimulation. **A, B** and **C** represent interspike interval (ISI) histograms during the 1st (minutes 31-35), 3rd (minutes 191-195) and 5th (minutes 291-295) hours of stimulation respectively. The ISIs are typically tall and narrow, a reflection of the regularity of the responses (CF Fig. 4.8).
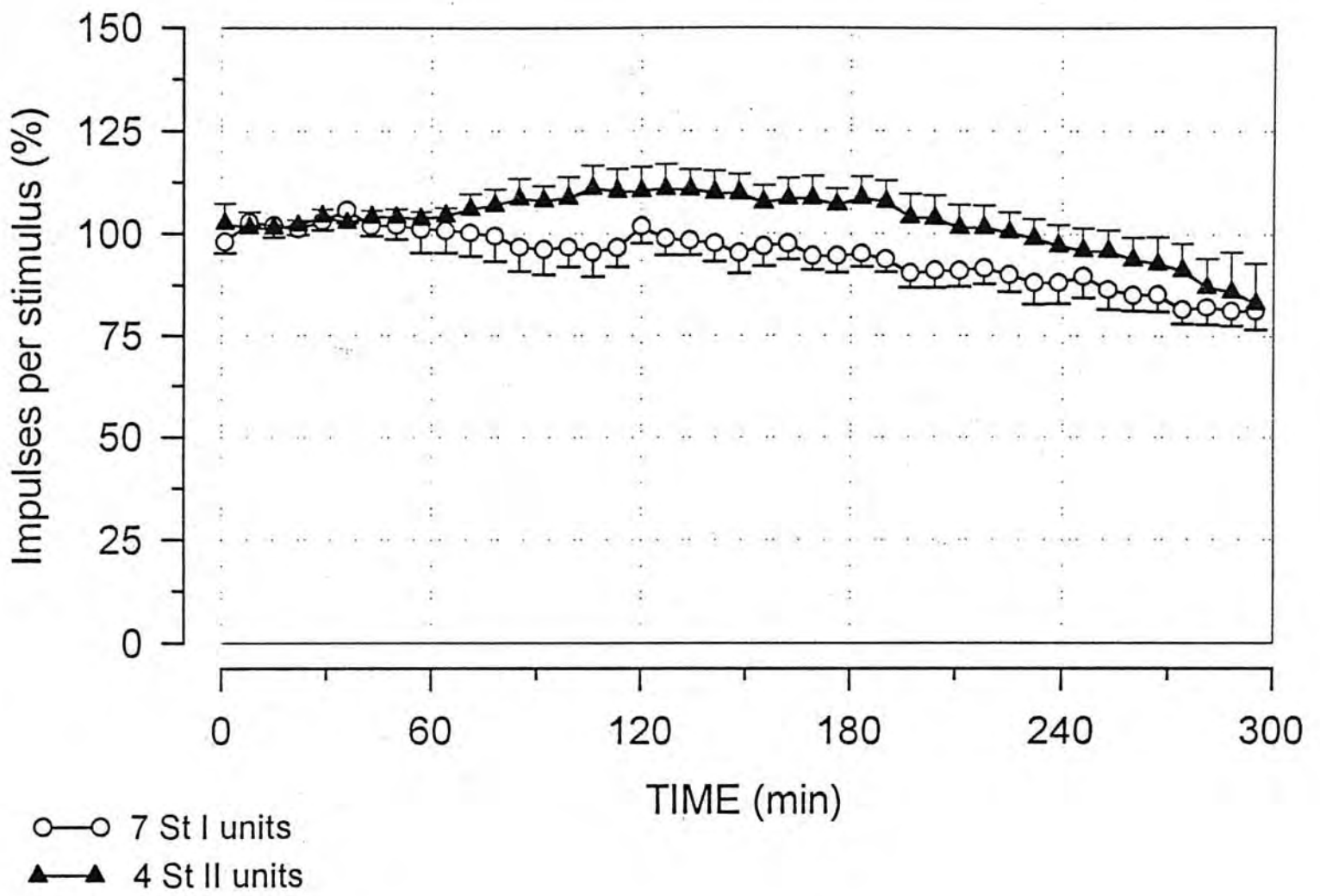
Figure 4.10

Stability of 7 St I and 4 St II receptors over 5 hours of stimulation (for 5 sec every 30 sec). Responses declined gradually by about 15 - 20% over the 5-hour period.
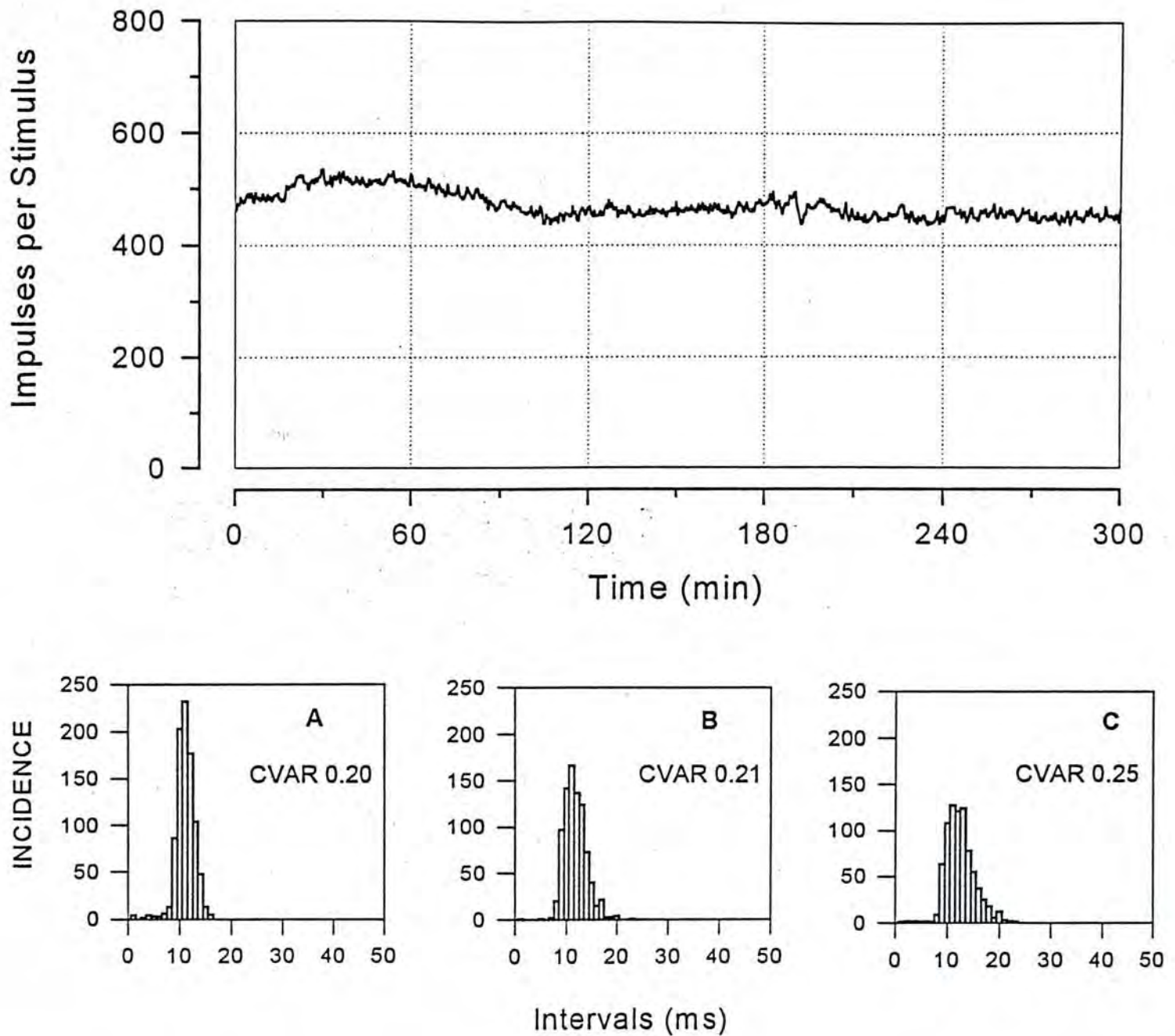
Figure 4.11

5 hours of stable single unit recording from a sinus hair after 24 hours of storage. **A**, **B** and **C** are interspike interval histograms during the 1st (minutes 31-35), 3rd (minutes 191-195) and 5th (minutes 291-295) hours of recording respectively. The stored hairs could not be functionally distinguished from the freshly dissected ones (CF stability of a similar unit from a freshly dissected hair, Fig. 4.8).
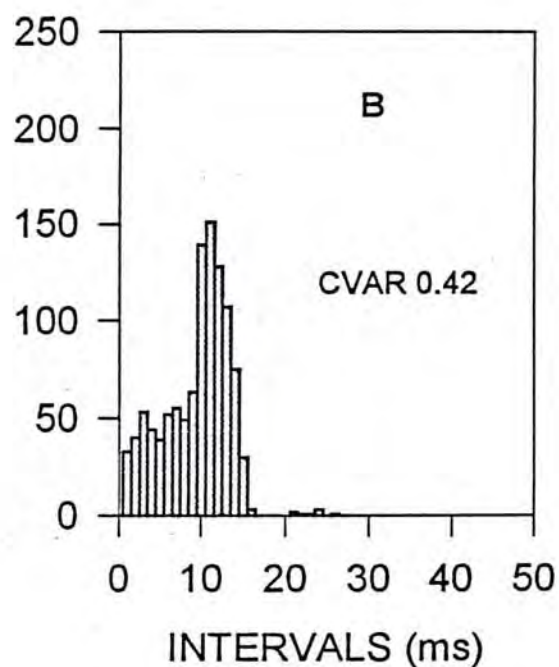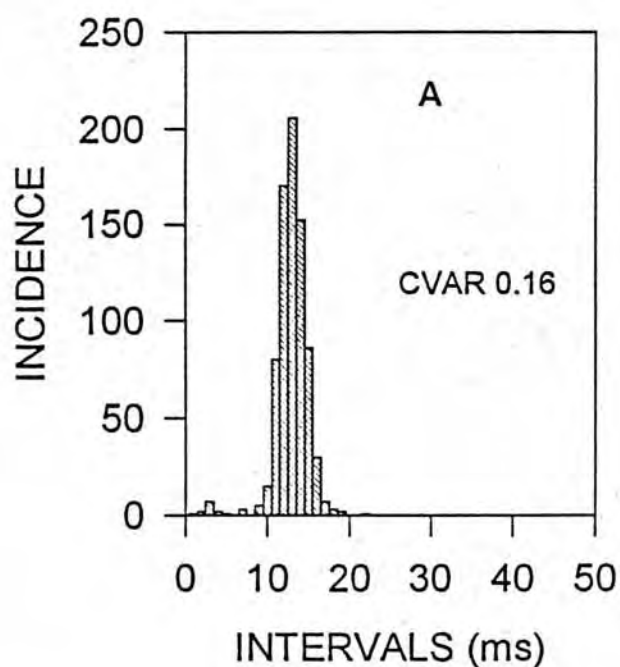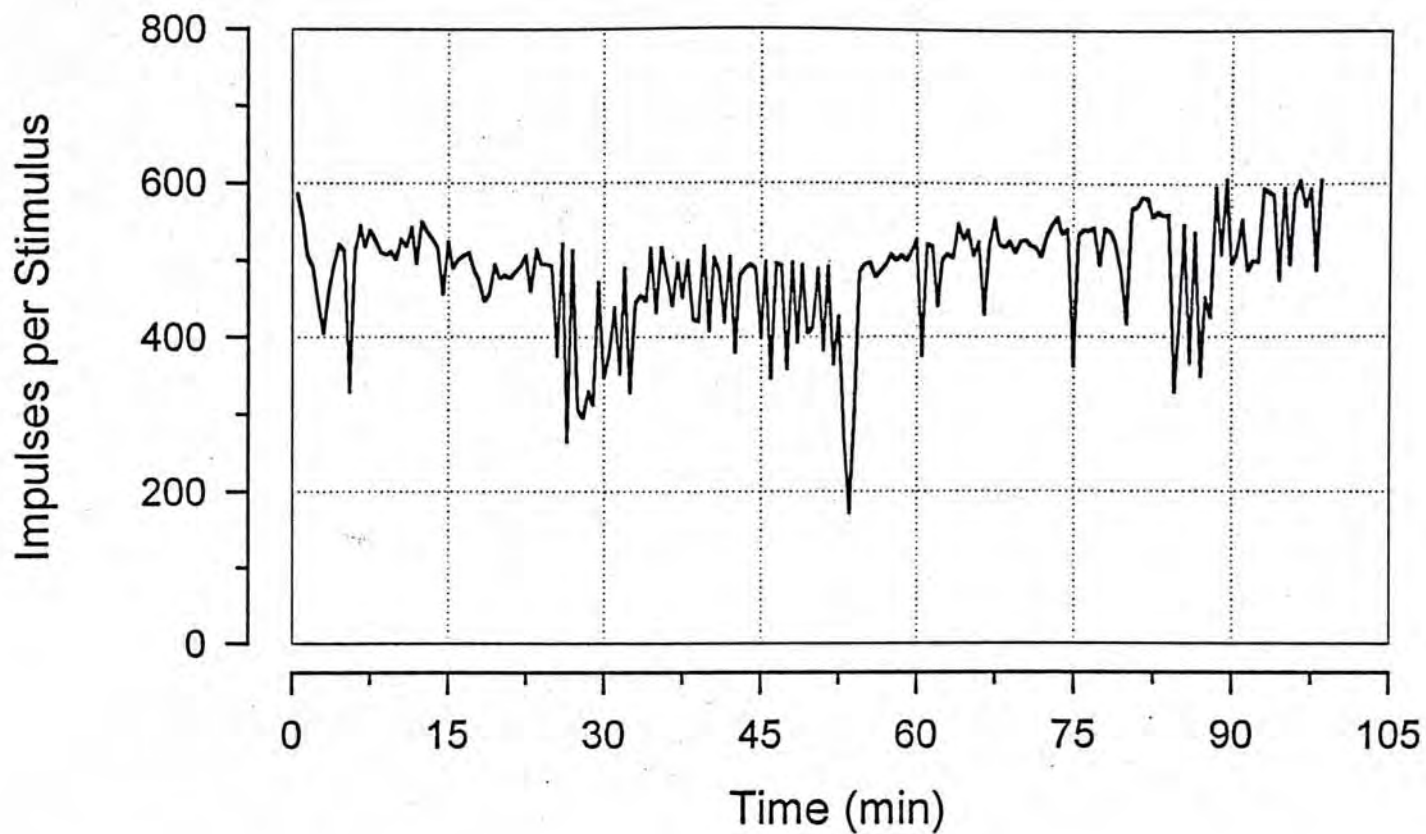
Figure 4.12

Shows a single unit recording from a sinus hair after 48 hours of storage in SIF at 4·C. Responses were highly variable. **A** and **B** are ISI histograms during the 1st (minutes 51-55) and 2nd (minutes 111-115) hours of recording respectively.
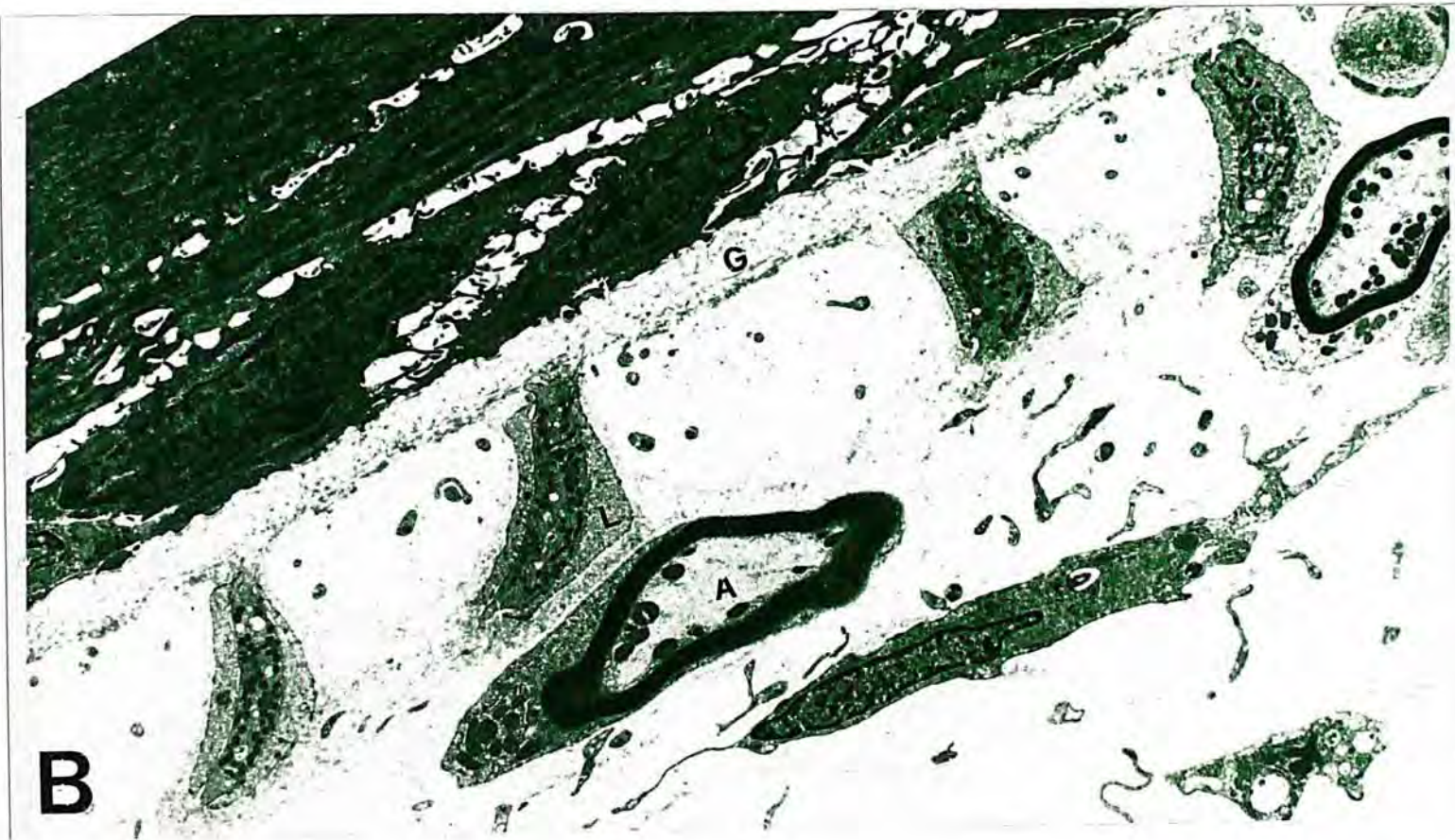
Figure 4.13
A. Transverse section at the level of the ring sinus of a freshly dissected vibrissal follicle. The Merkel cell (M), is typically oblong in shape with a lobulated nucleus and prominent dense cored granules which are polarized towards the nerve terminal (N) (17,000x). B. A view of the outer root sheath and the mesenchymal sheath separated by the glassy membrane (G). The lanceolate nerve endings (L) are each ensheathed between 2 sleeves of Schwann cell cytoplasm and are attached to the glassy membrane. There are 2 Merkel cells to the 4 lanceolates in this micrograph. A - myelinated axon (8,000x).
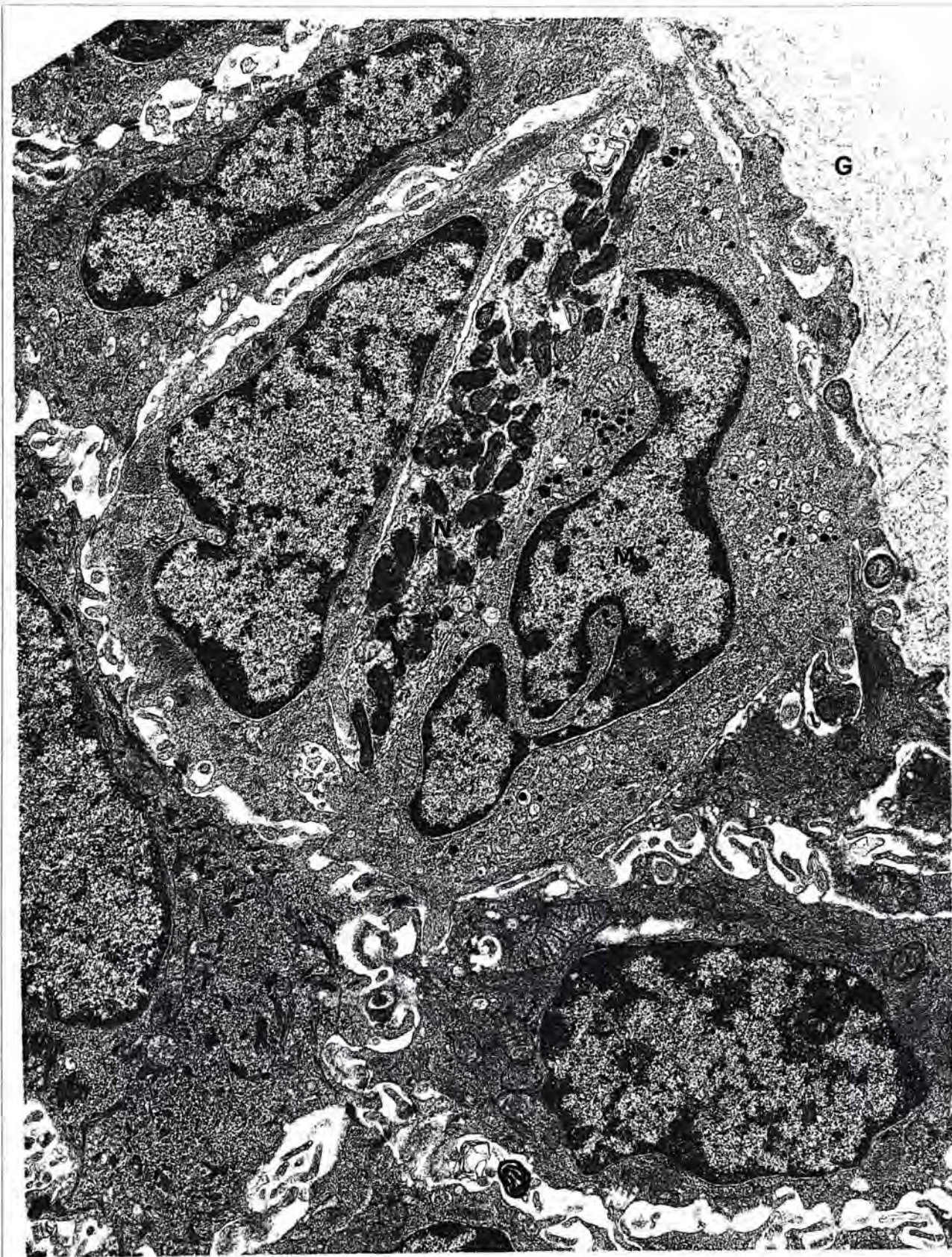
Figure 4.14

Section from a vibrissal follicle after 24hrs' storage at 4°C prior to 3hrs' normal recording. The Merkel cell (M) and nerve terminal (N) are healthy. The dense cored granules, some of which are empty, appear less polarized toward the nerve terminal. G - Glassy membrane (17,000x).
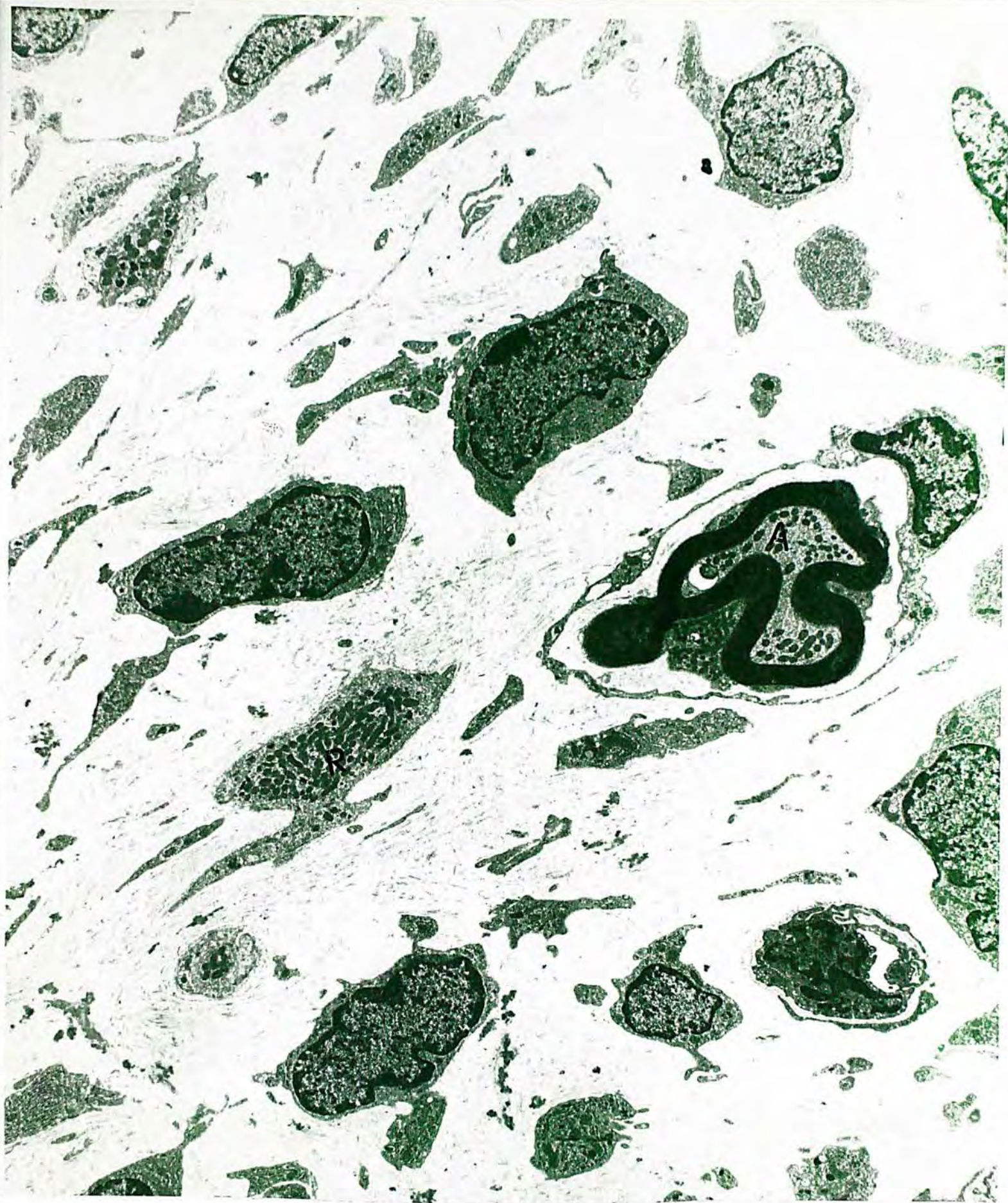
Figure 4.15

Section from a vibrissal follicle after 24hrs of storage at 4°C showing an area within the lower part of the ring sinus. The nerve endings and axons are normal. **R** - Ruffini ending, **A** - myelinated axon (8,000x).

4.3 PHARMACOLOGIC STUDIES

4.3.1 Effect of Quinacrine (QUIN)

Quinacrine, the antimalarial acridine derivative with fluorescent properties has been used as a fluorescent marker for Merkel cells in the skin and vibrissal follicles (Crowe & Whitear, 1978; Nurse *et al.* 1983). Using single Merkel cells isolated from rat foot pad glabrous skin, Yamashita *et al.* (1992), reported that QUIN blocked the sustained outward K current (I_{KD}) which they described in Merkel cells, with an IC_{50} of 10^{-5} M.

In the isolated skin-nerve preparation, it was however found that QUIN (up to 100μM) had no effect on the responsiveness of identified SA I mechanoreceptors to controlled mechanical stimulation (Senok & Baumann, 1993). It was therefore of great interest to test the effect of QUIN on the isolated vibrissal preparation, since the functional role of the K currents described by Yamashita *et al.* (1992), remained unclear.

QUIN, at concentrations up to 100μM, was tested on both St I and St II receptors by superfusing with QUIN-containing SIF for a maximum of 20 minutes.

St I receptors: 100μM QUIN resulted in complete inhibition of the St I receptors with recovery time varying from 30 - 45 minutes. The effect appeared to be dose-dependent with lower concentrations inhibiting responses to a lesser degree, with faster recovery times

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(Figure 4.16a). Both spontaneous (where present) and stimulated spikes were inhibited. Of 8 St I units tested only one remained relatively unaffected by 20 minutes of 100µM QUIN superfusion even on second application (Figure 4.16b).

St II receptors: Stimulated responses of St II receptors were little affected or slightly increased by QUIN (10, 100µM). Notably, a sustained spontaneous firing was introduced or increased when already present, recovering only slowly. 100µM QUIN resulted in varied responses. 3 out of 9 receptors examined had increased counts, especially the spontaneous firing (Figure 4.17); 5 St II receptors were inhibited while one remained unaffected.

The sustained increase in spontaneous firing due to QUIN could be inhibited by 10µM verapamil (Figure 4.17), the L-type Ca channel blocker, suggesting that it may be a calcium dependent process.
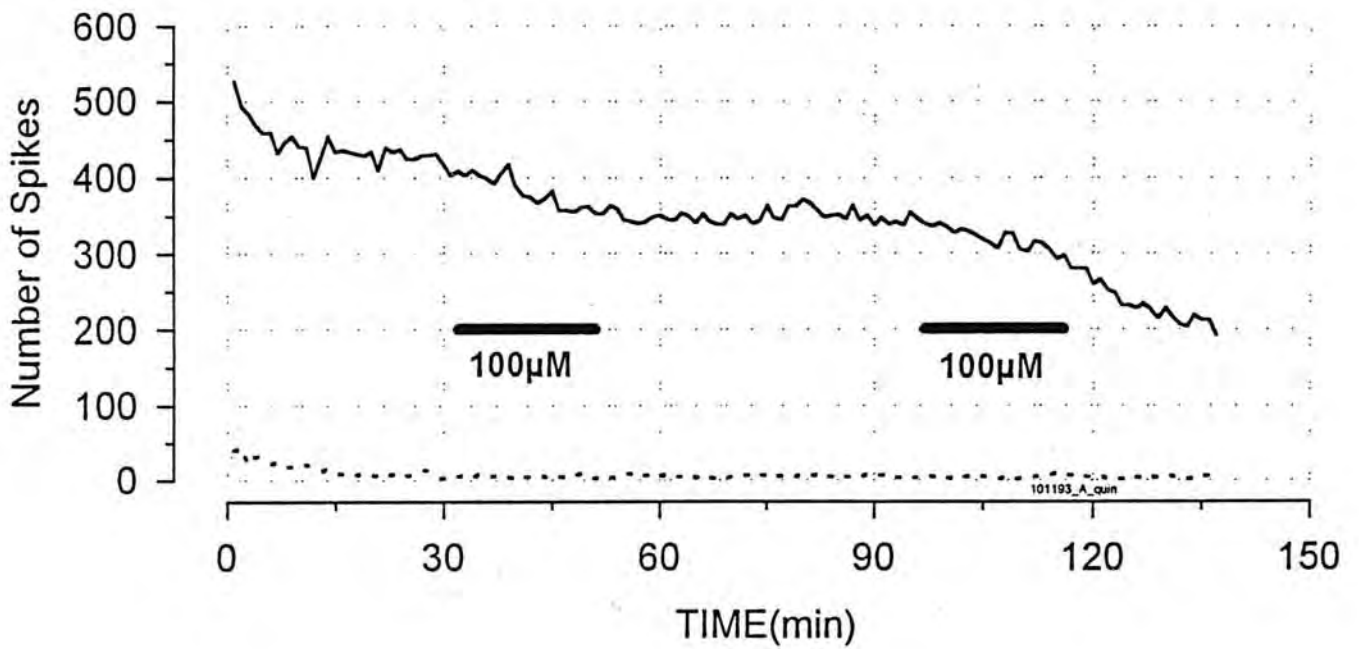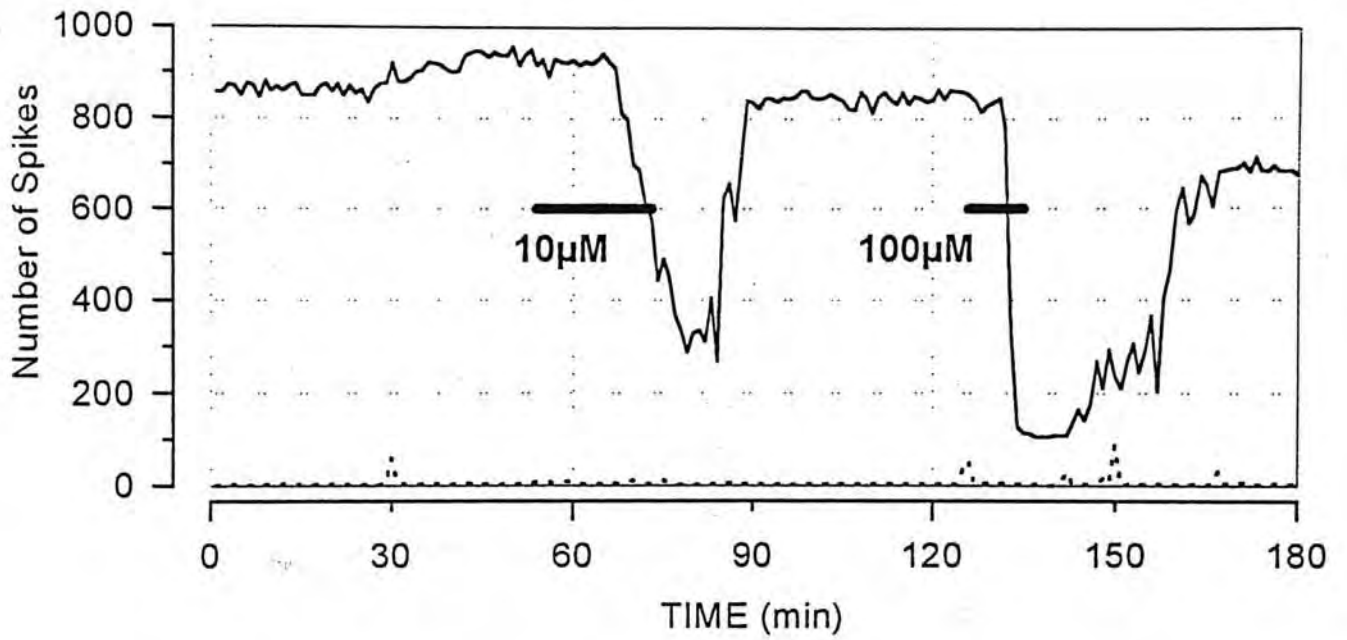
Stimulus spikes
Ongoing (spontaneous) activity
Quinacrine application

Figure 4.16

A. The effect of quinacrine on St I receptor responses is dose dependent. **B.** an St I receptor apparently resistant to quinacrine.
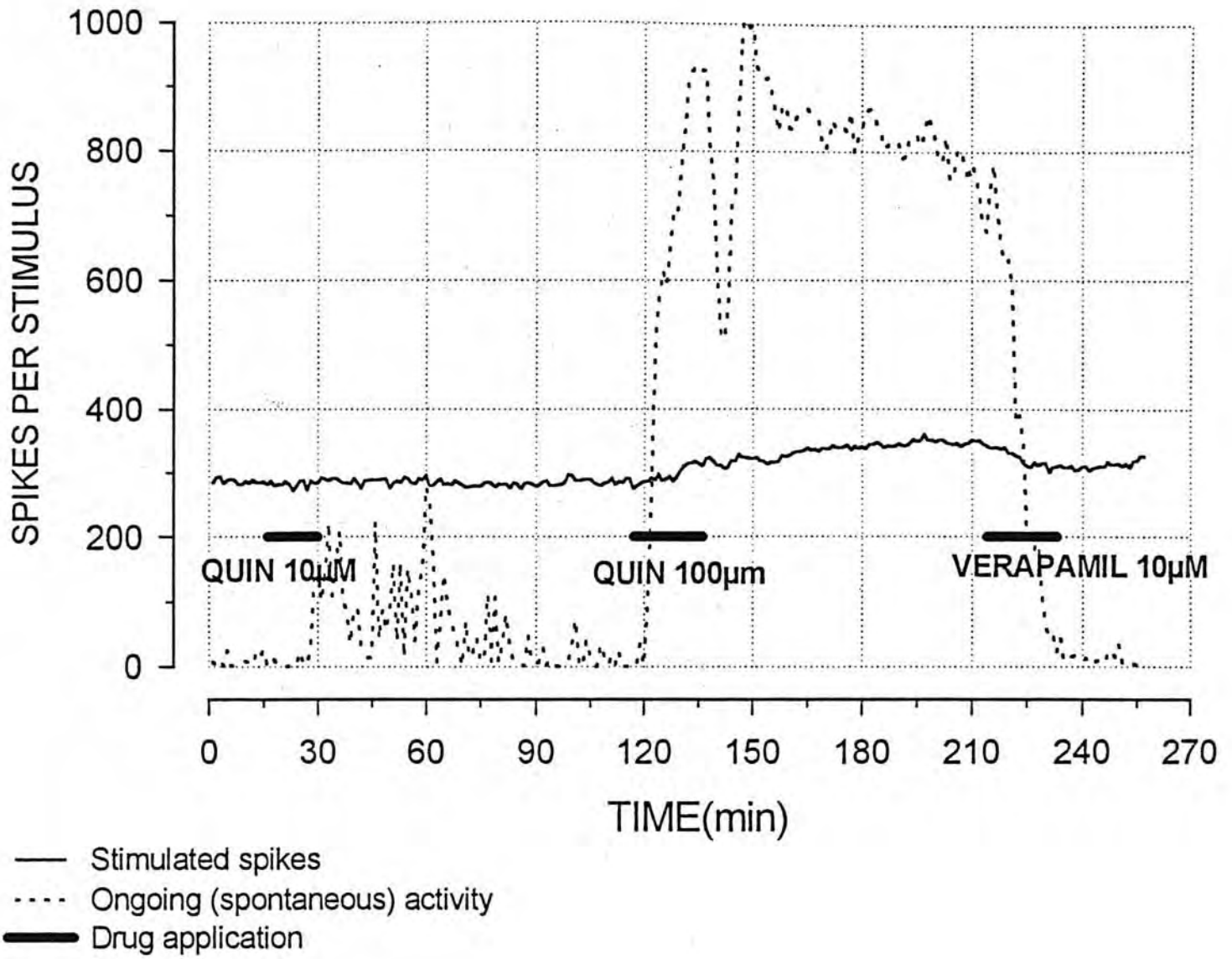
Figure 4.17

Effect of quinacrine on an St II receptor. There was a sustained elevation of spontaneous firing on superfusion of 100μM QUIN which could last for hours. The spontaneous activity was terminated by verapamil (CF Fig. 4.23 and 4.36).

4.3.2 Effect of Chloroquine (CQ)

Chloroquine, the 4-aminoquinoline derivative antimalarial with fluorescent properties has structural and functional

Chloroquine   Quinacrine
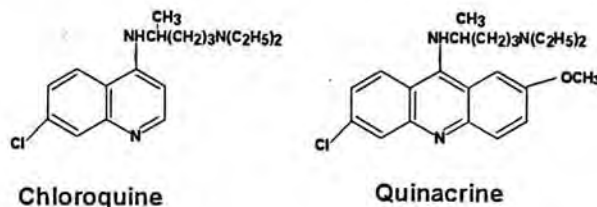
similarities to quinacrine. Both are selectively accumulated in certain peptide secreting cells and nerve terminals (Olson *et al.* 1976; Dencker *et al.* 1976; Ekelund *et al.* 1980).

In view of the equivocal results obtained with QUIN as described in the previous section, the effect of CQ was tested on St I and St II mechanoreceptors.

a)   **Effect of CQ on St I receptor responses:**

On application of CQ-containing SIF, St I responses were reversibly and dose dependently inhibited (Figure 4.18). 10µM CQ had virtually no effect while 300µM often resulted in complete inhibition of responses. Both stimulated and spontaneous firing (where present) were inhibited. Figure 4.19 shows the effect of 100µM CQ on 7 St I receptors ($p<0.0001$ for stimulated spikes, $p<0.001$ for spontaneous spikes). Responses usually took 30 - 45 minutes to recover. The pattern of CQ inhibition could be shown (in the same receptor) to be similar to that of QUIN (Figure 4.20). The magnitude of inhibition was highly variable between receptors tested (see Table 4.2).
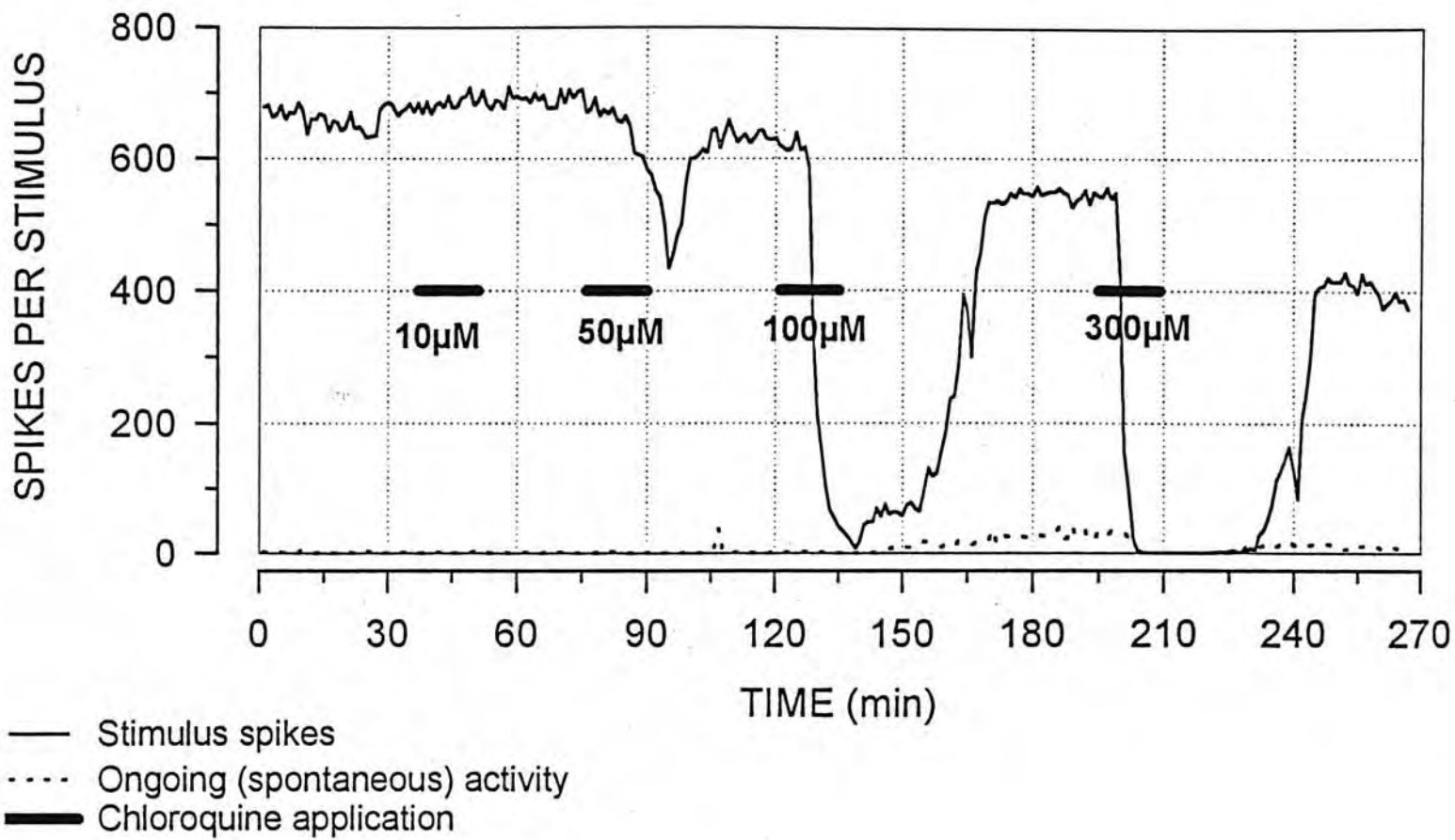
107

Figure 4.18

Effect of CQ on St I receptor responses. CQ dose dependently inhibited St I responses to mechanical stimulation. Note that the low level spontaneous activity later in the experiment was also inhibited by the next application of CQ. 300μM CQ caused a long-lasting, complete inhibition (CF Fig. 4.24).

Figure 4.19

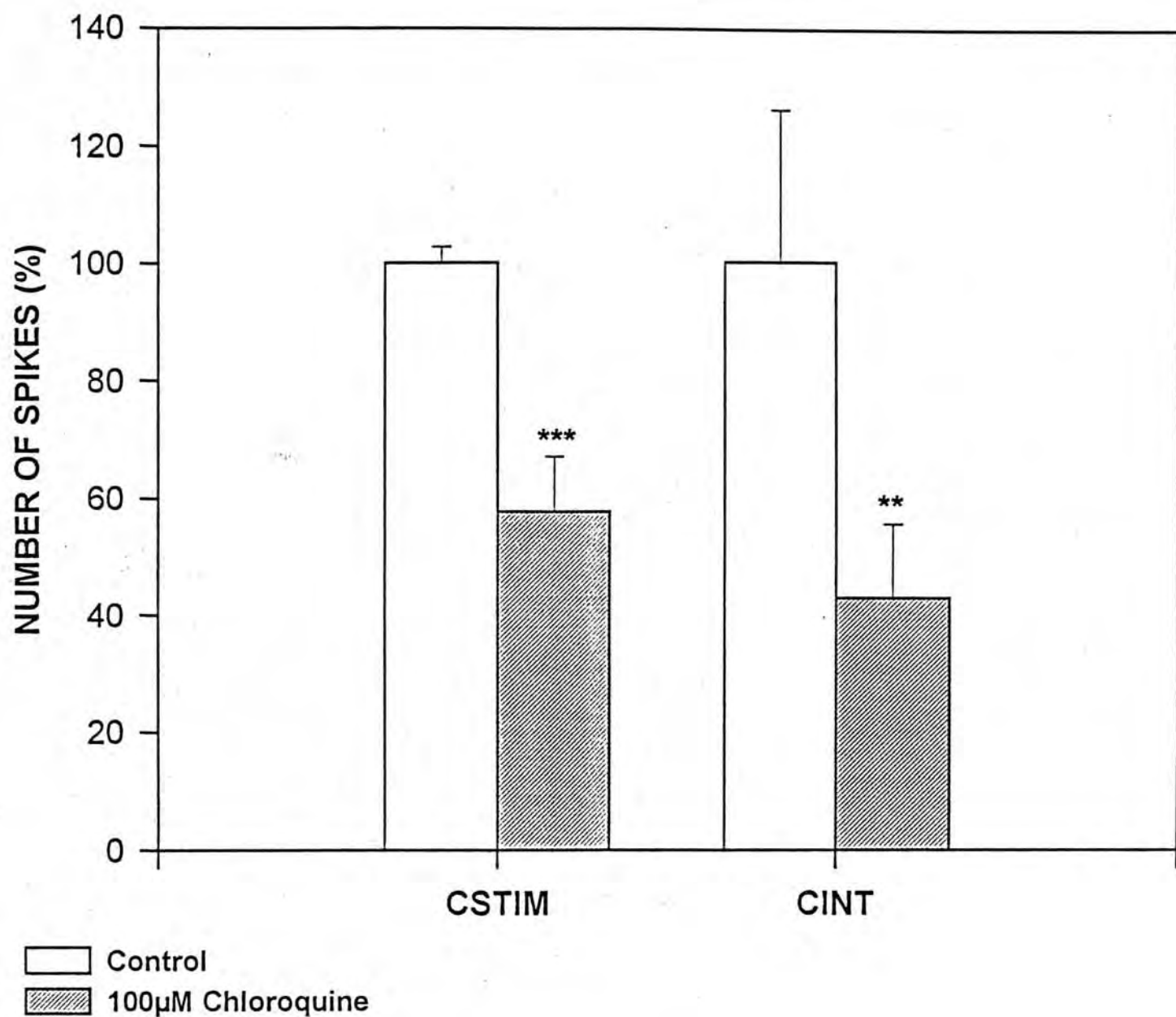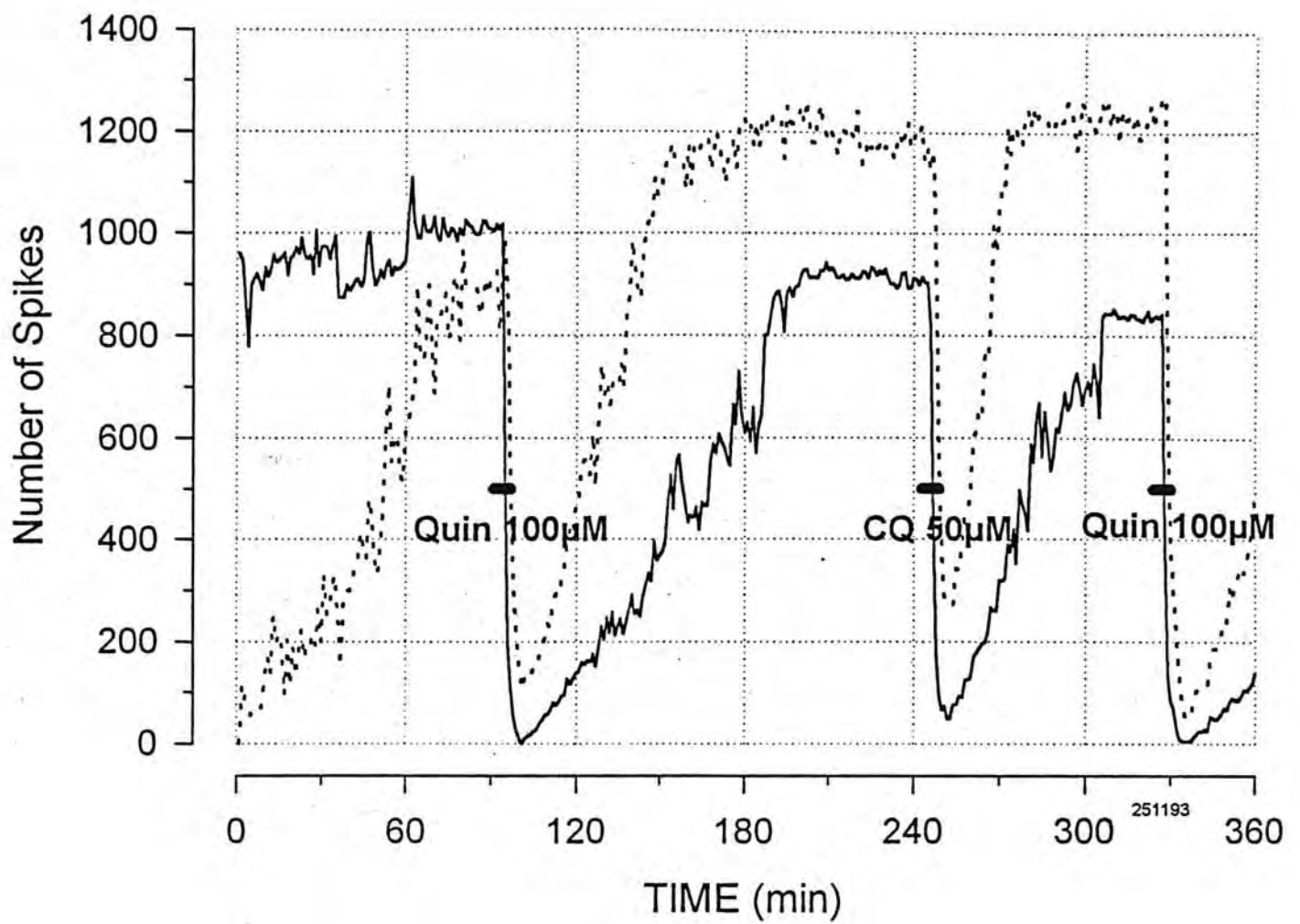Effect of 100µM CQ on 7 St I receptors showing inhibition of both stimulated (CSTIM) and ongoing (CINT) spikes. The 10 stimuli preceding drug application were taken as the control period. (***p<0.0001; **p<0.001).

Figure 4.20

Effect of QUIN and CQ on a single St I mechanoreceptor. Both the stimulated and spontaneous (relatively high in this particular unit) spikes were inhibited in like manner by both drugs..

Table 4.2 Effect of different concentrations of chloroquine on St I receptors.

% INHIBITION OF STIMULATED SPIKES

CQ conc.	RANGE*	MEAN	SD	*n*
50μM	3 - 50	24.7	15.6	6
100μM	20 - 98	61.75	29.31	8
300μM	90 - 100	95	3.9	5

% INHIBITION OF ONGOING SPIKES

CQ conc.	RANGE*	MEAN	SD	*n*
50μM	3 - 72	34.8	33.2	6
100μM	40 - 100	69.83	26.57	8
300μM	80 - 100	92.8	9.9	5

The magnitude of inhibition was highly variable between St. I mechanoreceptors tested but both stimulated and ongoing spikes (where present), were consistently inhibited. Note that complete inhibition was more consistently attained with 300μM CQ.

b)   Effect of CQ on St II receptor responses:

When CQ-containing SIF was superfused, St II receptor responses showed a slight but significant increase ($p<0.001$ n=6 for 100µM CQ; Figure 4.22). This effect was also dose dependent and long lasting (Figure 4.21). The effect of CQ on the spontaneous activity of St II receptors was more dramatic; receptors that were 'silent' before CQ application developed spontaneous firing often as high as 40 spikes/sec. In receptors with pre-existing spontaneous activity, CQ caused a further increase. CQ-induced (or enhanced) spontaneous firing was unrelenting and could persist at such higher levels for hours (e.g. Figures 4.22, 4.23). This CQ effect on St II responses could be blocked by 10µM verapamil (Figure 4.23), suggesting that it may be calcium dependent (CF the effect of QUIN above).

Concentrations of CQ $\geq$300µM could block both types of receptors. The characteristics of this block and recovery were however quite different in the two receptors. St I receptors completely inhibited by this high concentration of CQ (as shown in Figure 4.18), had a recovery time of 45 - 60 minutes while the St II responses returned within 10 - 15 minutes (Figure 4.24), overshooting the control (pre-CQ) counts.

Though qualitatively similar, the CQ effects were more specific than the QUIN effects, and could virtually distinguish between the two receptor types (St I and St II).

— Stimulated spikes

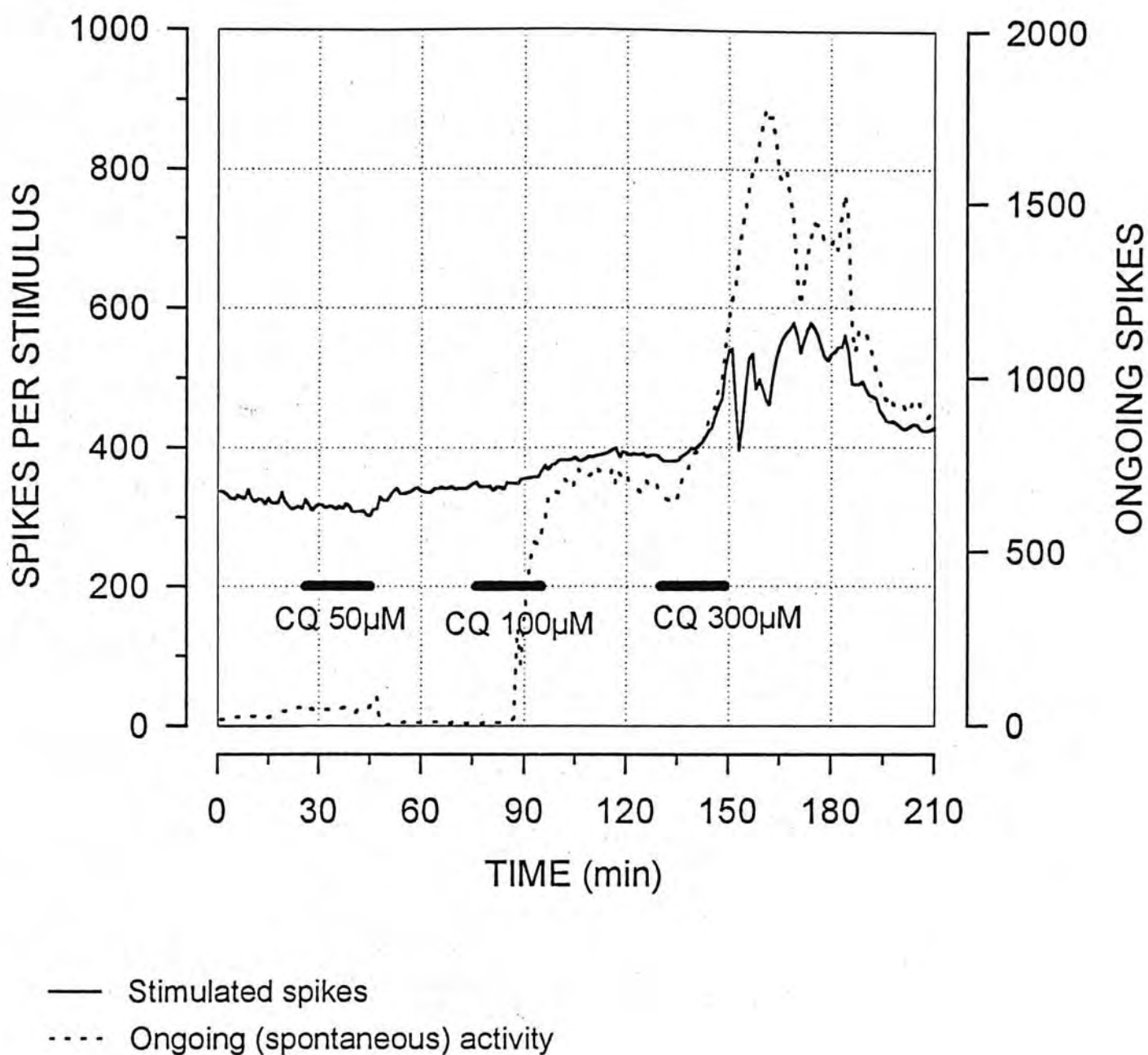···· Ongoing (spontaneous) activity

Figure 4.21

Effect of CQ on a single St II mechanoreceptor showing evidence of dose dependence.
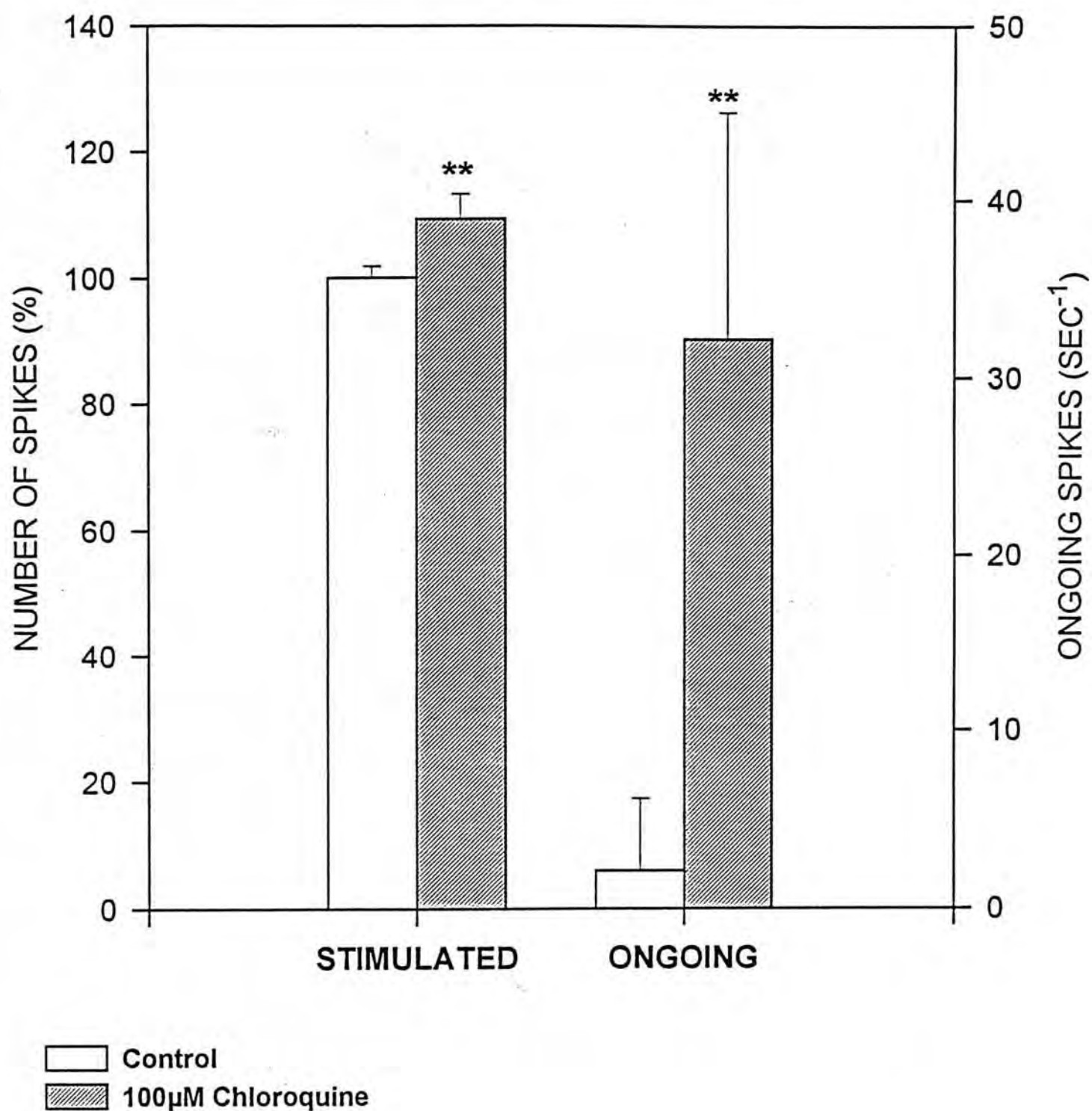
Figure 4.22

Effect of 100μM CQ on 6 St II mechanoreceptors. The moderate increase in stimulated spikes is a constant finding which is statistically significant. There is wide variation in the magnitude of the CQ-induced or enhanced spontaneous firing. **$p<0.001$.
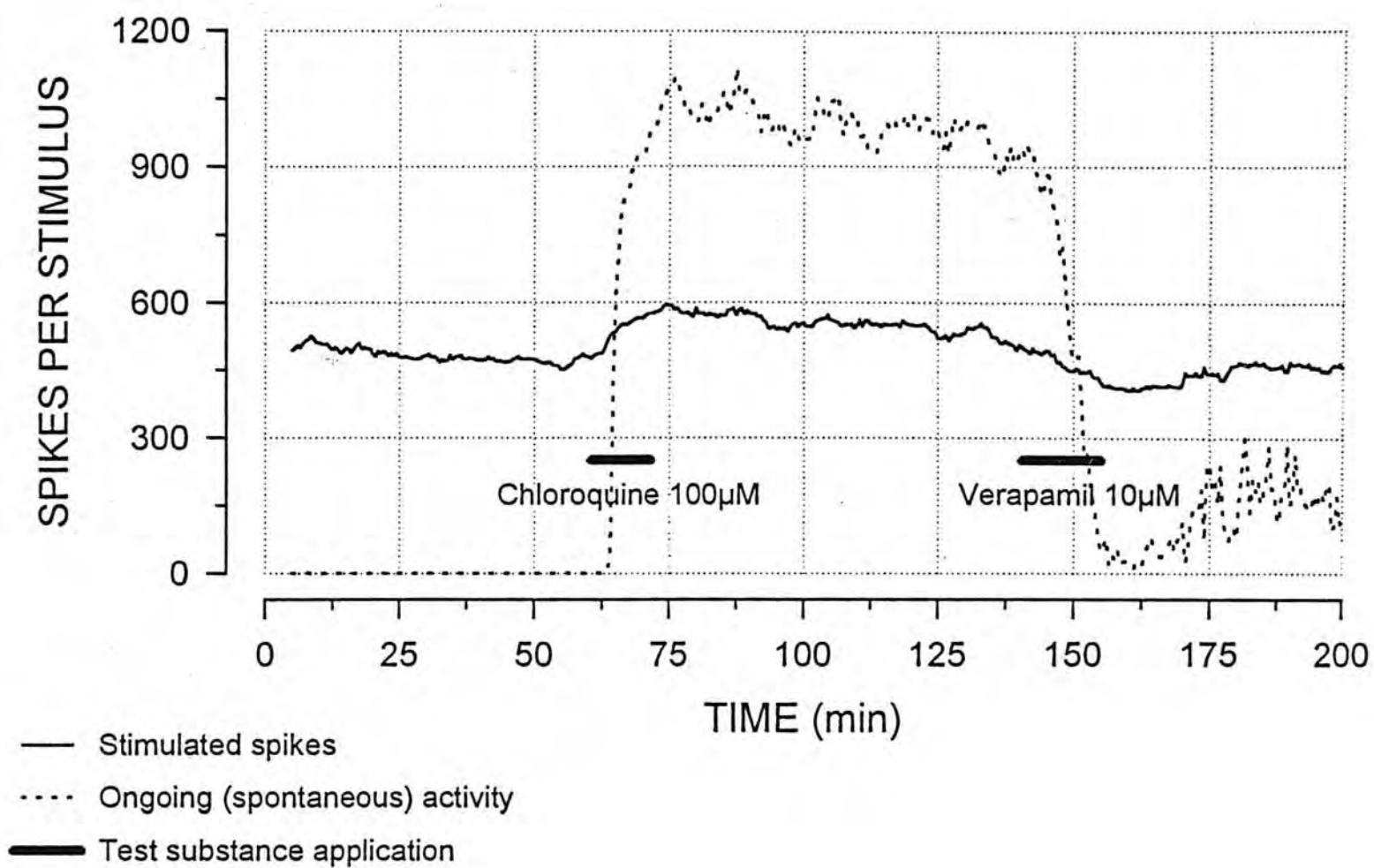
Figure 4.23

Effect of 100µM CQ on an originally 'silent' St II receptor showing the classic pattern of sharp, sustained rise in spontaneous firing with a more modest increase in stimulated responses. Note that the rise in stimulated firing preceded the sharp onset of spontaneous firing. Verapamil blocked the long lasting CQ effect.

Figure 4.24

Effect of high CQ concentration on an St II mechanoreceptor. The initial rise in activity is followed by a 'crash' with a relatively rapid (~15 min) recovery, overshooting the pre-CQ counts (CF Fig. 4.18). Inset ISI histogram showing that this was a typical St II receptor.

c)   Morphologic effects of CQ:

Electron microscopy showed that hairs fixed when responses started declining had pale Merkel cells with haloes round the dense cored granules (Figure 4.25). Hairs fixed when responses were severely depressed had swollen Merkel cells with evid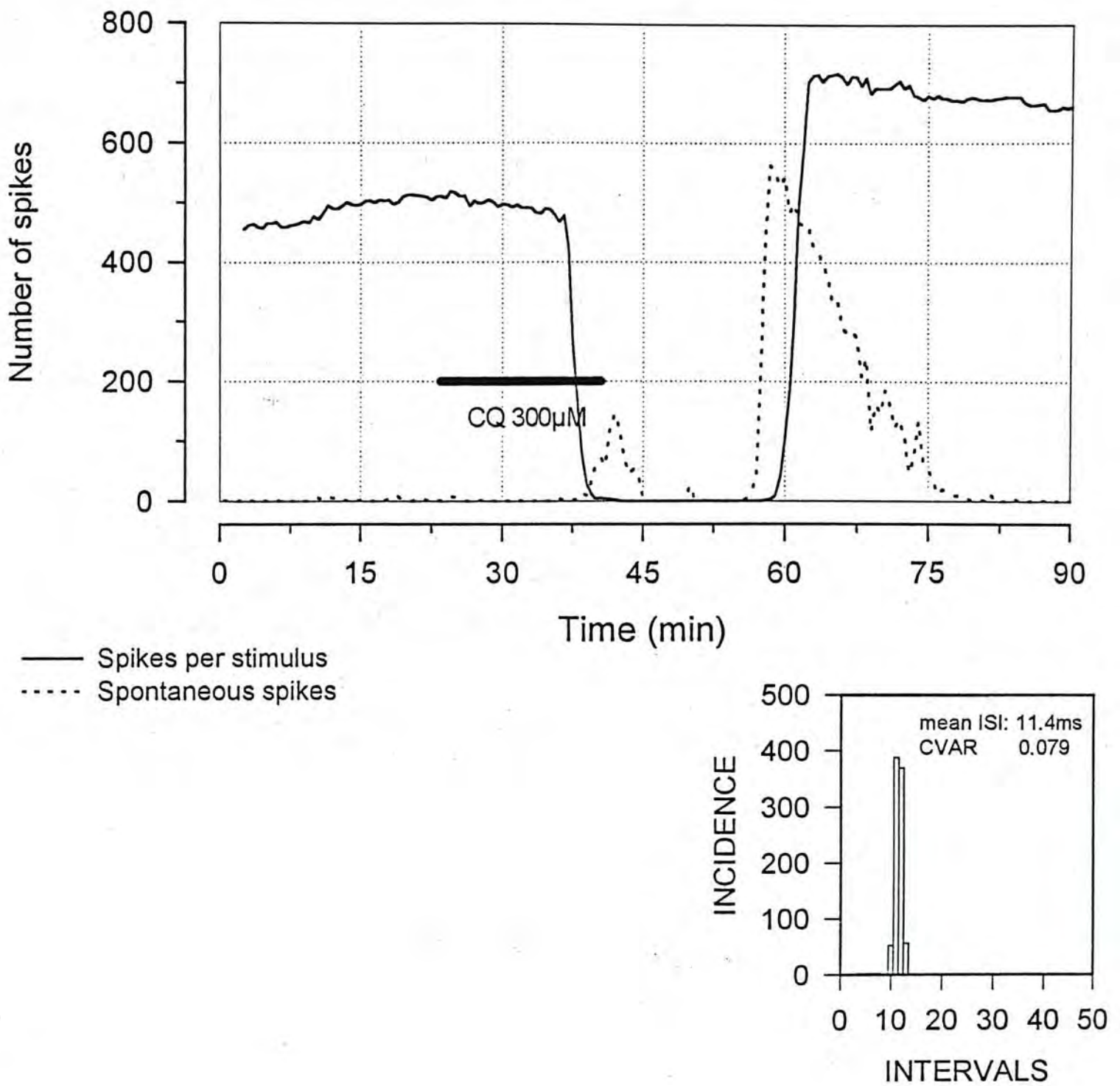ence of vacuolation similar to the toxic vacuoles CQ has been shown to induce in macrophages and fibroblasts (Fedorko et al., 1968a,b.). Very few, randomly distributed granules can be seen (Figure 4.26). Minimal changes were observed in nerve terminals associated with Merkel cells or in lanceolate nerve terminals nearby (Figure 4.27). After recovery, there were residual vacuoles associated with the dense cored granules some of which were still empty (Figure 4.27). Thus there was incomplete reversal of morphological changes within the 45 - 60 minutes recovery time used.
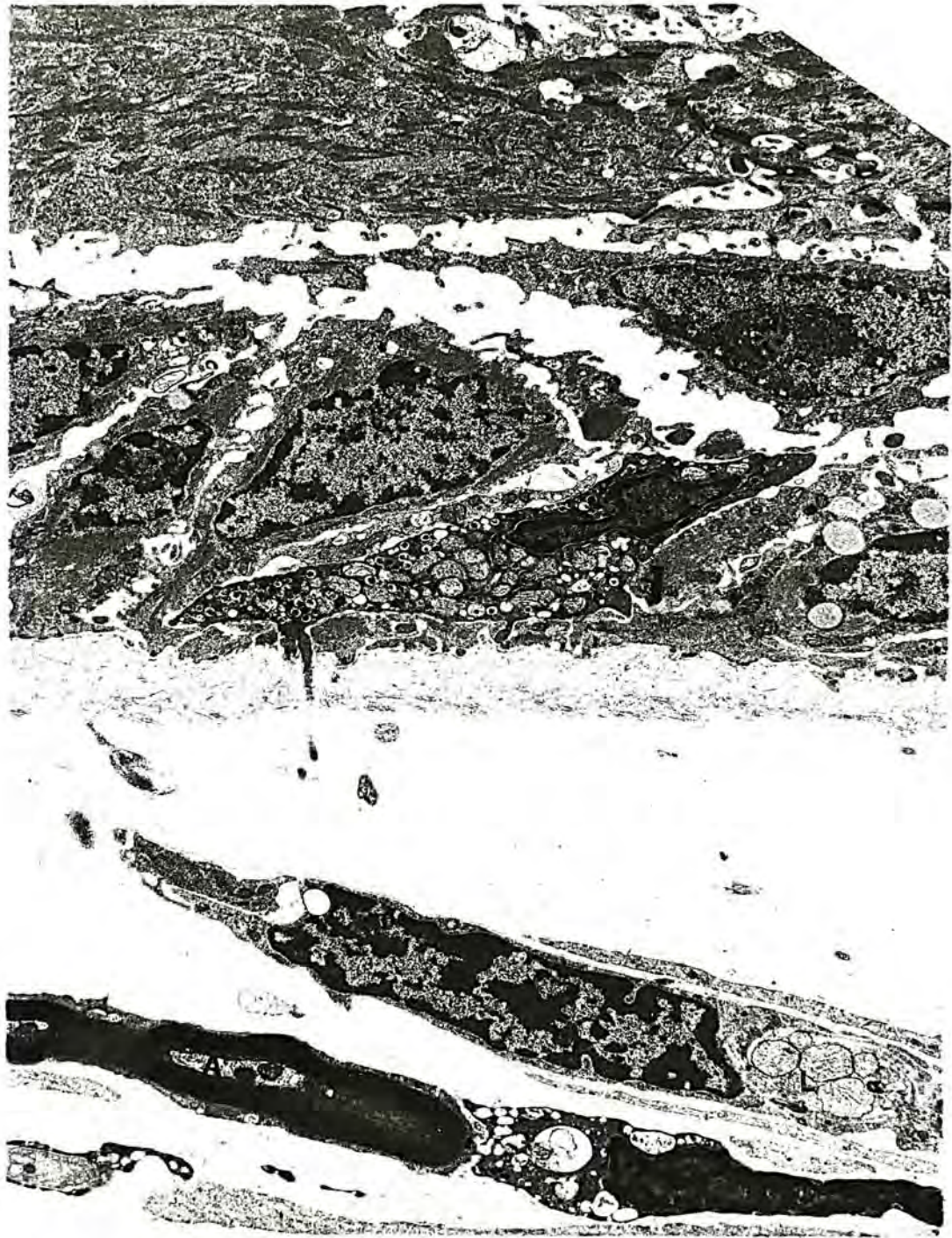
Figure 4.25

Morphologic effect of CQ. Follicle fixed when responses started going down. Merkel cells show evidence of haloes around the dense cored granules. The apposed nerve terminal is normal. Lanceolate endings (**L**) and myelinated axons (**A**) are also normal (8,000x).
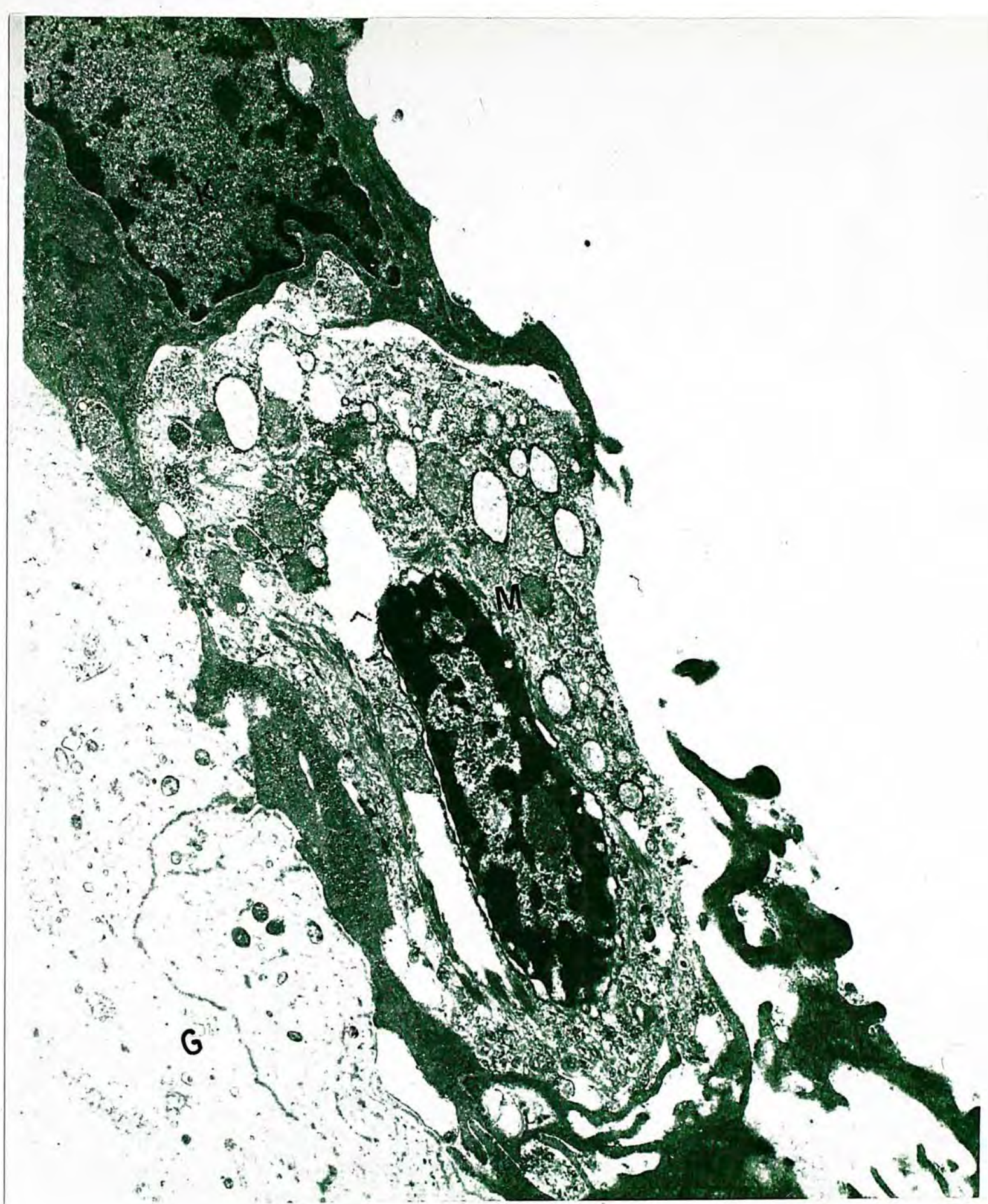
Figure 4.26

Effect of CQ. Follicle fixed after complete inhibition of receptor (St I) responses shows the presence of many vacuoles in Merkel cell (M) while the surrounding keratinocytes (K) are spared. There is little evidence of dense cored granules. The nerve ending shows partial destruction of the mitochondria but is otherwise normal. **G** - glassy membrane (17,000x).

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Figure 4.27

Effect of CQ. Follicle was fixed after about 80% recovery of responses. Granules are now present again within residual vacuoles which have the same distribution as the dense cored granules. The lanceolate terminal (arrow) is also normal (8,000x).

4.3.3 Effect of other lysosomotropic agents

The CQ (and qualitatively similar QUIN) effects observed raised the possibility that CQ was exerting its effect through its well known lysosomotrophic properties (de Duve *et al.* 1974), especially in the light of the observed vacuolation seen in the Merkel cells as described above. Other classic vacuole-forming lysosomotropic agents were therefore tested on the receptors.

a)   **Effect of NH_4Cl:**

SIF containing 10mM NH_4Cl was tested on both St I and St II preparations. 10mM NH_4Cl did not change the pH of the solution appreciably and as such there was no need for corrective measures.

On St I receptors, NH_4Cl depressed responses in a rather variable manner to 68.9 ± 5.9% of control values in 6 St I units examined ($p<0.001$). Figure 4.28a demonstrates how variable the responses were. About 80% recovery was achieved within 15 - 20 minutes of resuming normal SIF superfusion (Figure 4.28b).

The effect of NH_4Cl on St II receptors was so characteristic as to be diagnostic. During 20 minutes of superfusion, responses dropped rapidly to the lowest level within 5 minutes then gradually rose to stabilize at a higher level with an occasional smaller dip during the last 5 minutes. On discontinuing NH_4Cl, responses quickly recovered within 5 minutes, often briefly overshooting the control levels (Figures 4.29; 4.30a,b). Figure 4.31 compares the effects of NH_4Cl and CQ on

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the same St II receptor and shows that the effects are qualitatively different (as shown in figures 4.21, 4.23 for CQ, and figures 4.29,4.30 for NH$_4$Cl). Hence lysosomotropism is unlikely to be the main mechanism behind the observed CQ effects, at least on the St II.

Ultrastructural examination of NH$_4$Cl-treated specimens showed evidence of vacuolation in Merkel cells, especially around the dense cored granules. Specimens fixed after recovery still had some vacuoles with some empty granules. The changes being less severe than those observed for CQ (see figures 4.32, 4.33, 4.34).

The effect of NH$_4$Cl on the St II receptor ending could best be explained by its alkalinizing effect on intracellular pH. The first initial rapid drop in responses could be attributed to an initial rapid change in intracellular pH. This is then countered by the cellular regulatory mechanisms, producing the partial recovery in the presence of NH$_4$Cl. As soon as NH$_4$Cl is removed, the almost failing regulatory system of the nerve ending (note the second dip), is relieved and temporarily overcompensates. As a weak base, NH$_4$Cl diffuses rapidly in and out of the cells. The differential effect seen in the two receptors suggests that the site and/or mechanism(s) leading to action potential generation in the two slowly-adapting mechanoreceptors is fundamentally different.
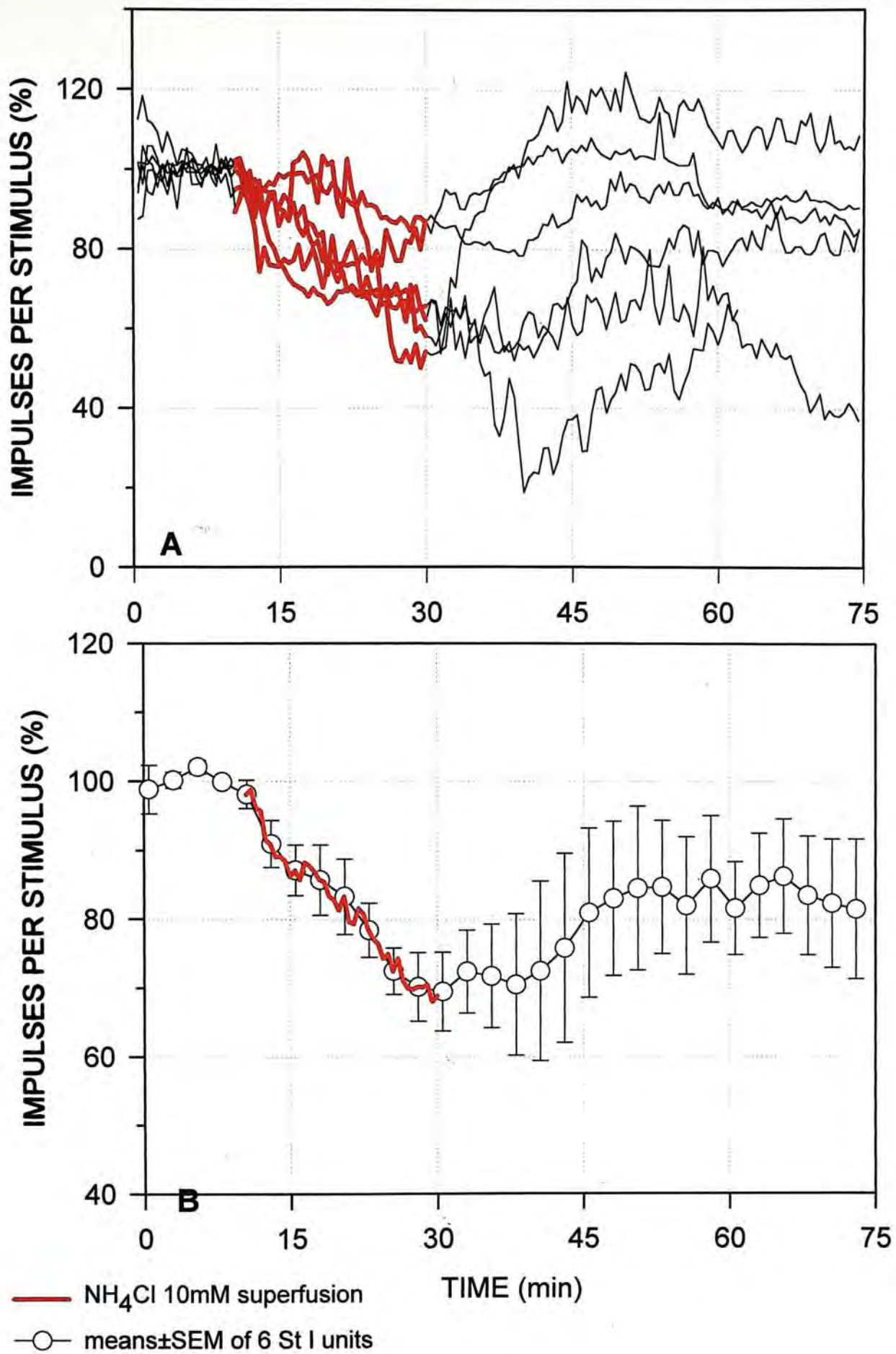
Figure 4.28

Effect of 10mM NH_4Cl on 6 St I receptors. **A.** Responses were varied with no particular pattern to the inhibition (CF Figures 4.29, 4.30). **B.** Mean ± SEM for the 6 St I units.
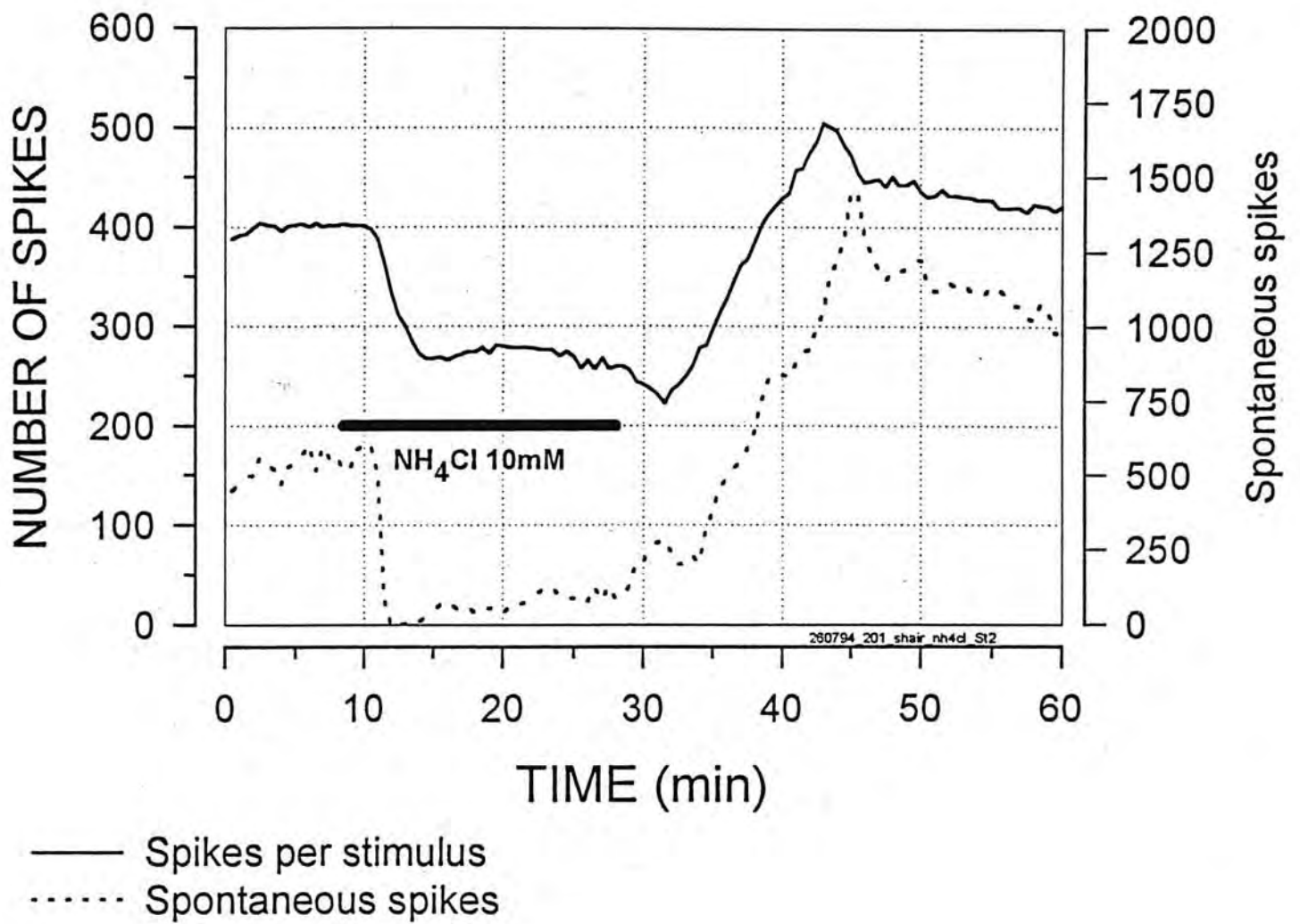
Figure 4.29

Effect of 10mM NH$_4$Cl on an St II receptor showing the typical, virtually diagnostic pattern of inhibition (CF effect on St I). Both Stimulated and spontaneous spikes are affected in the same way (CF effect of CQ on St II, Fig. 4.23).
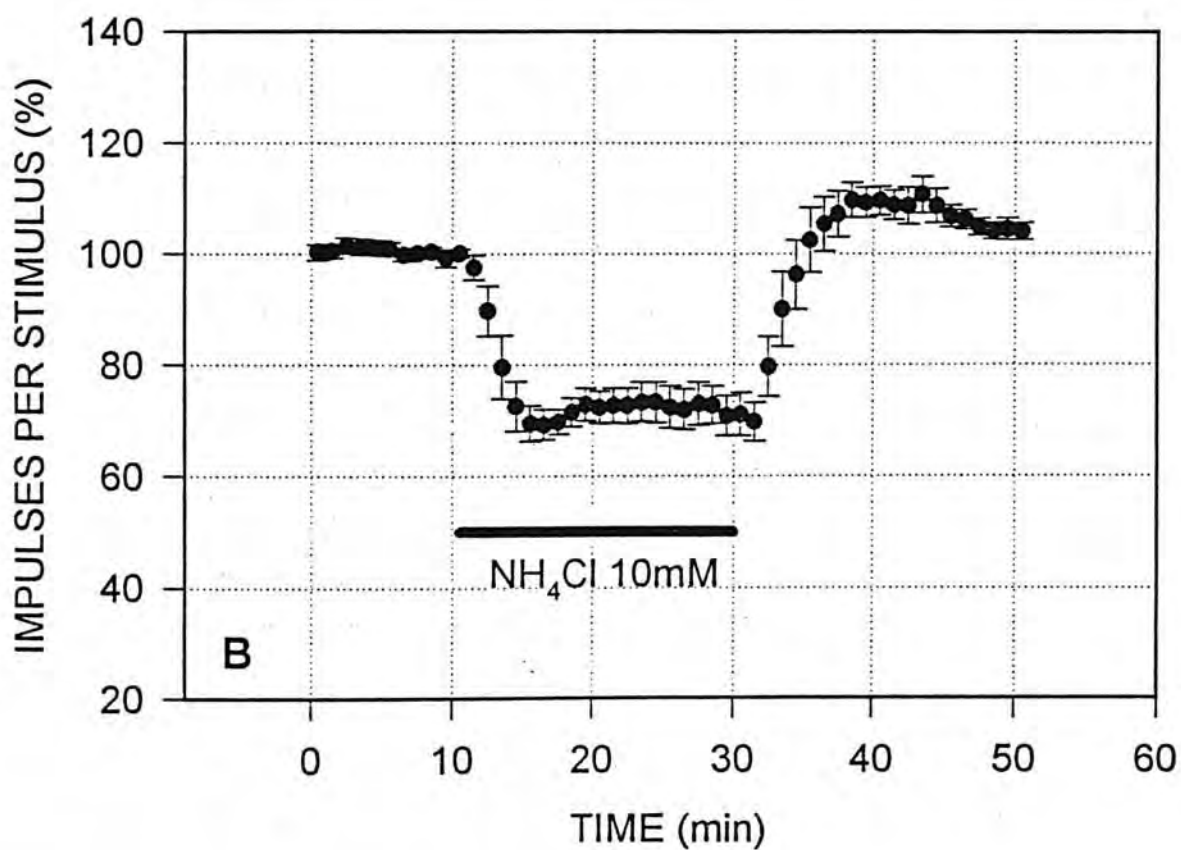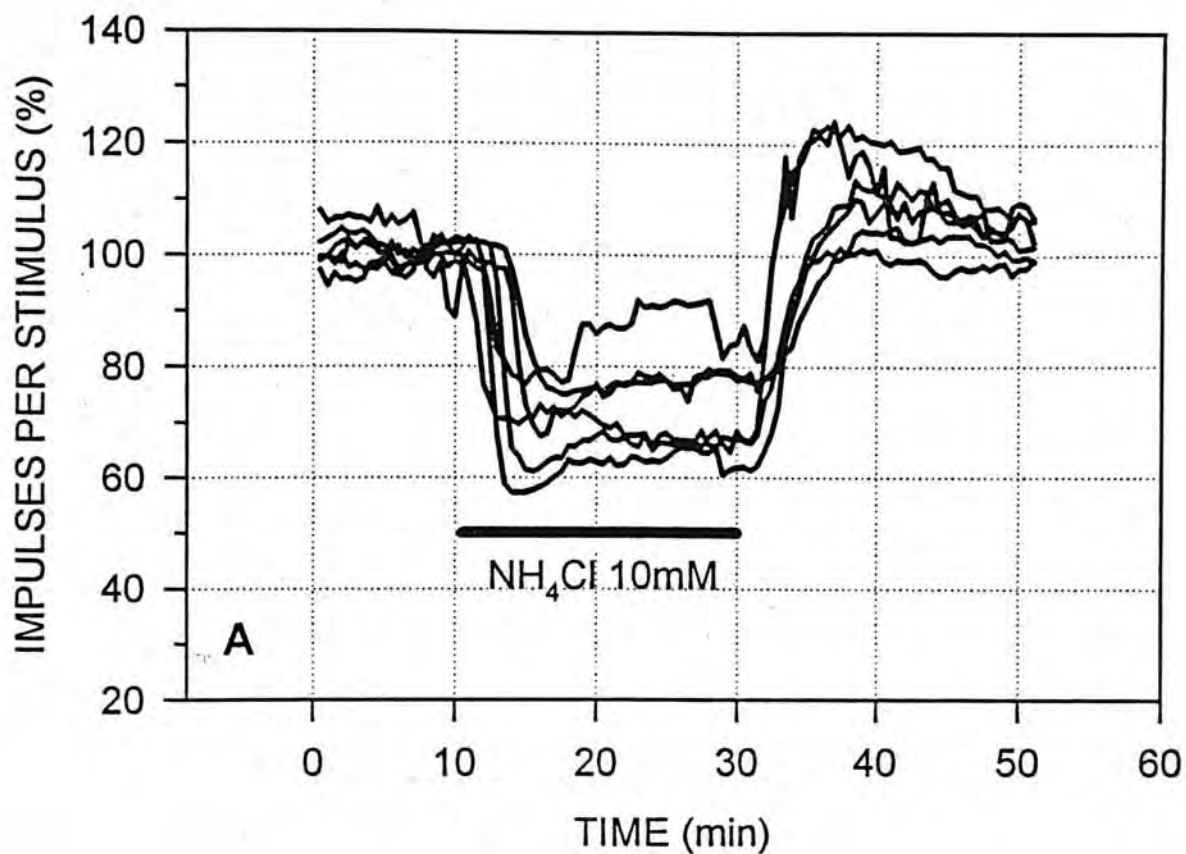
Figure 4.30

Effect of 10mM NH_4Cl on 6 St II receptors showing how highly reproducible the pattern of NH_4Cl-inhibition is among (**A**). Responses were inhibited to 71.10±3.46% ($p<0.0001$) of control at the end of 20 min of NH_4Cl superfusion (**B**).
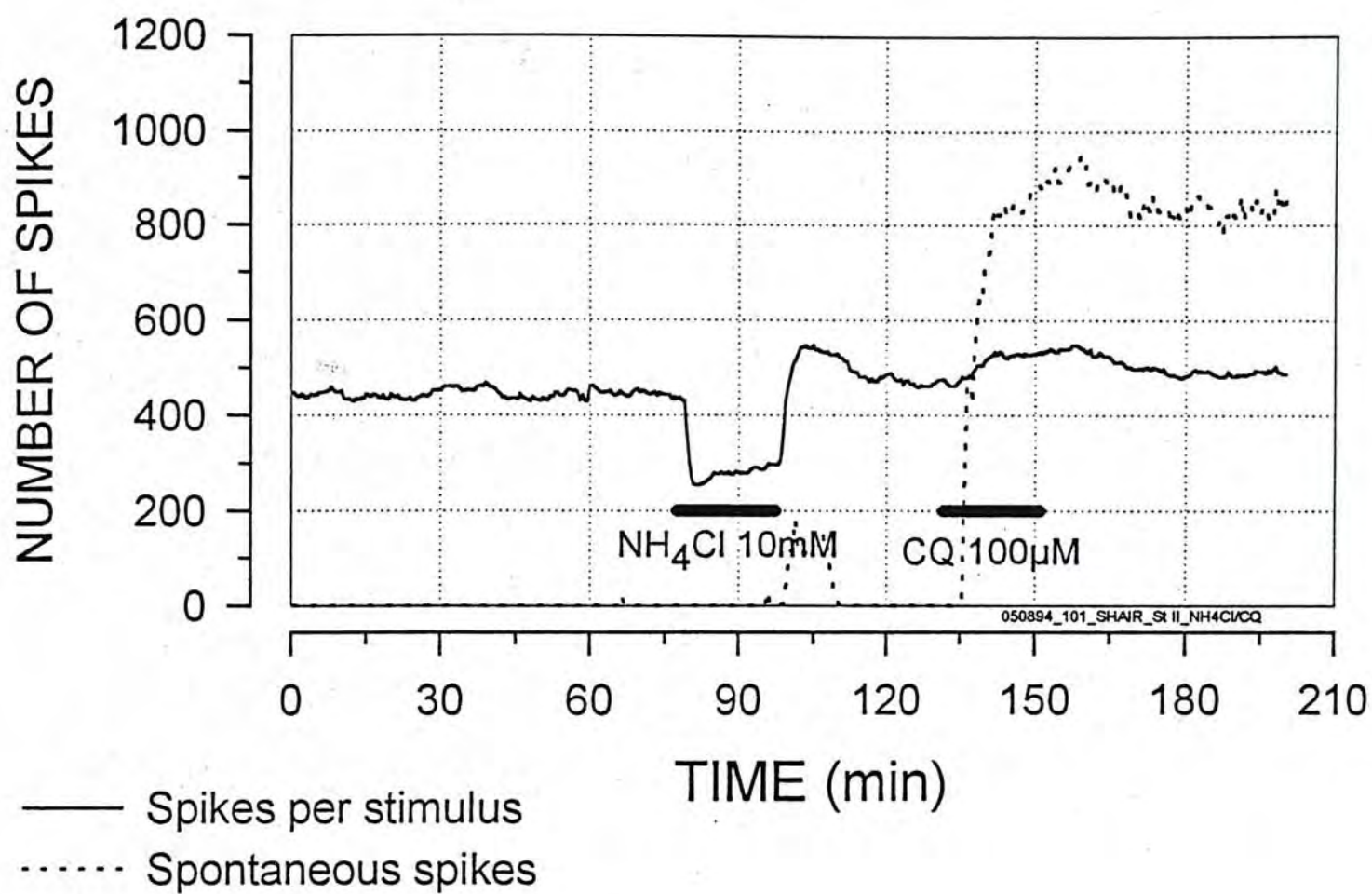
Figure 4.31

Effect of 10mM NH_4Cl and CQ (100µM) on the same St II receptor. The classic St II response to CQ could still be observed after treatment with NH_4Cl.
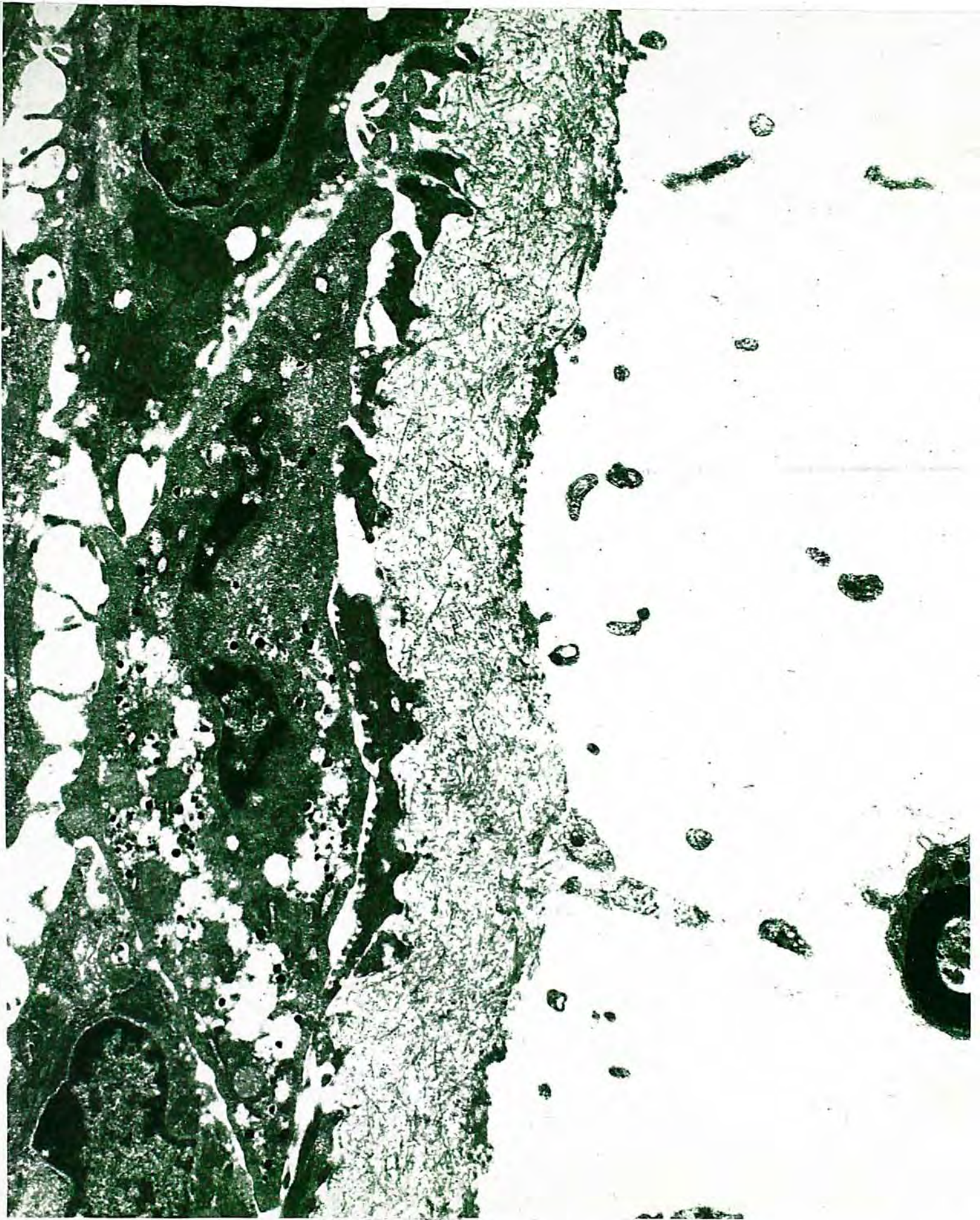
Figure 4.32

Effect of NH₄Cl. Treament with NH₄Cl produced vacuoles in the Merkel cells but not the surrounding keratinocytes. The vacuoles are concentrated at the same location as the dense cored granules. The myelinated axon on the right is normal (17,000x).
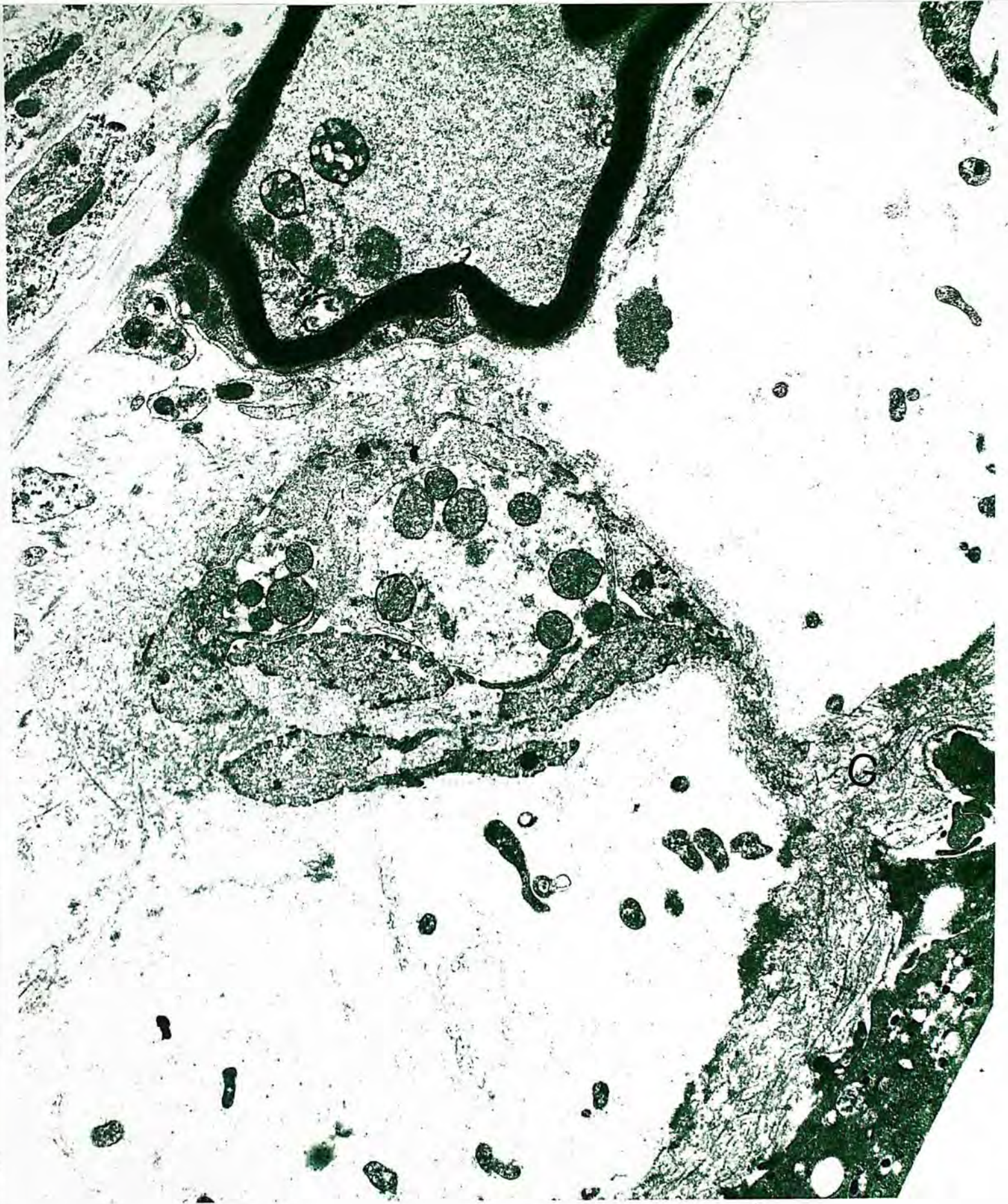
Figure 4.33

Effect of NH$_4$Cl. Micrograph shows a typical lanceolate terminal with attachment to the glassy membrane (G) which is normal. The myelinated axon above it is also normal while part of a Merkel cell shown (bottom right) has cytoplasmic vacuoles (17,000x).
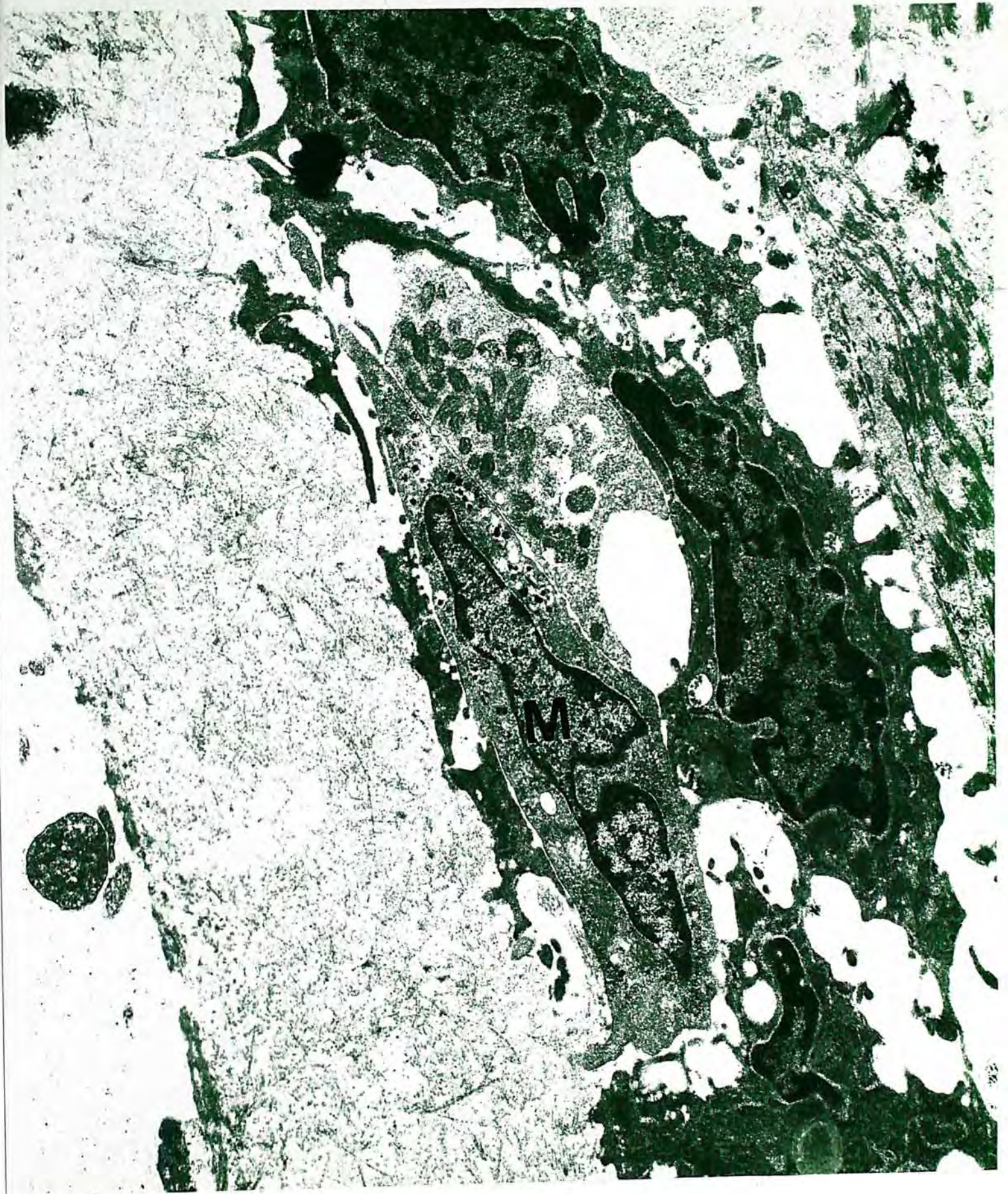
Figure 4.34

Recovery from NH$_4$Cl superfusion. Virtually complete recovery of the morphological changes are seen in this section with few residual vacuoles in the Merkel cell (M). The apposing nerve terminal is normal (17,000x).

b)   Effect of Methyamine:

Methylamine, another classic lysosomotrophic agent had little effect on St I units at 10mM concentrations but had a qualitatively similar (though less pronounced) effect on St II receptors (Figure 4.35).

c)   Effect of 3-methyladenine (3-MA):

The methylxanthine 3-MA, an inhibitor of autophagy (e.g. (Ciechanover *et al.* 1991; Sandvig & van Deurs, 1992), like CQ is also used as an inhibitor of cellular proteolysis (e.g. Bansal & MacGregor, 1992). Its effect on St I and St II receptors was also tested. At 100 - 200µM concentrations, 3-MA virtually reproduced the CQ effects on St II receptors (i.e. a slight rise in stimulated firing accompanied by a dramatic, sustained rise in spontaneous firing), while having no effect on St I receptors (see figures 4.36, 4.37).
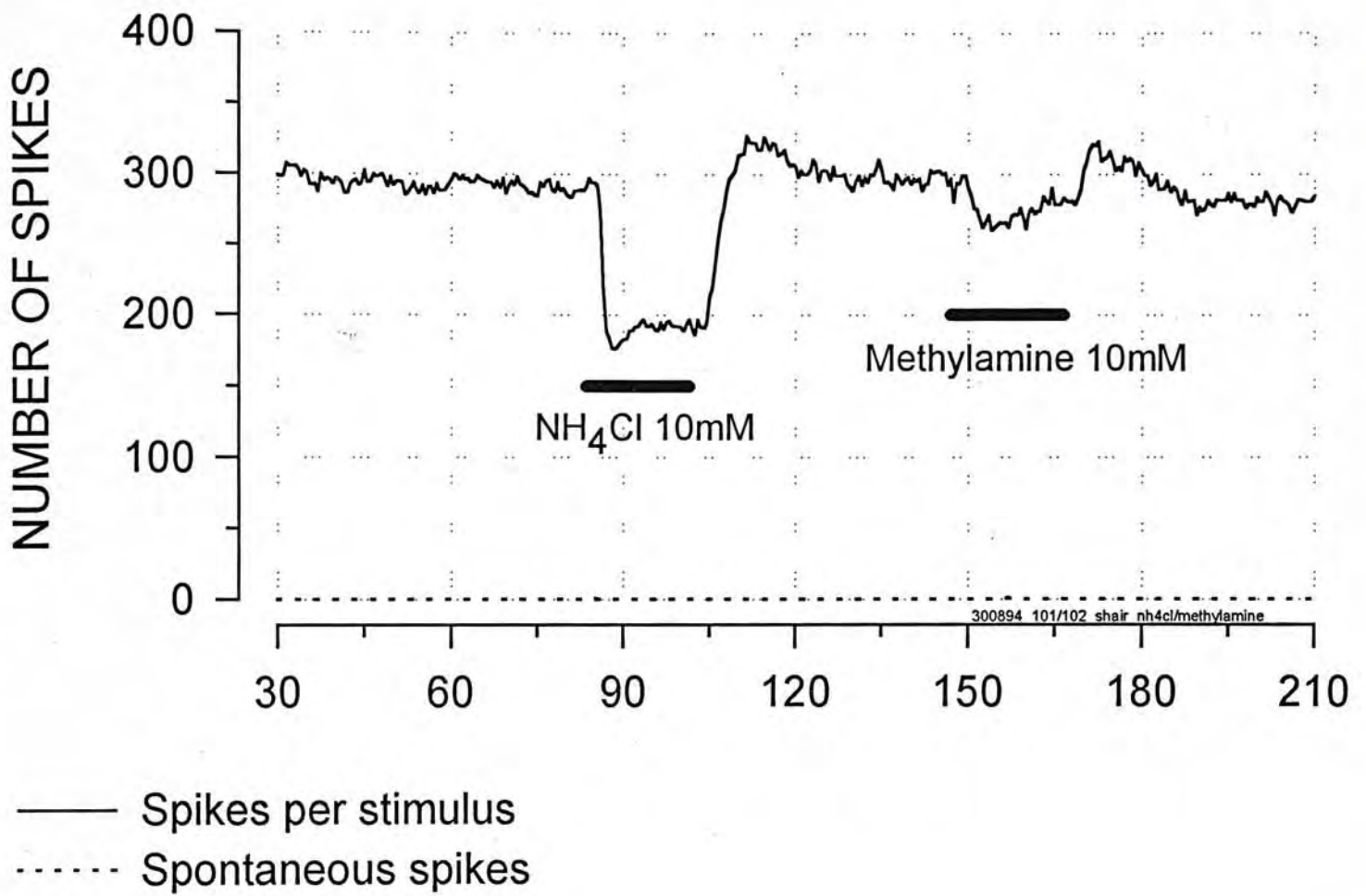
Figure 4.35

Effect of 10mM NH_4Cl and 10mM methylamine on the same St II receptor showing that the effect of methylamine on St II receptors is qualitatively similar to that of NH_4Cl.
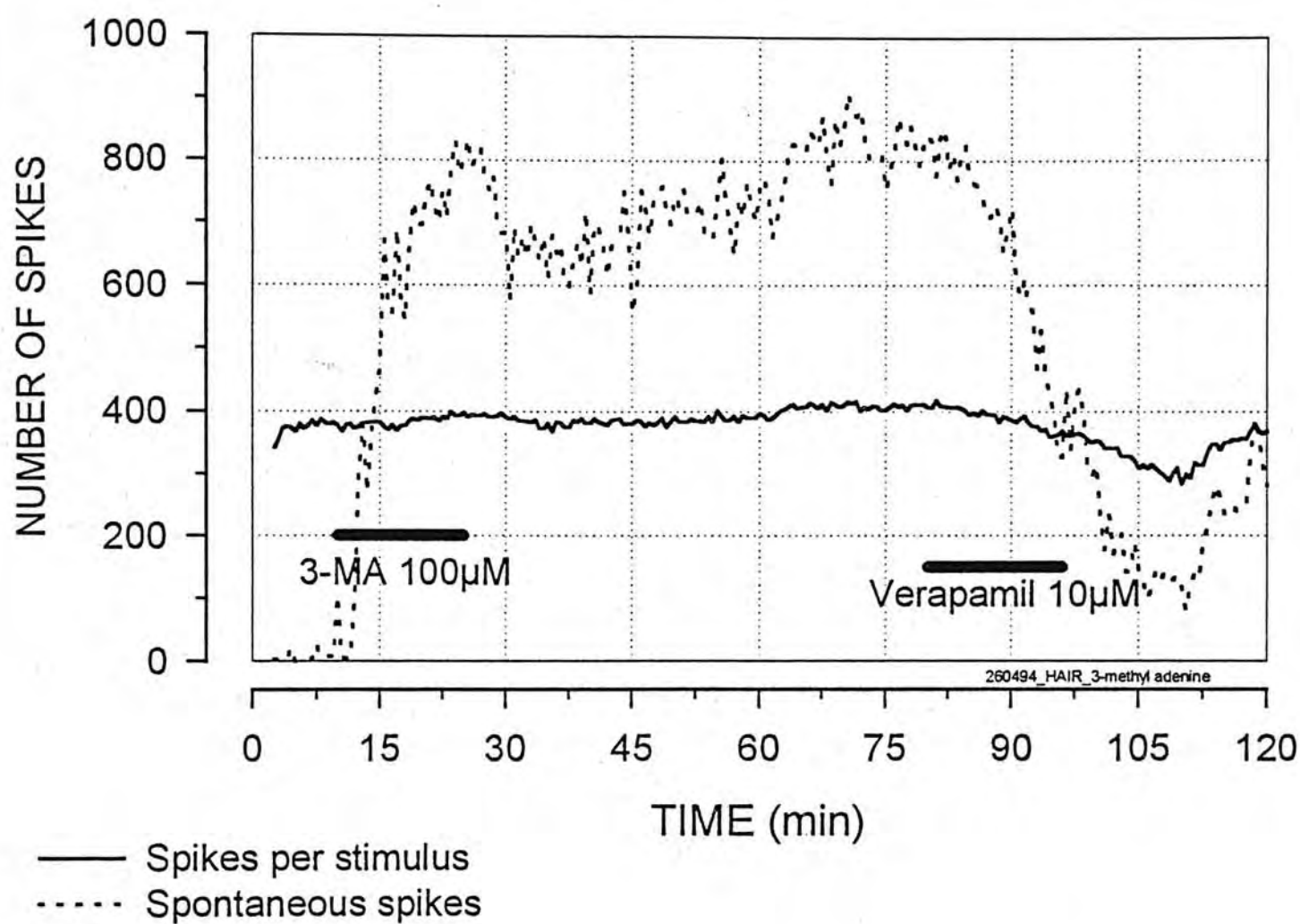
Figure 4.36

Effect of 100µM 3-methyladenine (3-MA) on St II receptor is virtually identical to that of CQ (CF Figure 4.23). The sustained effects were also sensitive to verapamil.
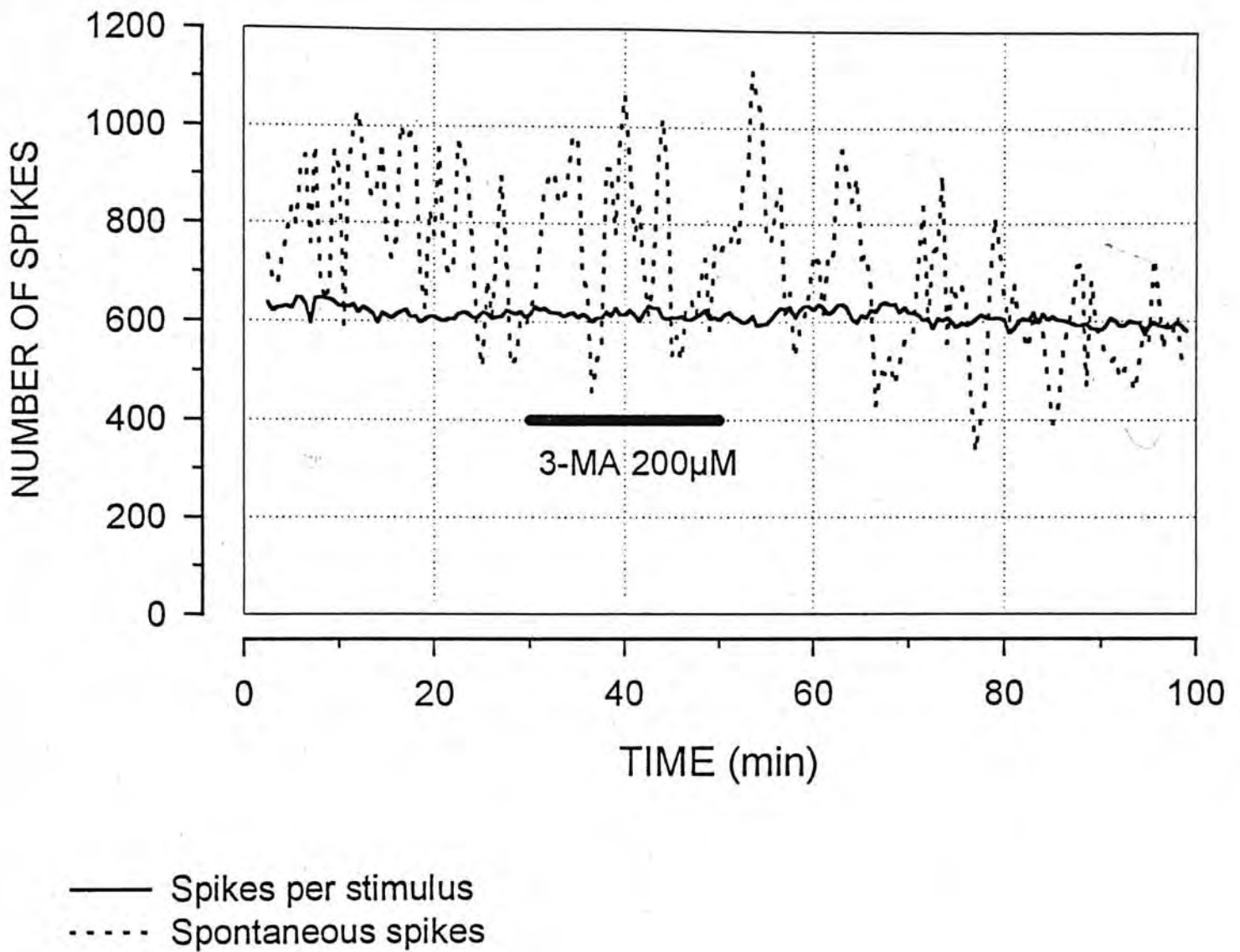
Figure 4.37

Effect of 200µM 3-methyladenine (3-MA) on St I receptor. 3-MA had no effect on both stimulated and spontaneous firing even at this higher concentration (CF Figure 4.36).

4.3.3 Experiments on Intracellular Ca^{2+} mobilisation

These experiments were embarked upon in part because of reports that suggested that CQ interfered with intracellular calcium mobilization. CQ was found to inhibit caffeine induced contraction in the isolated rat hemidiaphragm (Okwuasaba *et al.* 1990). This was therefore considered a possible mechanism for the observed CQ inhibition of the St I receptors.

The possibility that the transduction mechanism in St I receptors involved some form of intracellular Ca^{2+} mobilisation was therefore examined; the St II terminal again serving as a 'control' mechanoreceptor with no cellular attachment.

a)   Effect of caffeine:

Caffeine causes calcium induced calcium release (CICR) through the ryanodine receptor (RYR) intracellular calcium release pathway in intact and semi-intact systems (Ehrlich & Bezprozvanny, 1994).

Caffeine on St I: Caffeine caused a marked increase in responses to mechanical stimulation. The response was dose dependent (Figure 4.38) and varied widely in magnitude between receptors, ranging from about 50% to >150% of control values when 10mM caffeine was superfused for 10 minutes, (mean increase was $164 \pm 12.6\%$ (*p*<0.001) of control in 6 St I units examined). Caffeine

also produced a transient low level ongoing (spontaneous) activity which was often over before discontinuing caffeine (e.g. Figure 4.38).

The excitatory effect of caffeine appeared to be specifically coupled to mechanical stimulation rather than causing a general rise in $[Ca^{2+}]_i$. The transient low level increase in spontaneous activity (which was not always present) could be due to a rise in the basal inter-stimulus $[Ca^{2+}]_i$ sufficient to cause spontaneous firing.

When the percent change in dynamic and static counts were examined, it was found that caffeine had the greatest effect on the static phase. Before addition of caffeine, the %dynamic and %static counts stayed remarkably well together. Caffeine superfusion however increased static counts to a greater degree, leaving the static counts lagging behind (Figures 4.39 and 4.41b). This was also reflected by a drop in the dynamic-static ratio (Figure 4.39 inset). These findings suggest that caffeine is exerting its effect during a relatively late phase in the transduction process of the St I receptor.

To test the effect of competitively blocking calcium entry on the observed caffeine effect, Mg^{2+}-containing SIF was superfused for 10 minutes before addition of caffeine in the continued presence of Mg^{2+} for another 10 minutes. On its own, 5mM Mg^{2+} completely abolished responses to mechanical stimulation. When caffeine (10mM) was added after complete inhibition by Mg^{2+}, responses remained completely inhibited (Figure 4.40a). However when caffeine was added before complete inhibition of responses, the Mg^{2+}-induced

inhibition was temporarily halted (eg Figure 4.40d) or even reversed in some cases (e.g. Figures 4.40b,c); the degree of reversal being apparently related to the initial extent of inhibition by Mg^{2+}. This suggests that calcium entry is a prerequisite for caffeine's action, as one might expect for a CICR process.

Note that Mg^{2+} affected the dynamic and static counts to the same degree, until caffeine was added (Figure 4.41).
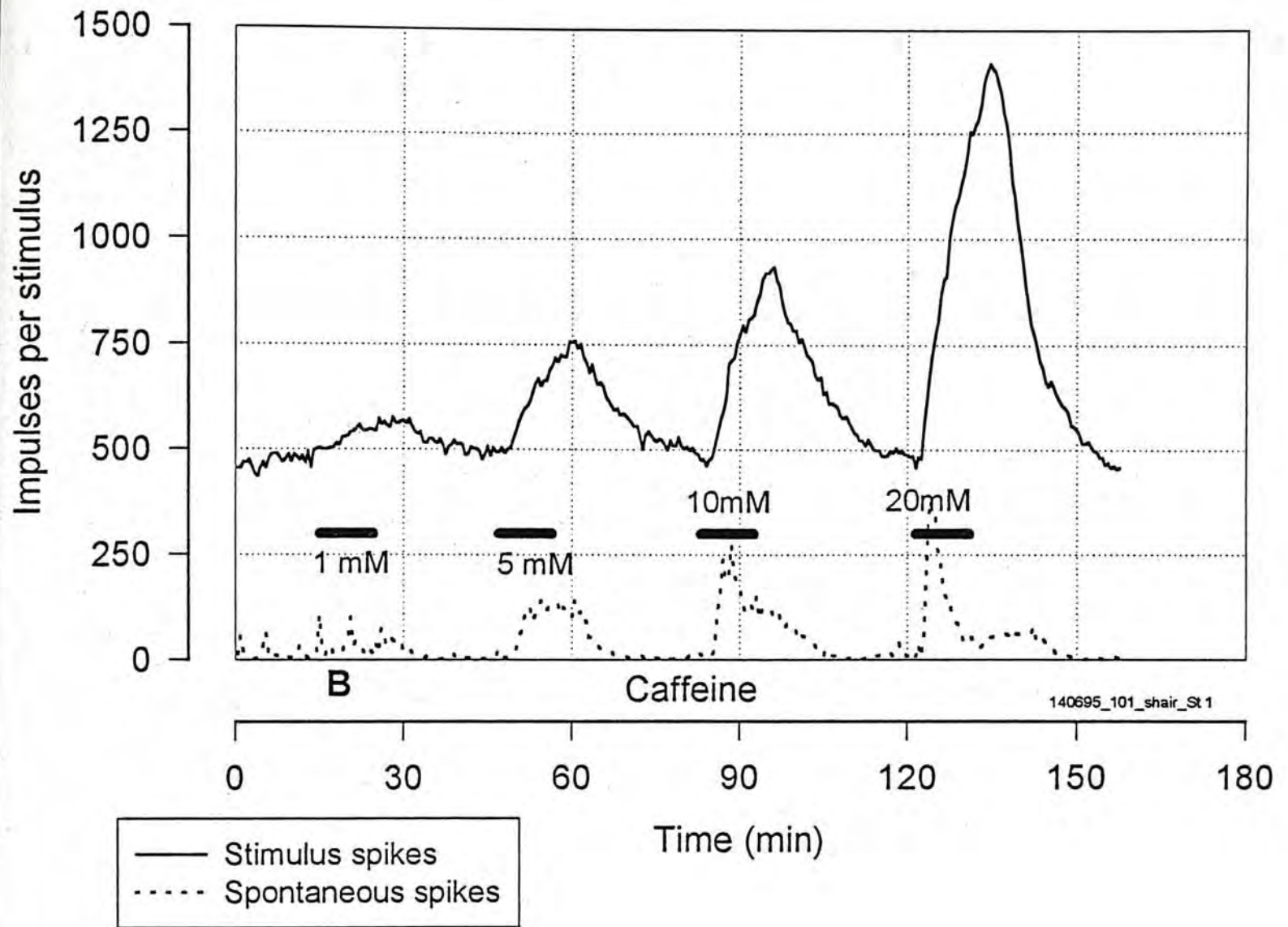
Figure 4.38

The effect of caffeine on St I receptor responses. The effect on receptor responses to mechanical stimulation is dose dependent. The associated transient low frequency spontaneous firing does not seem to depend very much on the concentration of caffeine (see also Fig. 4.42 in the St II).
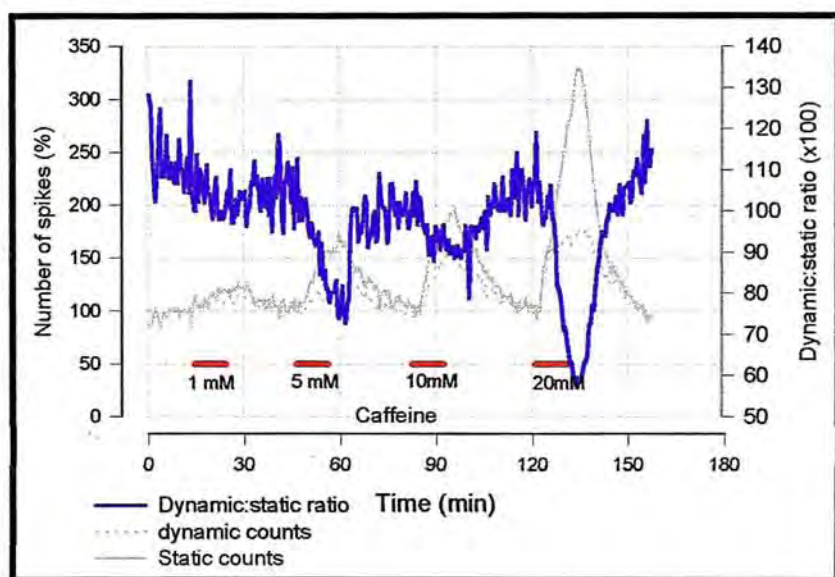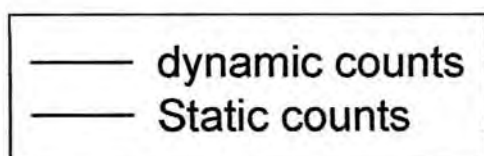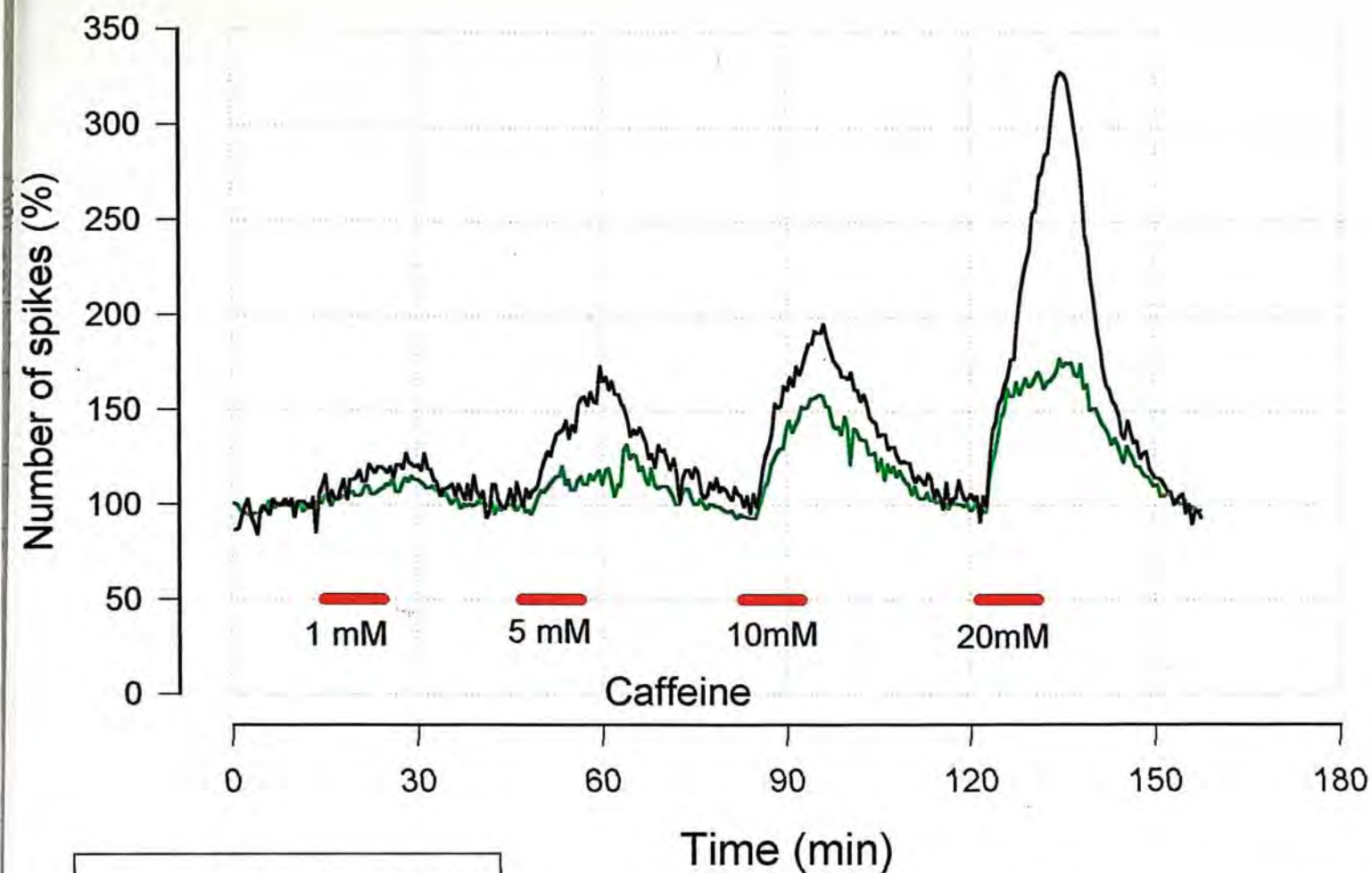
Figure 4.39

The effect of caffeine on St I receptor responses. % dynamic and % static counts are plotted together to show that during control periods, the responses during the two phases remain in tandem but break ranks when caffeine is superfused. Caffeine apparently had its greatest effect on the static phase. Inset - Dyn:Stat ratio (see Fig. 4.3) dropped when caffeine was superfused, further confirming that the static counts are affected to a greater extent than the dynamic.
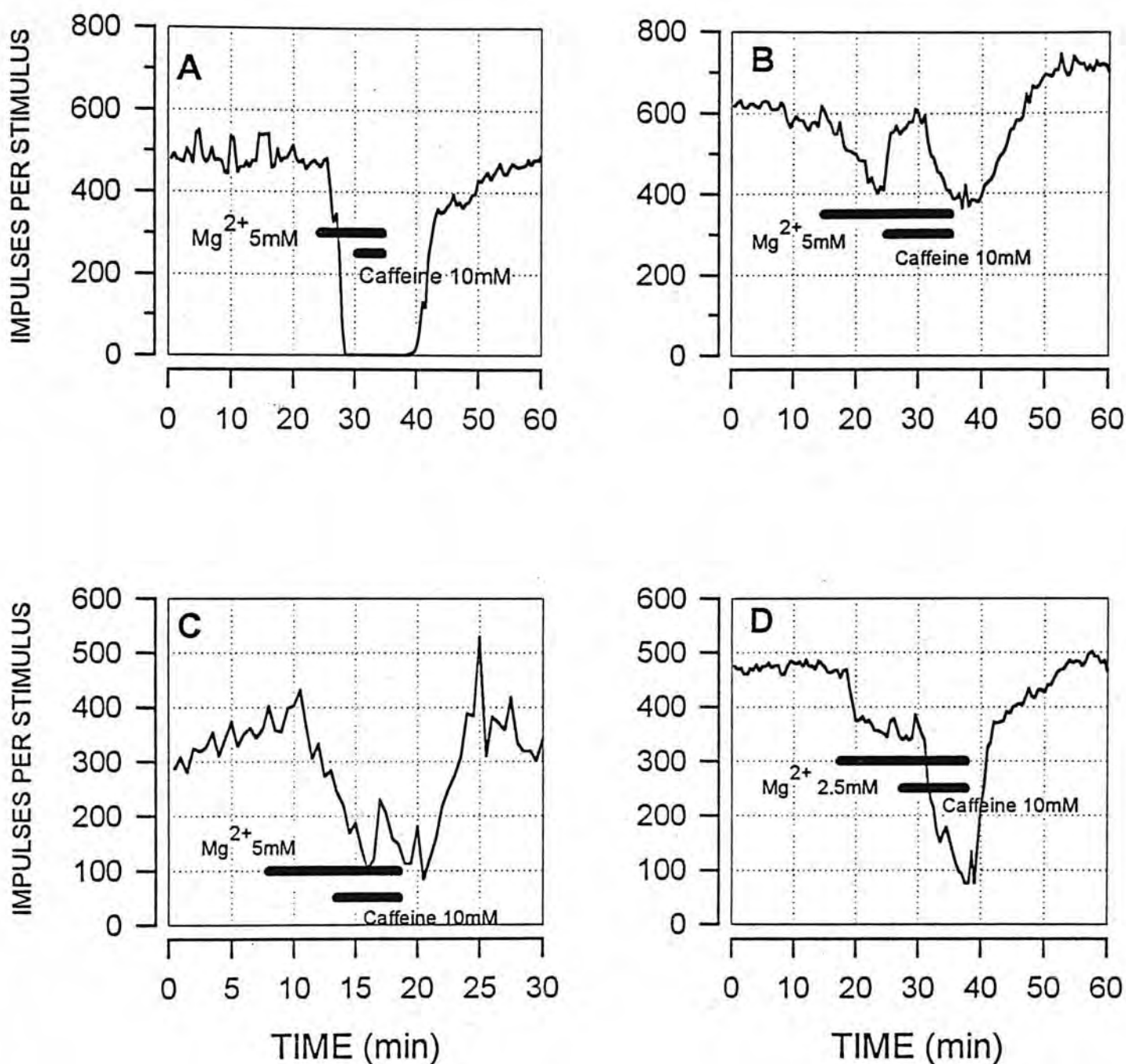
Figure 4.40

The effect of competitively blocking Ca²⁺ entry using Mg²⁺ on caffeine-induced excitation of St I receptors. **A.** Caffeine had no effect when used after complete inhibtion by 5mM Mg²⁺ . **B.** Mg²⁺ inhibition was temporarily reversed by caffeine, if applied before complete inhibition; apparently depending on the extent of initial inhibition by Mg²⁺ (**C**). **D.** Caffeine may only temporarily halt the progression of Mg²⁺ inhibition.
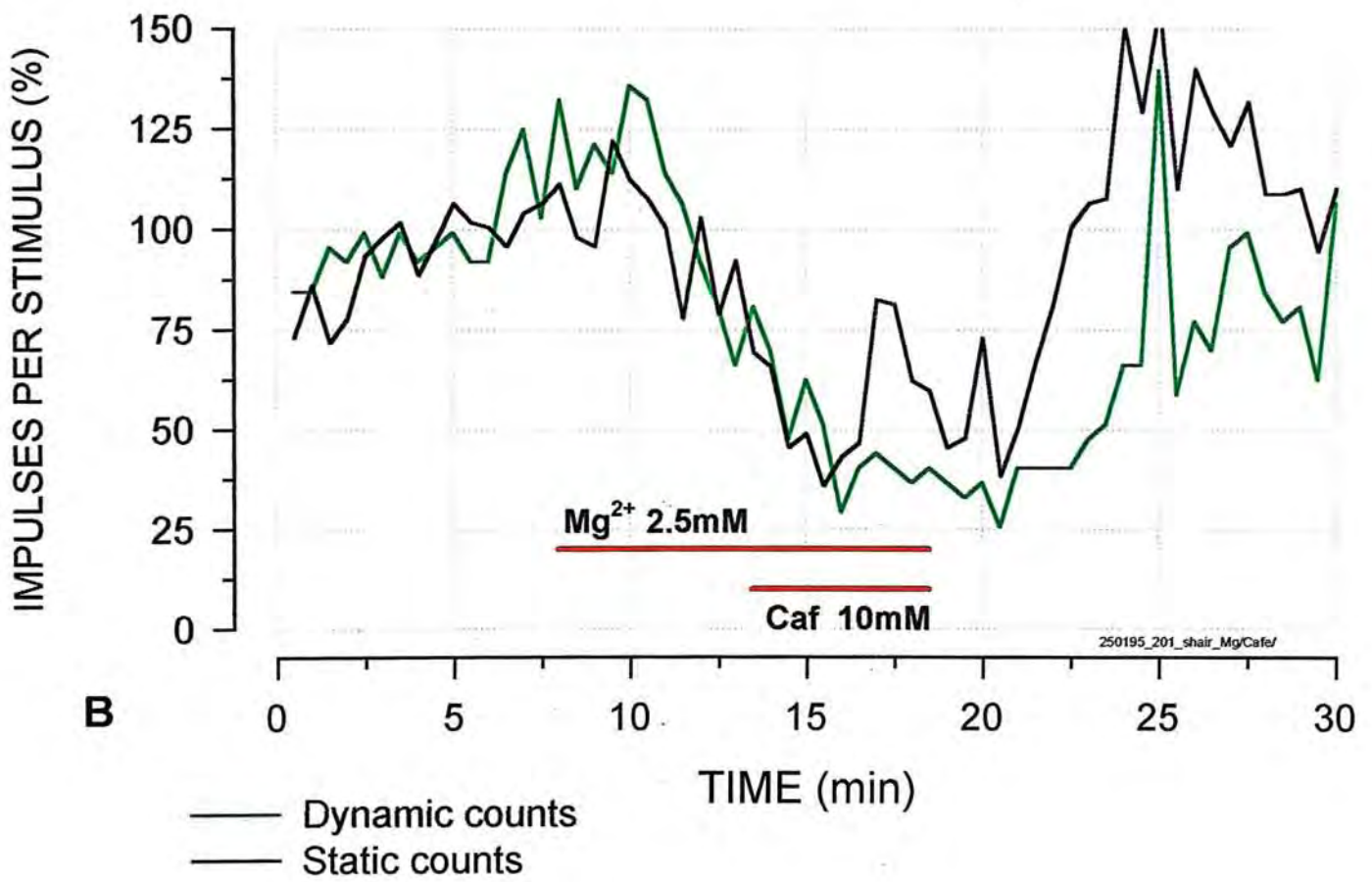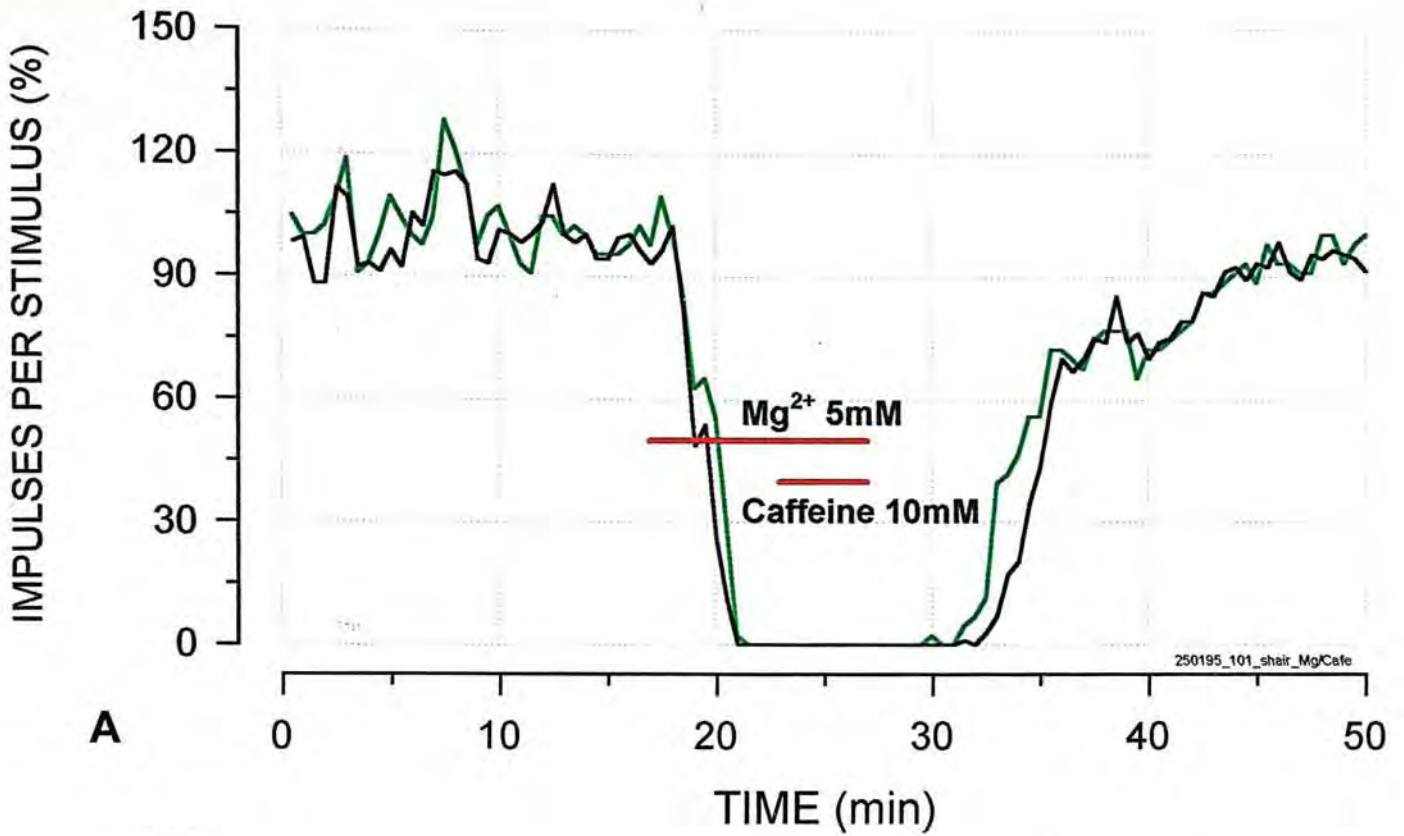
Figure 4.41

Mg^{2+} affected dynamic and static counts proportionately (falling phases of **A** and **B**) while caffeine affected static counts more (**B**). CF Fig. 4.39.

Caffeine on St II: St II receptor responses to mechanical stimulation were consistently inhibited by 10mM caffeine superfusion by 20 - 60% (mean ~35 ± 8% in 5 St II receptors superfused with 10mM caffeine for 15 minutes (Figure 4.44). 1mM caffeine had little effect while 5 and 10mM depressed responses to about the same extent, and 20mM almost completely the receptor responses (Figure 4.42). Like in the St I, caffeine also caused a transient increase in spontaneous firing of the St II receptors (Figures 4.42, 4.44).

Caffeine also seemed to depress the static responses to a greater extent than the dynamic, which was fairly resistant to inhibtion, even at the higher concentrations (Figure 4.43). The effect of 10mM caffeine on 6 St I and 5 St II receptors is compared in Figure 4.45.

The rather unexpected inhibition of St II responses by caffeine has provided another pharmacologic 'identifier' for St I and II receptors and further confirmed that the two receptors have fundamentally different transduction mechanisms. Caffeine has been shown to inhibit IP_3 generation and possibly functions (Missiaen _et al._ 1992; Bezprozvanny _et al._ 1994) at the millimolar concentrations that stimulate CICR. This suggests that the St II transduction process might involve the IP_3 system. Caffeine caused a transient increase in spontaneous firing in both receptors in spite of the opposite effects on stimulated responses. This may be a non-specific tendency of caffeine to increase the influx of calcium into cells (Bianchi, 1962; Bianchi, 1968).
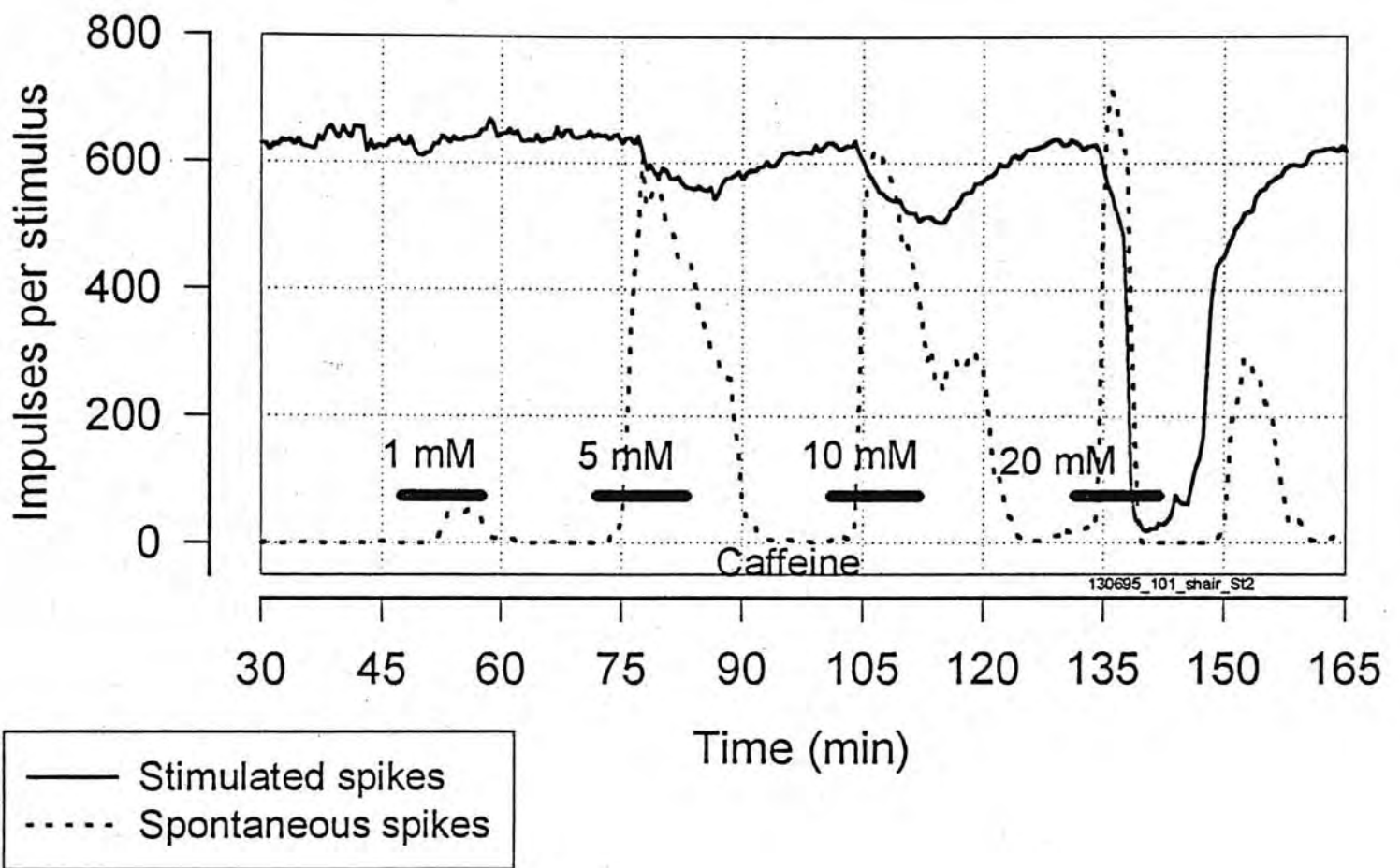
Figure 4.42

The effect of caffeine on St II receptor responses. The dose-response relationship is rather flat (CF St I). There is also spontaneous firing in response to caffeine in spite of the inhibition of stimulated responses.
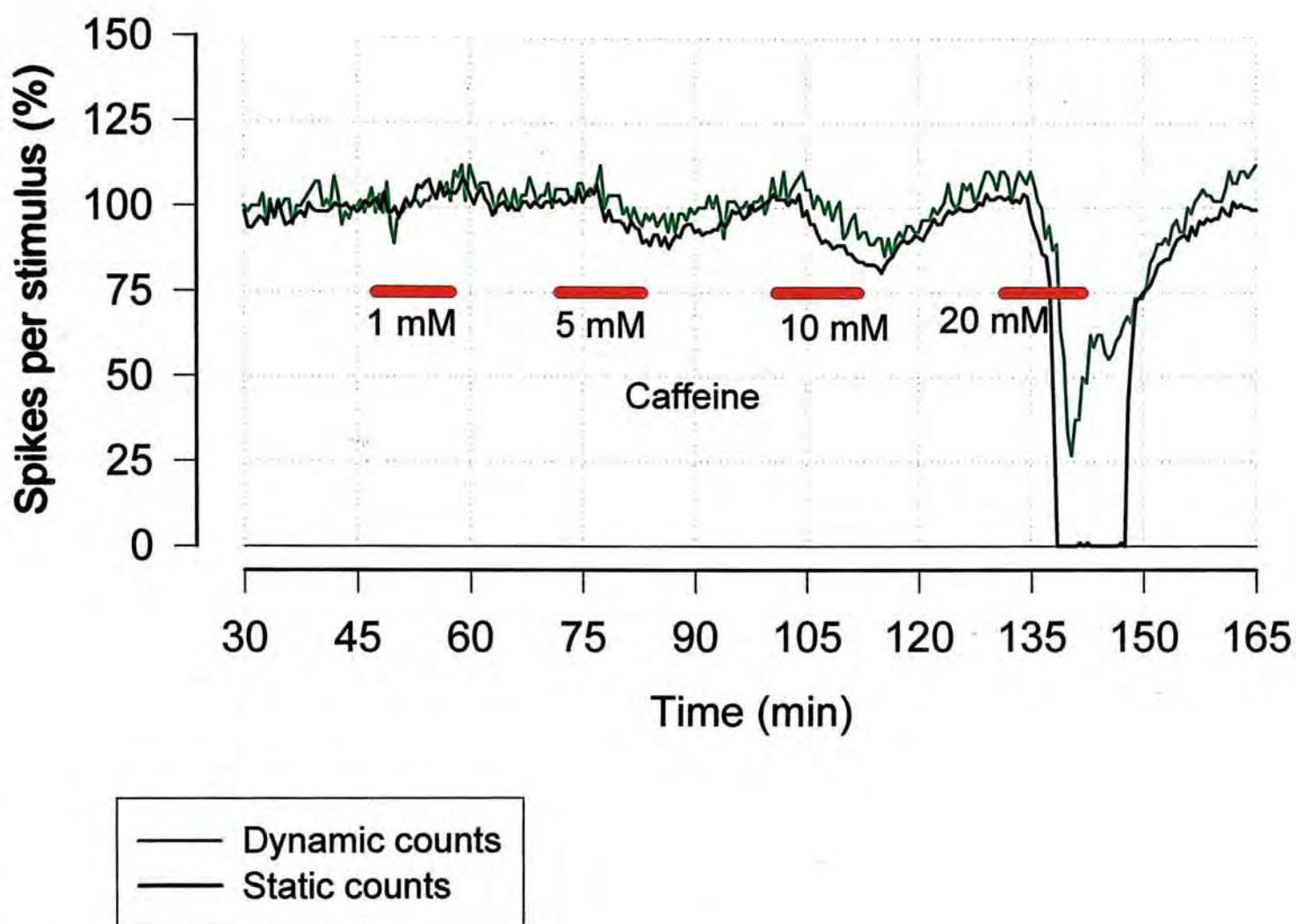
Figure 4.43

The effect of caffeine on St II receptor responses. Plotting the percent dynamic and percent static counts together show that the dynamic counts are relatively resistant to inhibition by caffeine, especially at the higher concentrations.
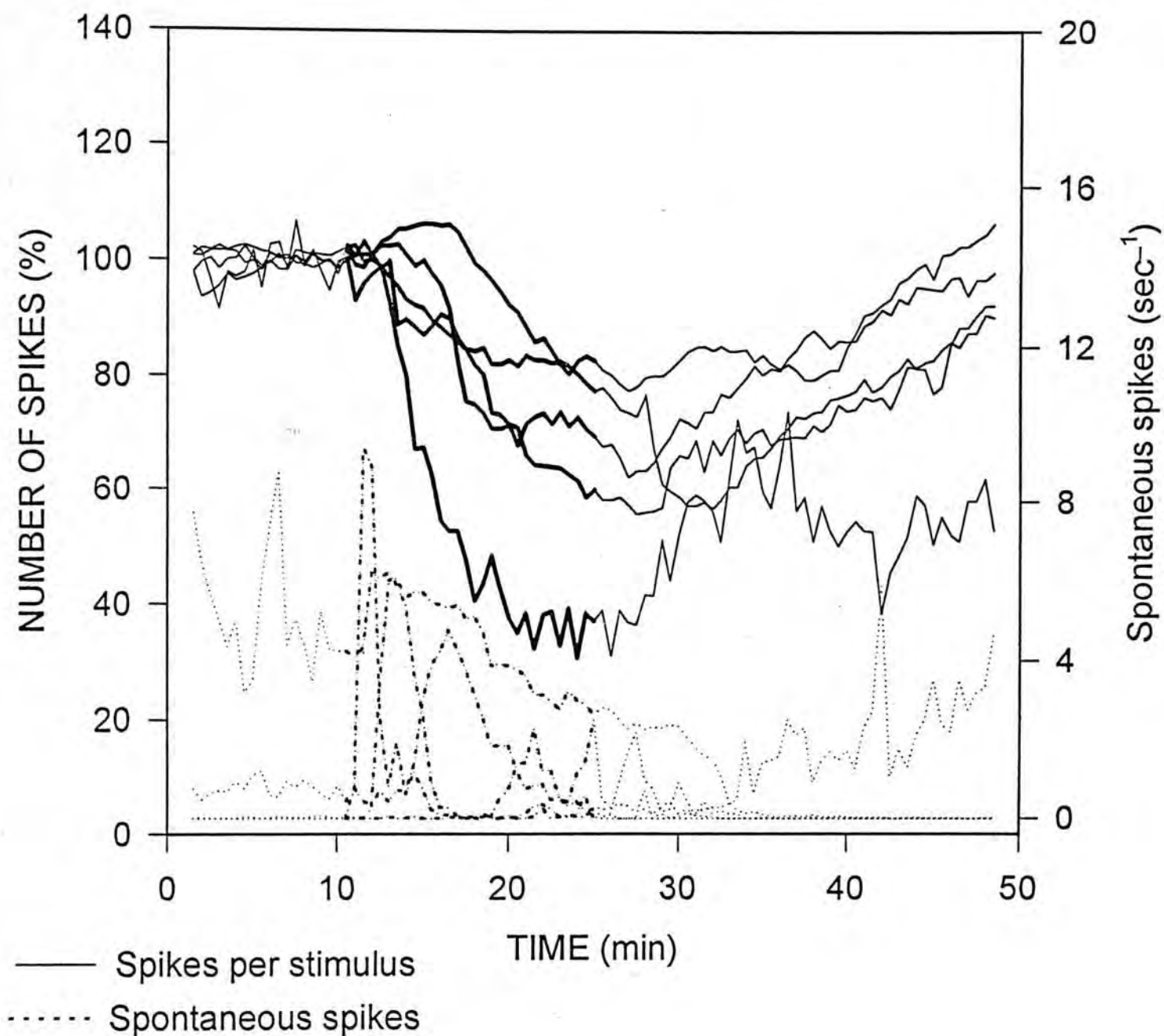
Figure 4.44

The effect of superfusing 10mM caffeine for 15 minutes on St II receptors. Although stimulated responses were inhibited, there was a transient increase in spontaneous firing, which drops before caffeine is removed, similar to that of St I receptors (Fig. 3.38). Darker segments in the traces represent the period of caffeine application.
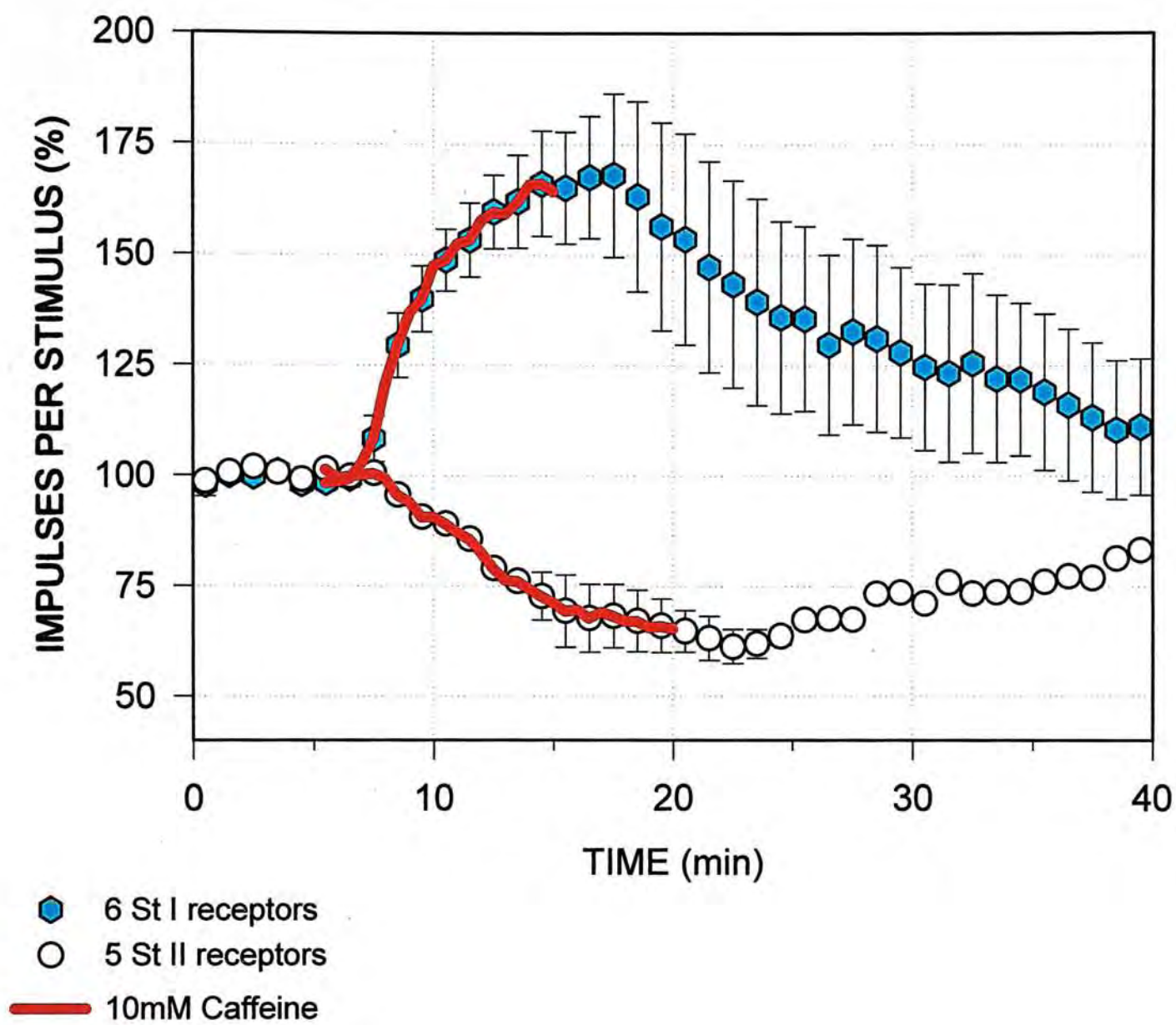
Figure 4.45

Effect of 10mM caffeine on 6 St I receptors (10 min) and 5 St II receptors (15 min).

b) Effect of Ryanodine:

The plant alkaloid ryanodine binds to the RYR channel and locks it in the open state, thus increasing the release of intracellular calcium from the stores (see (Ehrlich & Bezprozvanny, 1994; Pozzan et al. 1994) for recent reviews).

Ryanodine (1 - 10µM) increased responses to mechanical stimulation in the St I but had no effect on St II receptor responses. The increase in responses showed little tendency to recovery (Figure 4.46a). Superfusion of 10mM caffeine led to a further rise in responses which recovered to pre-ryanodine levels. Figure 4.47 shows that ryanodine (1µM) increased responses to $135.32 \pm 14.61\%$ of control values in 4 St I receptors ($p<0.05$). Figure 4.46b shows that 10µM ryanodine had no appreciable effect on the St II receptor which was subsequently responsive to caffeine in the expected manner as described above.

The sustained effect of ryanodine is expected since it binds strongly to the receptor (irreversibly so in the sarcoplasmic reticulum); these results however seem to suggest that caffeine is displacing ryanodine from the receptor.
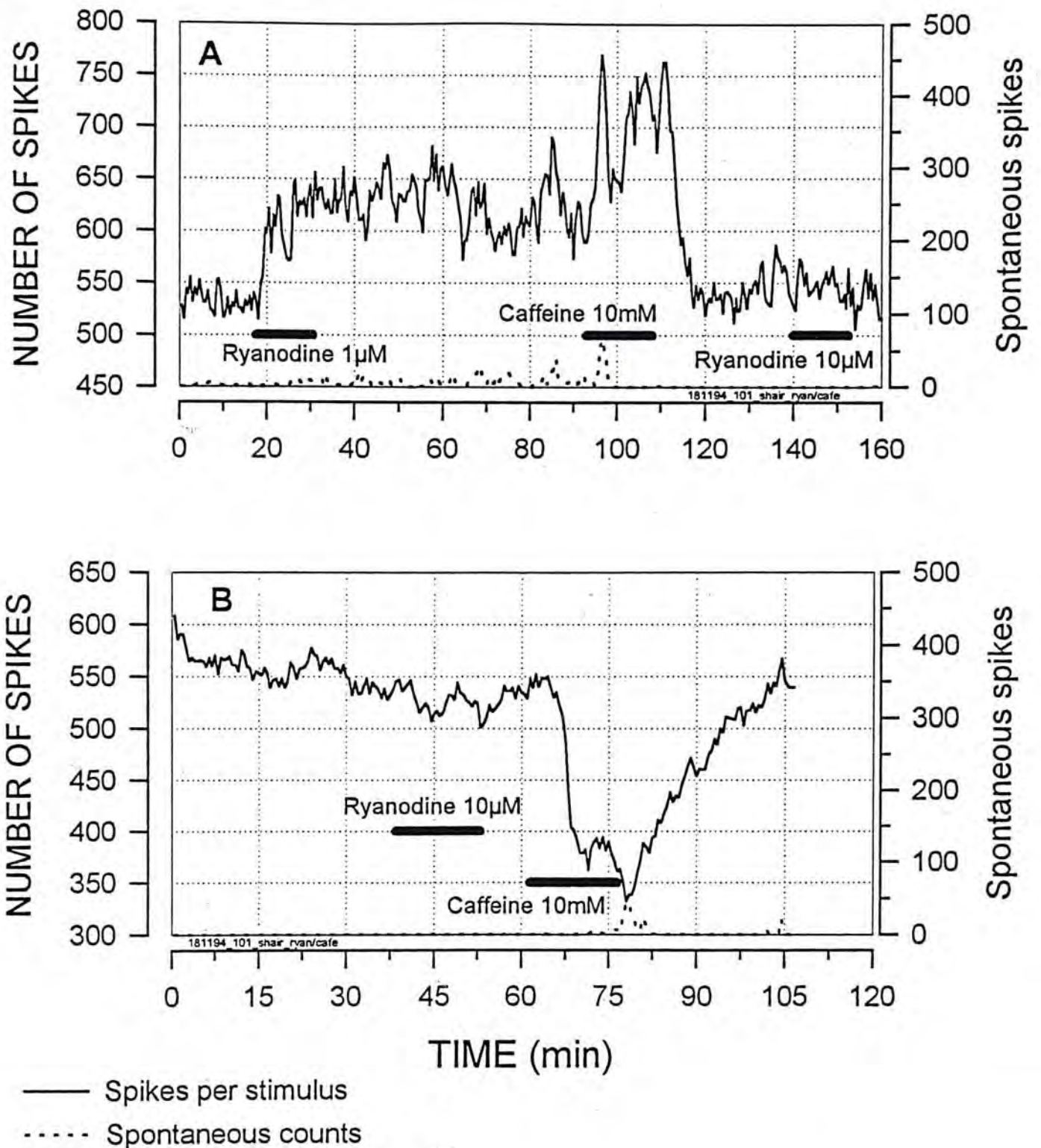
Figure 4.46

Effect of ryanodine and caffeine on single St I and St II receptors. Ryanodine caused a sustained increase in St I responses (**A**) while having no effect on the St II (**B**). 10mM caffeine subsequently increased (St I) and decreased (St II) responses as expected.
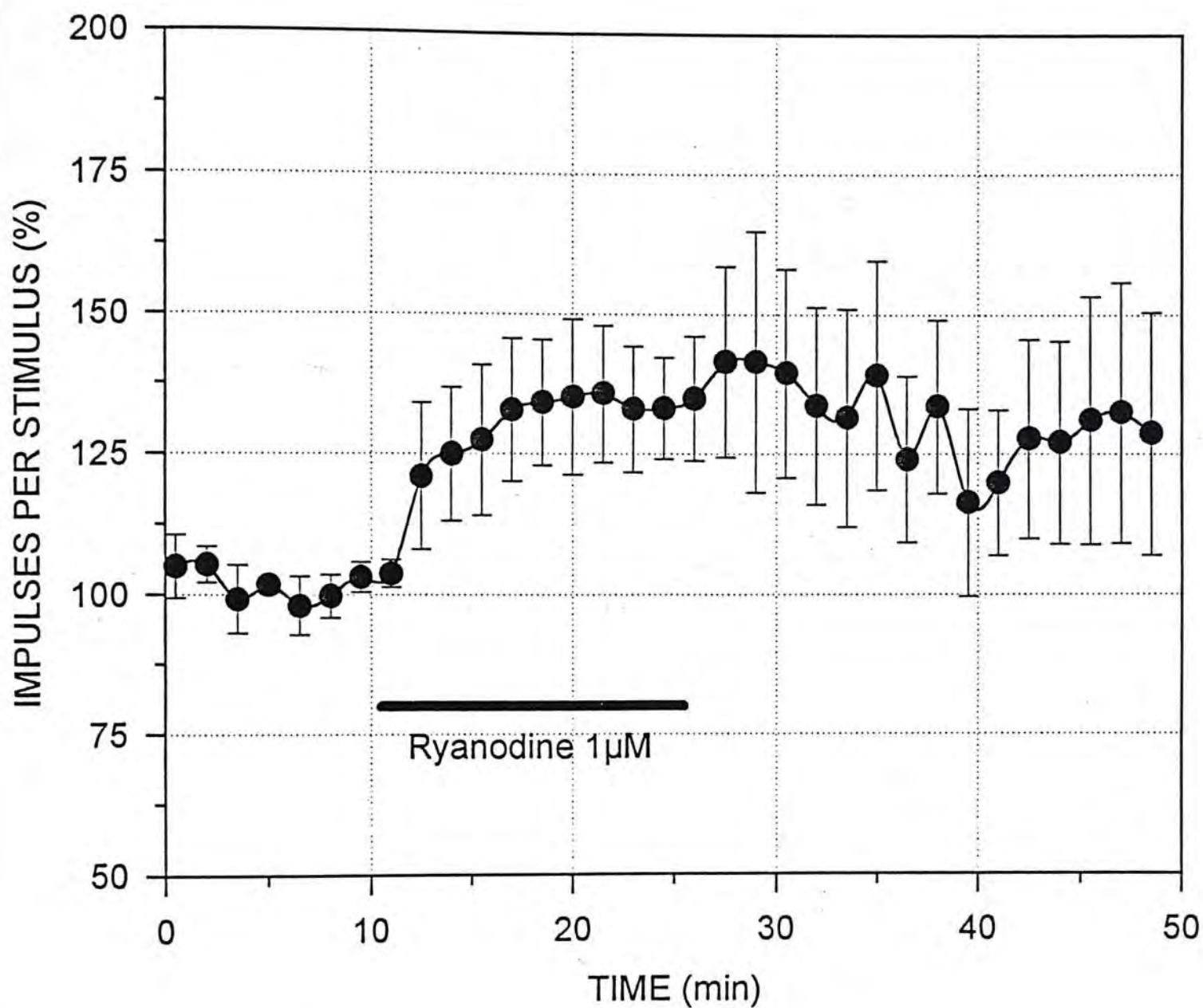
Figure 4.47

Effect of 15 minutes' superfusion of ryanodine on 4 St I receptors, showing the sustained increase in stimulated responses ($p<0.05$).

c) **Effect of Heparin:**

Heparin is used to inhibit intracellular calcium mobilization through the IP_3 second messenger pathway which it competitively inhibits (Ghosh et al. 1988; Taylor & Richardson, 1991). Heparin was subsequently shown to activate the ryanodine receptor, thereby activating calcium release through the ryanodine receptor (Ritov et al. 1985; Bezprozvanny et al. 1993).

Heparin was hesitantly applied in the present study because this polyanion was not expected to cross the cell membrane. It was tried on the receptors nonetheless.

Heparin was found to increase the responses of St I receptors to mechanical stimulation in a dose dependent manner (Figure 4.48). Superfusion of 1.5mg/ml (equivalent to 2100 units) of heparin in 6 St I receptors for 10 minutes resulted in ~40% increase in responses ($p<0.01$), with a range of about 30 - 70% (Figure 4.49). Unlike caffeine, heparin did not increase ongoing activity (e.g. Figures 4.48 and 4.50).

St II receptors were not affected by heparin ($p=0.14$ for 7 St II receptors tested with 1.5mg/ml heparin, Figure 4.49).

If heparin were acting intracellularly then the effect on St I responses would not be unexpected (see above), but it would argue against IP_3 involvement in St II mechanoelectric transduction, unless it was able to cross the membrane in Merkel cells but not the nerve terminals.

Heparin and other polyanions do not cross the cell membrane however (Knaus *et al.* 1990; Lacinova *et al.* 1993). Heparin must therefore be acting extracellularly, possibly as an agonist of L-type Ca^{2+} channels in the St I (see Knaus *et al.* 1990; Knaus *et al.* 1992).

Pentosan polysulfate (PPS), another polyanion with similar action as heparin on IP_3-induced intracellular Ca^{2+} mobilisation, had no effect on St I receptors (Figure 4.51). This suggests that the extracellular action of heparin on the St I is not shared by other polyanions but is specific to heparin (e.g. Lacinova *et al.* 1993).
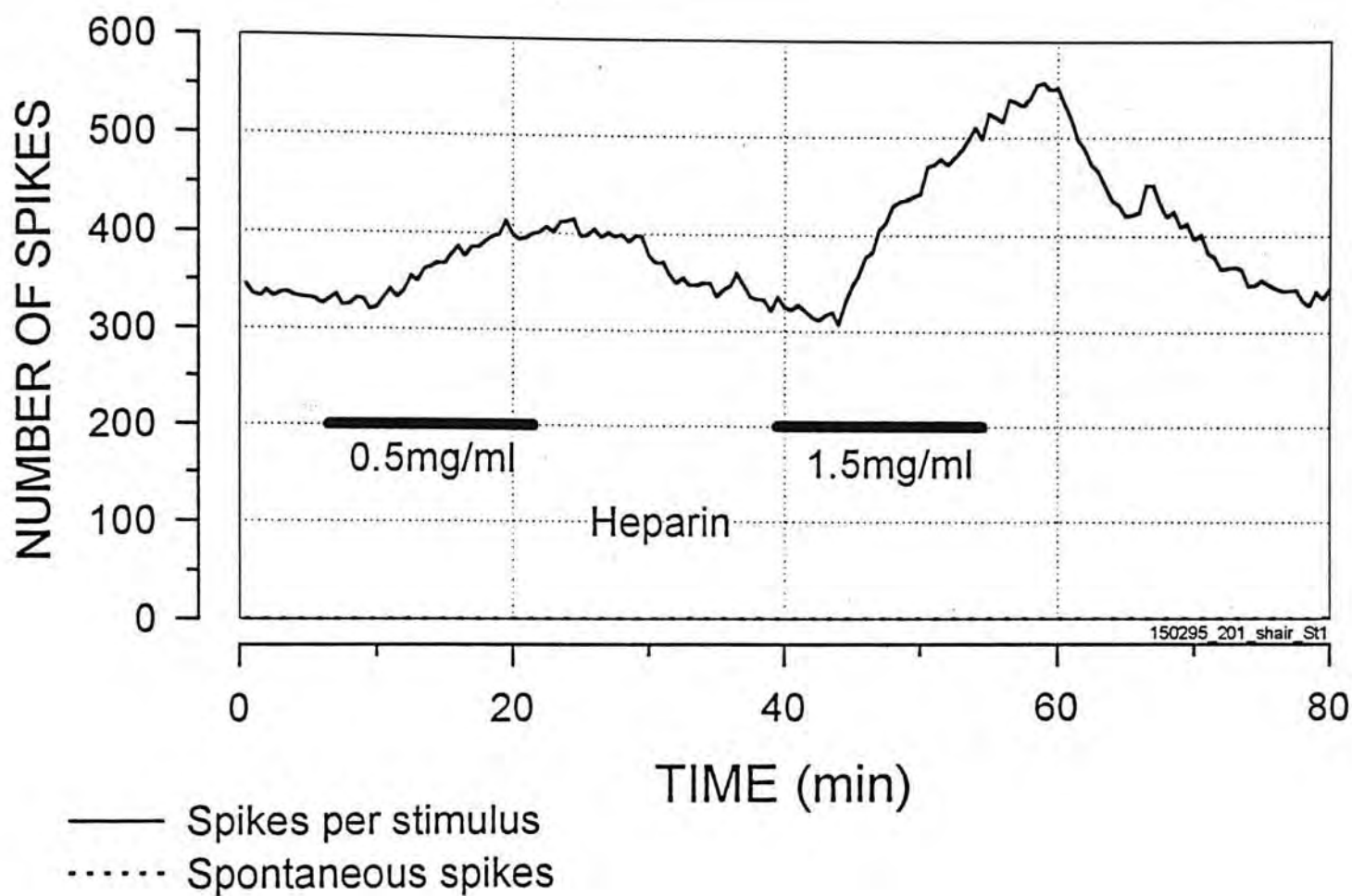
Figure 4.48

Effect of heparin on the responses of a single St I receptor to controlled mechanical stimulation showing dose dependence. Unlike caffeine, heparin did not affect spontaneous firing.
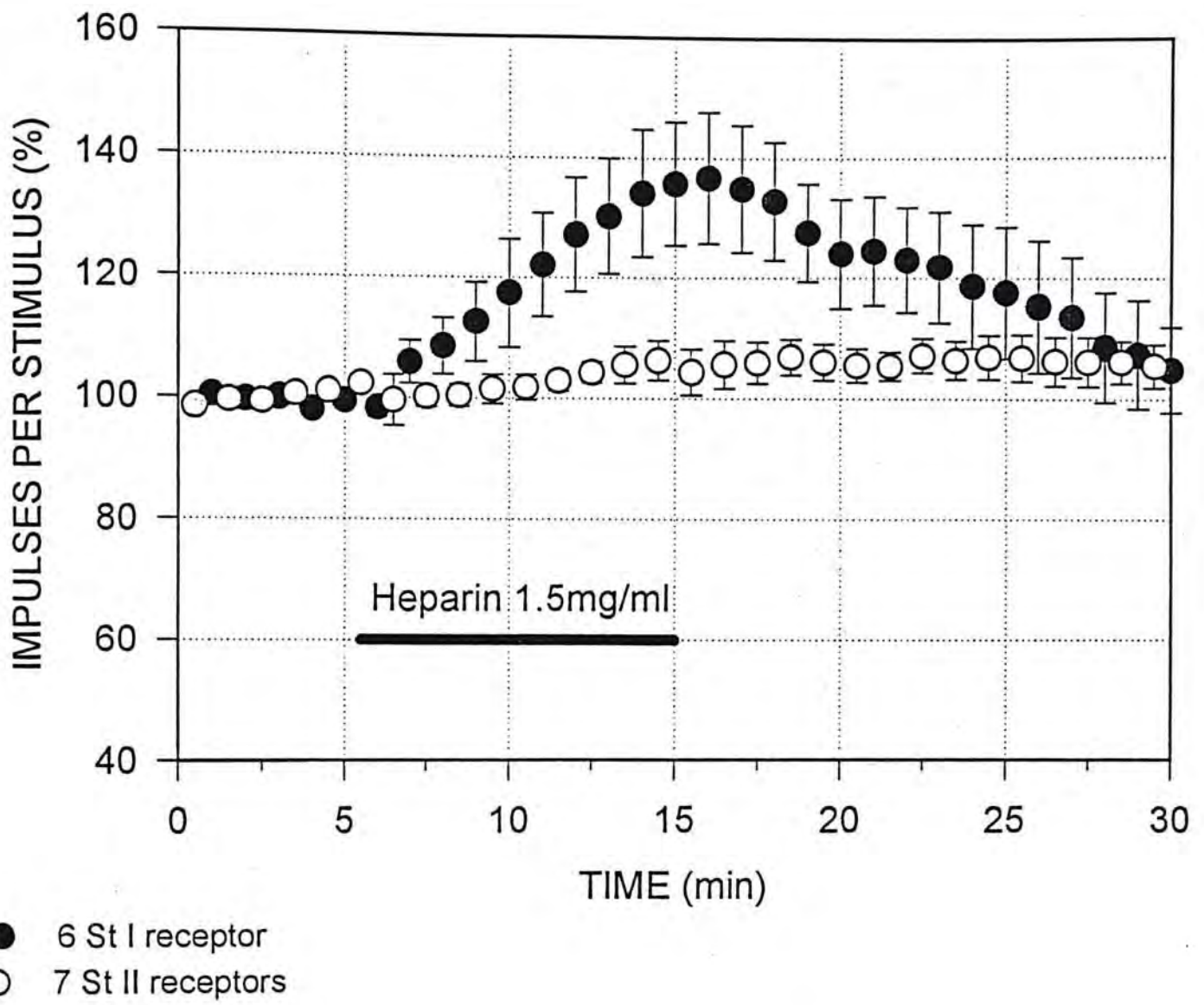
Figure 4.49

Effect of 10 minutes' superfusion of 1.5mg/ml heparin on the responses of 6 St I ($p<0.01$) and 7 St II receptors to controlled mechanical stimulation. Heparin had no effect on the St II receptors ($p=0.14$).
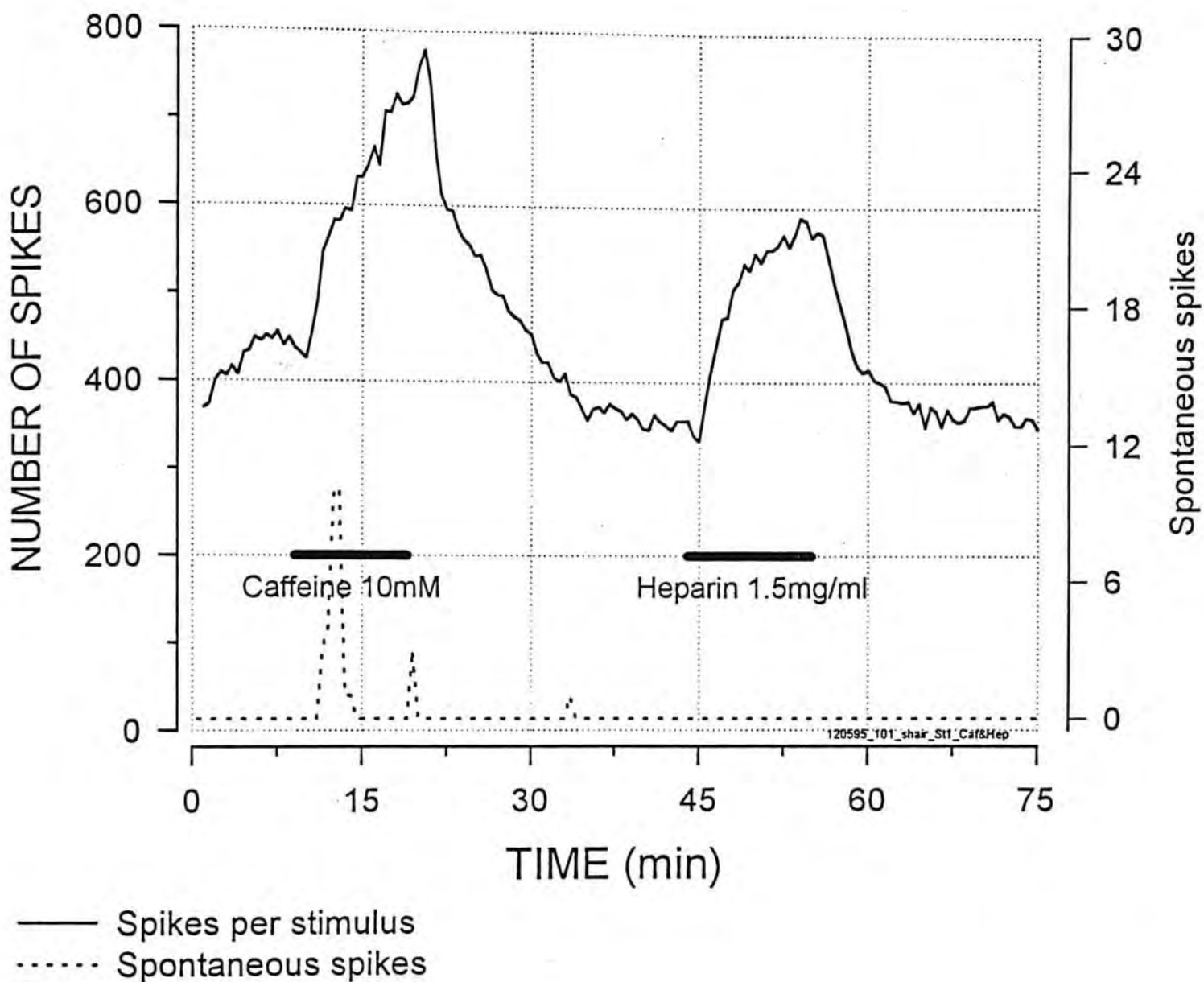
Figure 4.50

Caffeine (10mM) and Heparin (1.5mg/ml) increase responses in an St I receptor. Heparin did not effect spontaneous activity in the receptor.
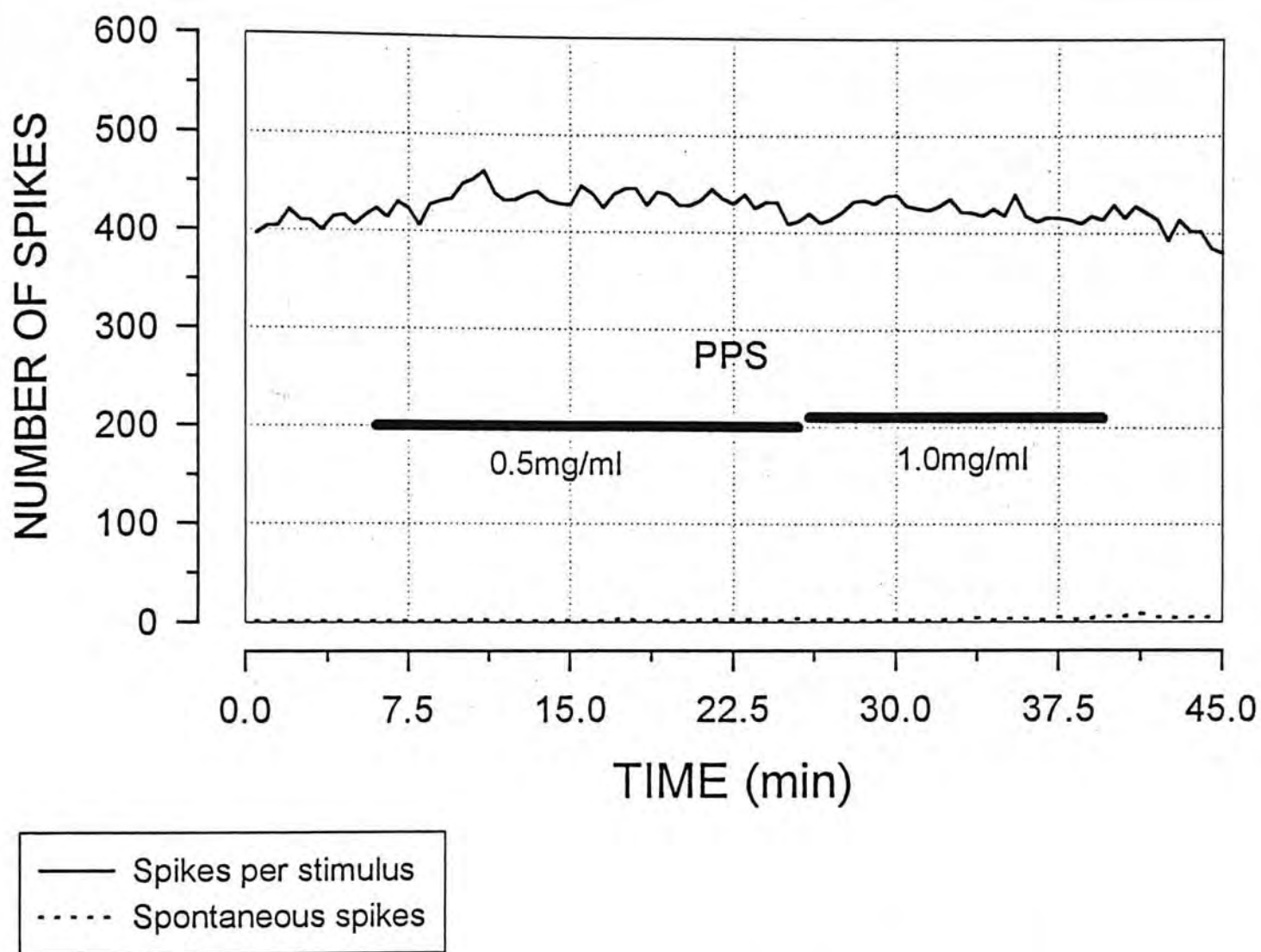
Figure 4.51

Pentosan polysulfate (PPS) had no effect on St I receptor function.

d) **Effect of Procaine:**

Procaine HCl, better known for its action as a local anaesthetic is used as a specific inhibitor of CICR (see Galione *et al.* 1991; Khoyi *et al.* 1993; Lee, 1993; Huang, 1995).

Procaine inhibited St I receptor responses in a dose-dependent manner. Near maximal inhibition was attained with 100μM procaine Fig. 4.52a). In 4 St I units, mean responses were depressed to <25% of control values (Figure 4.55). Procaine (100μM) also blocked the excitatory effect of caffeine (10mM) when they were superfused together. There was an aborted attempt to increase counts, with responses settling down at about 80% of control values (Figure 4.53). Thus the two compounds were antagonistic to each other, suggesting that they are acting at the same site. Caffeine (10mM) alone increased counts by about 90% while procaine (100μM) alone decreased responses by about 80% in the same receptor (Figure 4.53).

The responses of St II receptors were little affected by up to 100μM procaine superfusion. 50μM procaine had no effect on responses while 100μM produced about 10% drop in responses (Figures 4.52b, 4.55). When present, spontaneous activity in St II receptors was silenced by 100μM procaine.

St II receptors were completely blocked by procaine at concentrations above 100μM (Figure 4.54). Recovery was however rapid and rather abrupt (within 10 minutes, compared to 30 - 45 for St I). This is most likely a manifestation of the local anaethetic action of procaine rather than a specific action on the receptor.

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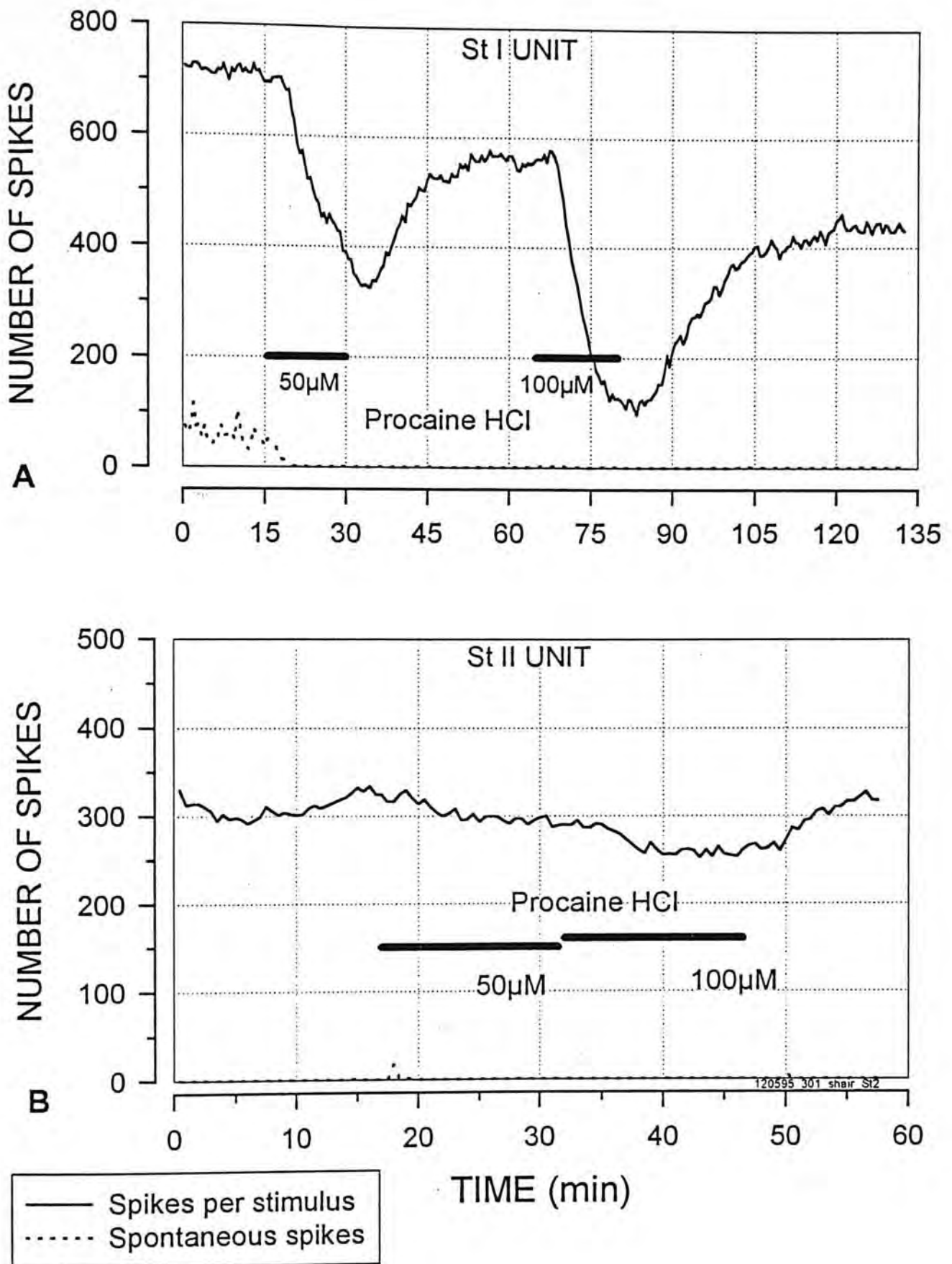
Figure 4.52

Effect of procaine HCl on single St I and St II units. **A** St I receptor dose dependently inhibited. **B** St II receptor resistant to procaine even with 30 minutes' continuous superfusion of the same concentrations as in the St I. Note the relatively rapid recovery in the St II receptor.
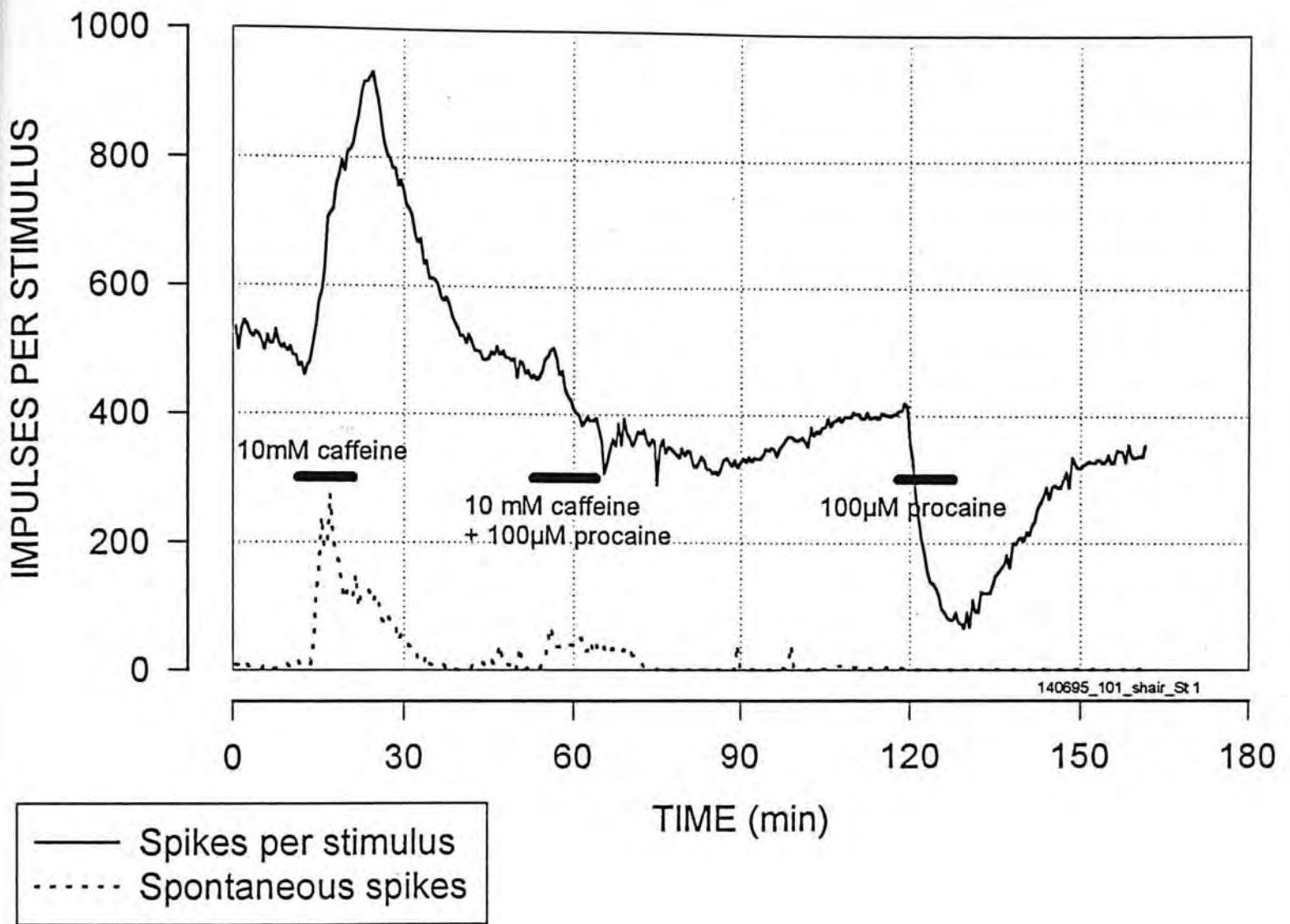
Figure 4.53

Effect of 10mM caffeine and 100μM procaine HCI on St I responses. Procaine prevented the caffeine-induced excitation while caffeine prevented procaine's inhibitory action. Both stimulated and spontaneous firing are similarly affected.
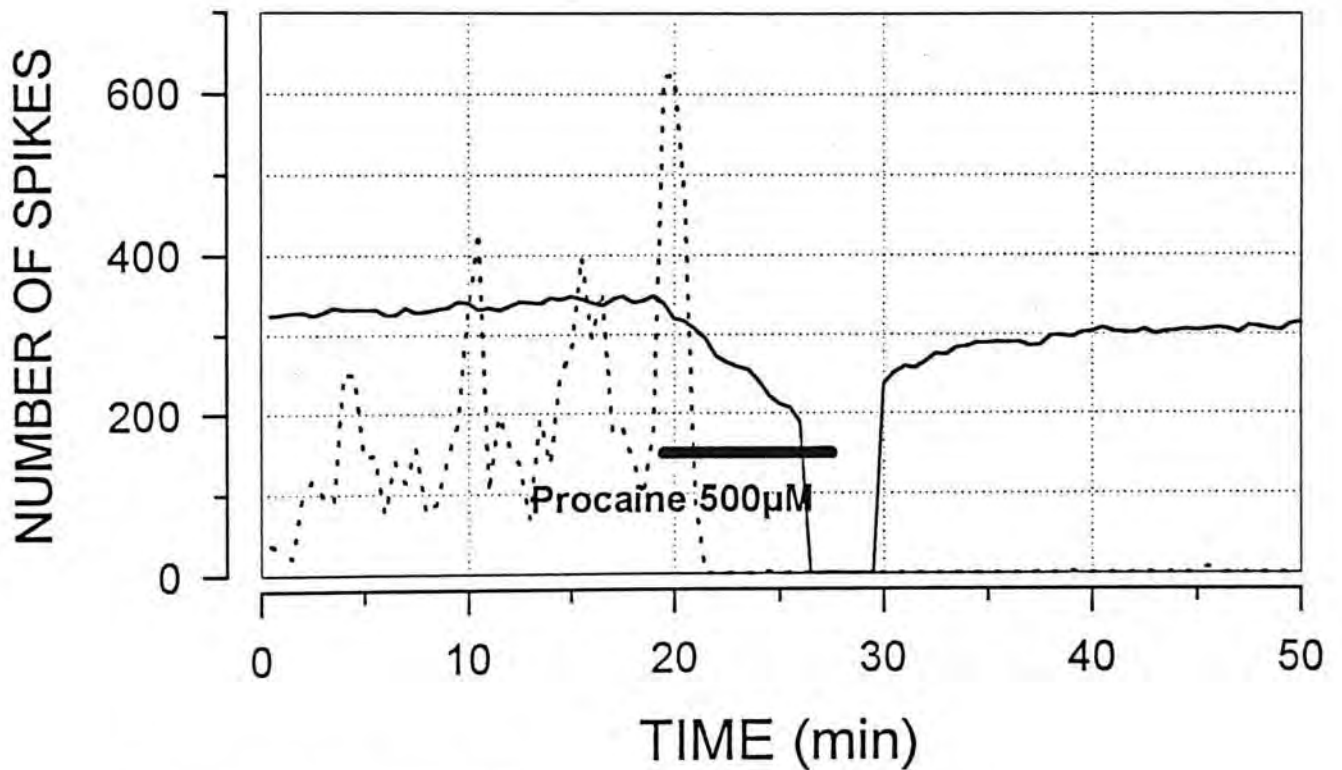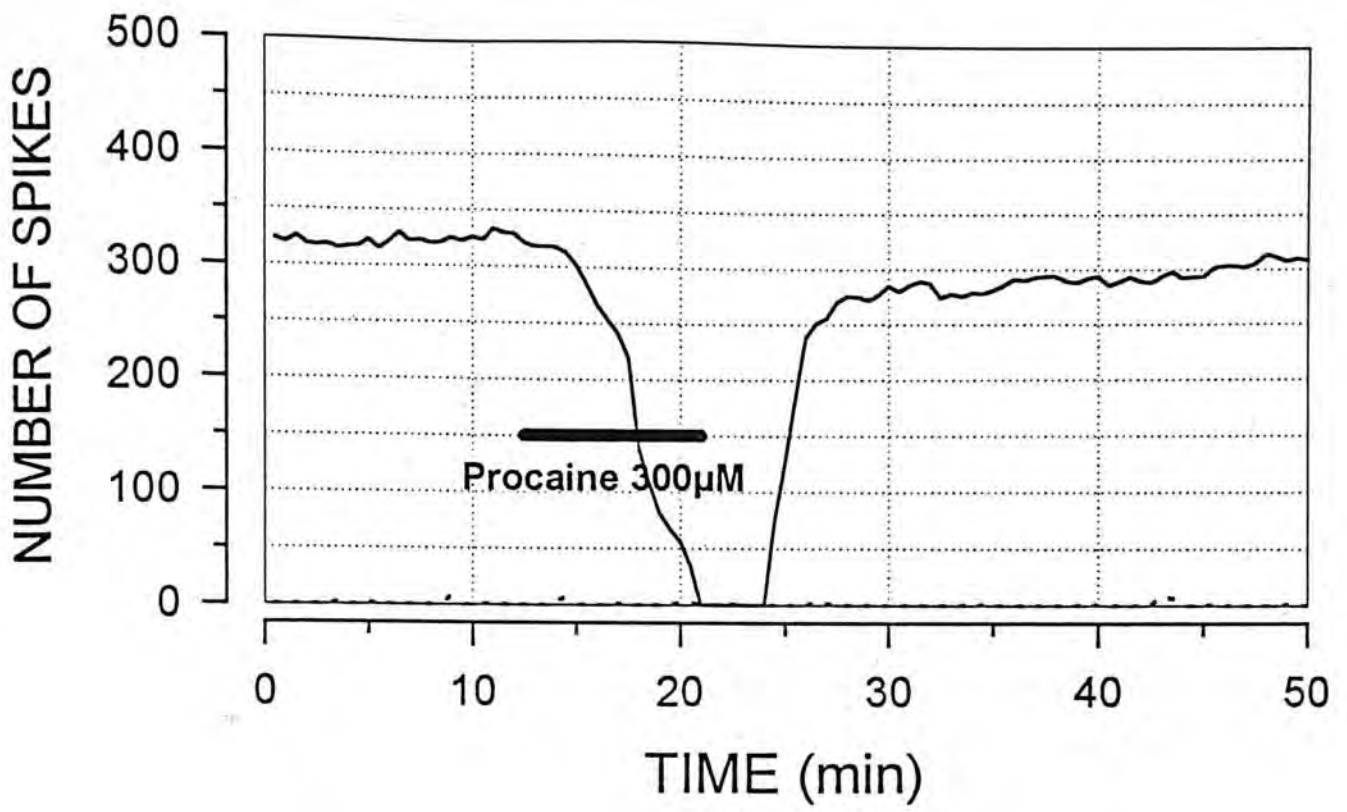
Figure 4.54

High concentrations of procaine produce a complete inhibiton of St II responses. The recovery is however rapid (<10 minutes). This is similar to the effect of high concentrations of CQ on the St II (Fig. 4.24) and is most likely a manifestation of the local anaesthetic properties of the two drugs.

O   4 St I receptors

●   4 St II receptors

··········   Individual experiments
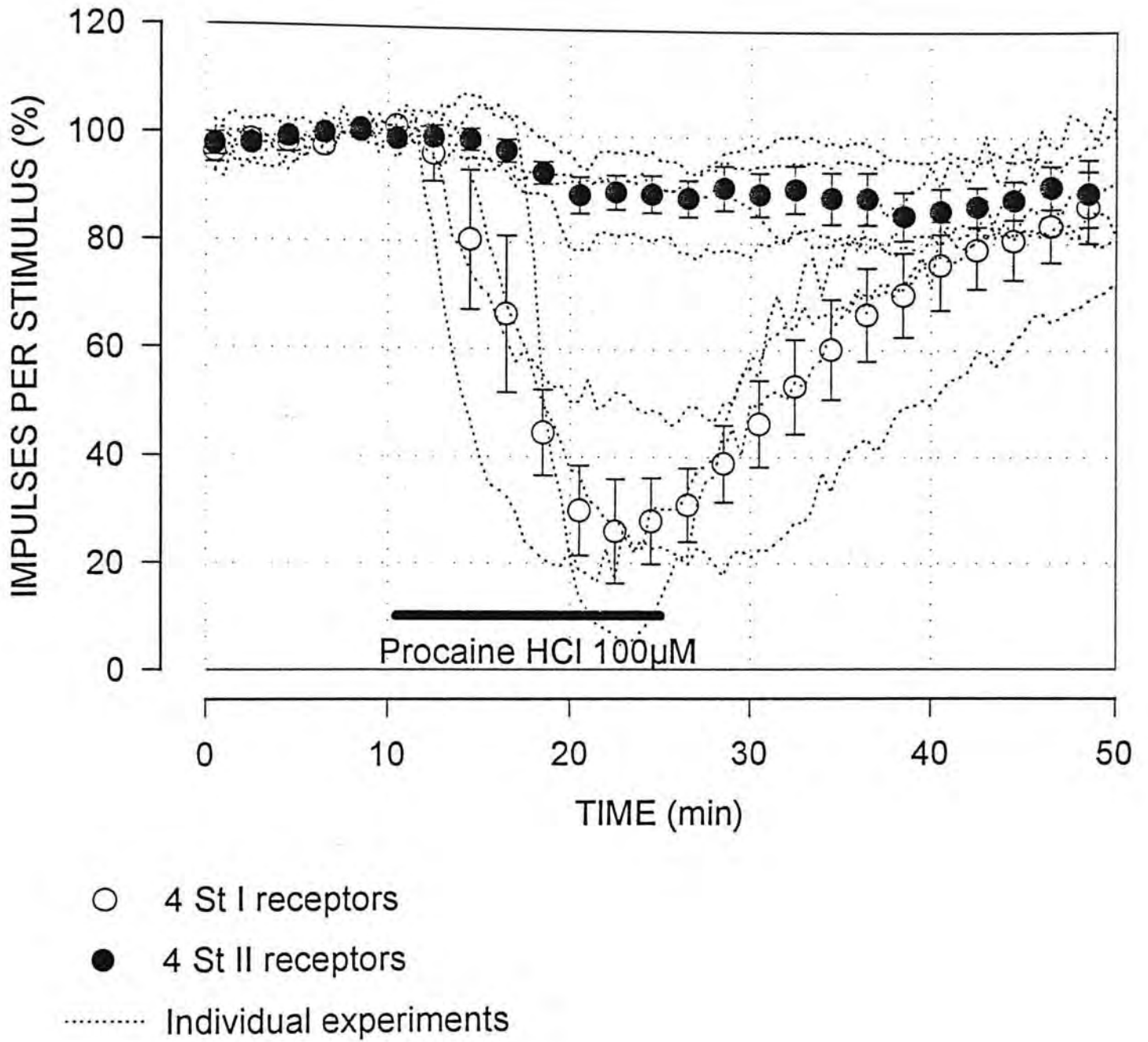
Figure 4.55

Effect of 100µM procaine HCl superfusion on the responses of 4 St I and 4 St II units. St I responses were inhibited by almost 80% while St II responses were inhibited by about 10%.

Chapter 5
DISCUSSION

5.1   OUTCOME OF PROJECT

i.   **Development and perfection of the technique** for functionally studying the mechanoreceptors of rat vibrissae *in vitro*. The study has been restricted to the slowly adapting receptors but any of the other nerve endings in the vibrissa could be studied using the preparation.

ii.   **Characterisation of the vibrissal slowly adapting mechanoreceptors** on the basis of response properties. This had been difficult for some workers (see literature review). The finding that the St II type of responses may constitute up to 80% of the slowly adapting responses recorded might explain why the casual observer whose principal interest is not in the vibrissal receptors themselves might find it difficult to make such a distinction.

Pharmacological characterisation: The pharmacological studies have provided the first qualitative 'identifiers' for Merkel cell receptors. In this regard, the findings that chloroquine and caffeine affect St I and St II receptors in a qualitatively opposite manner are of particular interest. Apart from the obvious implications of these findings on the proposed transduction mechanisms of these receptors,

there is now little doubt that the identification of the receptors on the basis of response characteristics alone, as done by Gottschaldt *et al.* (1973) and in this project, is valid.

iii. **Evidence for a calcium induced calcium release (CICR)** mechanism through the ryanodine (RYR) intracellular calcium release channel has been provided for the St I receptor with some suggestion that an IP3 mediated mechanism might operate in the St II receptor.

The implications of these findings on the current views on the physiology of the mechanoreceptors in question are discussed in this chapter. In particular, a review of the current model of mechano-electric transduction in Merkel cell endings (St I) is suggested in the light of the new data. Also, further investigation that might provide conclusive evidence and hopefully resolve the Merkel cell controversy is suggested.

5.2 THE ISOLATED VIBRISSAL PREPARATION

Single unit receptor responses could be recorded from slowly adapting mechanoreceptors for at least 5 hours from isolated vibrissal follicles even from hair follicles that had been dissected over 24 hours earlier. The receptors still looked normal under electron microscopy following 24 hours' storage and a few hours of recording. The preparation, as developed and used for these studies therefore appears to be quite stable. The ability of the receptors to respond to mechanical stimuli applied by bending the sinus hair shaft intermittently for long periods of time would suggest that the stimulus applied was appropriate and that such responses were a valid measure of the function of these receptors.

Direction sensitivity: This was observed in the slowly adapting mechanoreceptors in this preparation. Directional sensitivity in vibrissal mechanoreceptors has been described by several authors (e.g. Fitzgerald, 1940; Zucker & Welker, 1969; Hahn, 1971; Gottschaldt *et al.*, 1973; Waite, 1973; Lichtenstein et al., 1990; Waite & Jacquin, 1992) The receptors responded best when the hair shaft was displaced from the resting position towards one direction but not the other (we used only one plane of stimulation in two opposite directions). Gottschaldt and co-workers (1973) however found only positional sensitivity in St II receptors.

By slitting open the thick sinus body capsule enclosing the hair follicle, the preparation has provided excellent access to the erstwhile inaccessible receptors. This was especially useful for attempts at using pharmacological agents with a view to furthering the understanding of the transduction mechanisms of the receptors in their native location.

After overcoming the initial technical difficulties of microdissecting the sinus hairs, the preparation was found to be very convenient. One could get as many hairs as needed from one animal and if need be, even store them overnight for later use.

The receptors in the preparation were found to be remarkably robust. Even without splitting the sinus body capsule, single unit responses could be recorded for hours, i.e. splitting the capsule was not an absolute requirement except for the obvious difficulty when a drug needed to be superfused. Apparently, the receptors could still get sufficient nutrients through the capsule and the non-circulating blood in the blood sinus. This compares well with reports that even O_2 diffusion through the skin was apparently sufficient to prevent hypoxia in cutaneous SAI mechanoreceptors (Iggo & Findlater, 1984) It is interesting to note that the blood within the sinus did not clot as long as the sinus remained intact.

A possible problem arising from this apparent ability of the receptors to survive so well is that a receptor under study may actually lie directly underneath, pinned to the silgard platform. Thus while this

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receptor may continue to give stable responses to the cyclical controlled displacements used, it may not be so accessible to superfused drugs. It is thus important to try as much as possible to lay the capsule open from top to bottom. As stated in the *Methods* section, one got the distinct impression that there were more receptors on the side of the nerve entry and that slitting the capsule just beside the DVN entry gave better results in drug superfusion experiments, since one was more likely to have the receptors under investigation being located on the surface rather than underneath.

5.3 THE VIBRISSAL SLOWLY ADAPTING MECHANORECEPTORS

A clear distinction was possible between the sub-types of the slowly adapting mechanoreceptors in the preparation based entirely on their response properties. This is in line with the findings of Gottschaldt *et al.* (1973) in the cat. To the best of our knowledge the present study is the first to successfully characterise the vibrissal slowly adatping mechanoreceptors *in vitro*. Furthermore, this study has demonstrated that mechanoreceptors distinguished on the basis of response properties, responded differently to certain pharmacological agents.

The St I receptor: The irregularly firing St I receptor is generally accepted as the Merkel cell receptor in vibrissae though this is yet to be directly demonstrated. The typical discharge pattern associated with cutaneous SA I receptors has been shown to originate from Merkel nerve endings in the skin (Iggo & Muir, 1969). No other cutaneous mechanoreceptor is known to give this irregular slowly adapting discharge. Gottschaldt and co-workers (1973) thus proposed the correlation between the irregularly firing St I receptors and the vibrissal Merkel nerve endings. Other features of the responses of the St I including the greater dynamic sensitivity, lower tendency for spontaneous discharge and vibration sensitivity are all in keeping with

described response properties of Merkel cell receptors (Iggo, 1968; Iggo & Muir, 1969; Merzenich & Harrington, 1969; Kenton et al., 1971; Vickery et al., 1992).

The St II ending: The anatomical substrate for the St II receptor is not as clear cut. Gottschaldt and co-workers (1973) proposed that their St II receptor responses originated from the straight and branched lanceolate terminals as had been earlier described in Andres' (1966) electron microscopical studies. Many references to lanceolate endings (or palisades) simply refer to them as rapidly adapting (e.g. Rice et al., 1986; Lichtenstein et al., 1990; Halata, 1993). Andres and von Düring (1973) however distinguish between the lanceolates found in down and guard hairs and those of vibrissae. The latter are longer with a more complex internal structure, each terminal being supplied by a single axon. Brown and Iggo (1967) found only rapidly adapting responses in the down and guard hairs (see literature review); the lanceolates in these hairs are rapidly adapting. Andres and von Düring (1973) suggested that the arrangement of the lanceolates in the down hairs and guard hairs, which resembles that of some lamellated receptors, might account for their rapidly adapting responses.

It was found in the present study that the St II receptor responses arose further down in the hair follicle than the St I. Most of the cavernous sinus area below the point of entry of the DVN could be

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excised with no change in the relative availability of either of the two receptors. It is thus unlikely that the St II receptors are located in the cavernous sinus. That leaves only the ring sinus area to consider. The only receptors that could numerically compete with Merkel cell endings in the ring sinus area are the lanceolate terminals. Therefore one cannot but agree that the lanceolate endings are the source of the recorded St II receptor responses (Gottschaldt *et al.*, 1973).

Spontaneous discharge: Spontaneous discharge was observed in both types of slowly adapting mechanoreceptors though more frequently in the St II. The pattern of spontaneous discharge was found to be characteristic for each of the two receptors. It cannot be ruled out that some of the spontaneous discharge we observed was due to the way the hair follicles were pinned to the dissecting platform.

'Staccato' firing: The slowly adapting mechanoreceptor with a 'staccato' firing pattern of responses found in the present study has not been described before. The pattern of discharge however resembles the burst pattern described in 'cold' receptors of primate skin (Kenshalo & Galegos, 1967; Iggo, 1969). The receptors were quite distinct and could be recorded from for hours. It is not clear what the anatomical substrate for this receptor may be but one is inclined to think that this might be a variant of the St I. Although Merkel cells typically arise as elongated parallel endings branching directly from

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the parent afferent nerve fibre in vibrissae (e.g. Rice *et al.*, 1993; Fundin et al., 1994); Fundin et al. (1994) have described, in addition, some Merkel cells arising serially. The number and arrangement of Merkel cells in the St I unit might be related to the degree of irregularity of the receptor responses. The 'staccato' unit may thus be one extreme in the widely varying degree of irregularity of responses of St I firing which this study also observed. Furthermore, it could be shown that ISIs monitored online could shift from typically 'staccato' to look very 'St I-like', only the CVAR remaining relatively high. Unfortunately, typical 'staccato' receptors were too few and far between for any meaningful systematic pharmacological testing.

Gottschaldt et al. (1973) described a subtype of the St II which they called the St IIb. This receptor, the responses of which they ascribed to the branched lanceolate terminals, had spikes which were not as regular as the St II but more irregular than the St I. The present study did not sub-categorise the St II receptors into IIa and IIb since the more regular St I units (CVAR < 0.150) which could have been confused with the St II, were found to be excited by caffeine just like their more irregularly firing kin.

SA II vs St II: While the vibrissal St I receptors are analogous to the SA I receptors in the skin, the vibrissal St II receptors are not analogous to the cutaneous SA II. The SA II receptors have been shown to be Ruffini endings (Chambers *et al.*, 1972). Ruffini endings

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have been described in vibrissae of monkeys (Halata & Munger, 1980) and rats (Andres & von Düring, 1973; Halata, 1975 and the EMs from some of the specimens in this study); though Rice *et al.* (1993) contend that they are absent in rat sinus hairs. Since Ruffini endings are slowly adapting with a regular discharge pattern and a tendency to spontaneous discharge and stretch responsiveness (Chambers *et al.*, 1972; Burgess & Perl 1973; Biemesderfer *et al.*, 1978), their contribution in the present study is unclear. It may well be that their responses are functionally indistinguishable from those of the predominant lanceolate endings and might have contributed to the population of St II receptors recorded from in this study. This is however not a major drawback since our line of interest is mainly the Merkel cell endings.

Relative number of St I and St II endings: One is not aware of any reports quantitatively comparing the relative number of nerve fibres in vibrissae innervating lanceolates and Merkel nerve endings in rats or other rodents. In the monkey, where branched lanceolates were not observed, Halata and Munger (1980) reported a total of 10 - 20 lanceolate terminals per vibrissa against 50 - 65 fibres for Merkel nerve endings (the order was Merkel cell endings>>lanceolates>Ruffini>simple (lamellated) corpuscles). We estimated that a greater proportion of the slowly adapting responses in the isolated rat vibrissa were of the St II variety, the reverse of the

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situation in the monkey. This apparent skewed distribution of the receptors may be responsible, at least in part, for the inability of some investigators to distinguish between the subtypes of the rat vibrissal slowly adapting mechanoreceptor responses (e.g. Zucker & Welker, 1969; Gibson & Welker, 1983b; Lichtenstein *et al.*, 1990).

5.4 MECHANISM OF CHLOROQUINE INHIBTION OF MERKEL CELL RECEPTORS

Chloroquine and quinacrine were found to inhibit Merkel cell receptor responses to mechanical stimulation while having the reverse effect on St II receptors. This is the first report of a functional qualitative differnce between Merkel cell endings and another slowly adapting receptor. This is clear evidence that mechanoelectric transduction in St I and St II receptors occurs by different mechanisms. Morphologically, chloroquine's vacuolating effects (e.g. Fedorko *et al.* 1968a,b; de Duve *et al.* 1974) were mostly confined to Merkel cells with the surrounding keratinocytes remaining relatively vacuole-free and the neurite terminals making contact with Merkel cells little affected. Other nerve terminals in chloroquine-treated preparations were also unaffected. These findings, coupled with the fact that CQ and QUIN are known to accumulate in certain peptide secreting cells and nerve endings including Merkel cells (Dencker *et al.*, 1976; Crowe

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& Whitear, 1978; Alund & Olson, 1979; Ekelund, *et al.*, 1980; Nurse, *et al.*, 1983), suggest that Merkel cells were the most probable site of the observed action of chloroquine on St I function.

The exact mechanism by which CQ exerts its inhibitory action on St I mechanoreceptor function is unclear but the some possibilities are considered below:

i.   *CQ is a lysosomotropic agent:* As a lysosomotropic agent (de Duve *et al.* 1974), CQ inactivates lysosomal function and by so doing interferes with processes that require lysosomal degradation (Izzo *et al.*, 1990; Schwartz *et al.*, 1991; Caporaso *et al.*, 1992), including inhibition of exo- or endocytosis (e.g.   Kalina & Socher, 1991; Korolenko *et al.*, 1992). By this mechanism, CQ might be inhibiting the processing and release of the putative neurotransmitter in the Merkel cells in a manner similar to that of the known CQ inhibition of hormonal secretion from the pituitary gland (Moore *et al.,* 1983; Schwartz et al., 1991).

The data from NH_4Cl a classic lysosomotropic agent, show a rather variable inhibition of St I function by 10mM NH_4Cl (the inhibition by chloroquine was also quite variable in magnitude as shown in table 3.1). There was also evidence of vacuolation of Merkel cells in NH_4Cl-treated specimens which, like for CQ was not completely reversed by the time receptor responses had recovered. NH_4Cl also produced a highly reproducible pattern of inhibition of St II receptor

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responses which could be explained by the expected effect of NH_4Cl on intracellular pH (the pH of the superfusing solution itself was maintained at 7.4). Thus functionally, NH_4Cl inhibited both receptor types, though the pattern of inhibition varied in its characteristics. This differential effect of NH_4Cl on the St I and St II receptors suggests that CQ is acting by more than one mechanism - the lysosomotropic effect alone does not explain the action on the two receptor types.

When the effects of other lysosomotropic agents like methylamine and 3-MA (both of which had no effect on St I receptor function) are taken into account, it becomes less likely that a lysosomotropic action was primarily responsible for the effect of CQ on St I receptors either. The fact that after receptor function had recovered, CQ-treated Merkel cells were still replete with the physical evidence of CQ's lysosomotropism (the vacuolation) would seem to also suggest that this was not the primary mechanism of the inhibition. However the prolonged action of CQ on the St I receptors may be related to the fact that the Merkel cells actively concentrated the drug. Cells exposed to CQ have been shown to avidly take up the compound within minutes, attaining a high (several fold of the external) concentration (see de Duve *et al.*, 1974). It thus took longer for the concentration of the drug in the cell to drop below the effective inhibitory levels.

ii. *CQ affects intracellular Ca^{2+} pools:* There are several reports of CQ action on extracellular as well as intracellular calcium pools. (i) CQ was found to inhibit caffeine-induced mobilisation of intracellular Ca^{2+} in the rat hemidiaphragm (Okwuasaba *et al.*, 1990). (ii) It was found to decrease calcium channel currents in intact frog atrial trabeculae (Filippov *et al.*, 1989). (iii) CQ inhibited calcium currents in *Paramecium calkinsi* (Barry & Bernal, 1993); and (iv) Tona and co-workers (1990) suggested that the depressant effects of CQ on the isolated guinea pig heart involved a superficial calcium pool.

In the light of the later findings of evidence for a CICR mechanism in St I receptors in this study (discussed below), the CQ inhibition of caffeine-induced mobilisation of intracellular calcium in rat skeletal muscle (Okwuasaba *et al.*, 1990), is of particular interest. A CICR mechanism operates in excitation-contraction coupling (see the recent review by Meissner, 1994). It appears probable therefore that CQ is behaving (in the St I) as an inhibitor of intracellular calcium mobilisation. In the St II, the fact that the sustained CQ-induced spontaneous firing was sensitive to verapamil, would at least suggest a calcium-related mechanism in the St II receptor as well.

iii. *CQ is a local anaesthetic:* Chloroquine has local anaesthetic properties (Ayiteh-Smith & Vartanian, 1975; Horrobin & Manku, 1977; Horrobin *et al.*, 1977 and personal experience of use in patient care), and as such, can block nerve transmission. This is clearly not likely to

be the mechanism of the observed CQ inhibition of St I function since the same concentrations of CQ had an excitatory effect on St II receptors. Both receptors are supplied by large diameter myelinated afferent nerve fibres (see *Literature Review*).

At high concentrations CQ blocked St II responses completely (e.g. Figure 3.24). Unlike the St I however, St II receptor function was quickly restored within 15 - 20 minutes of stopping CQ superfusion, overshooting the pre-CQ counts. The same concentration of CQ (e.g. Figure 3.18) on an St I receptor also causing complete inhibition, took over 45 minutes to attain about 80% recovery after withdrawing CQ from the superfusing solution. An overshoot was not observed in St I receptors treated with CQ. The inhibition of both receptors at the higher concentrations can be accounted for by the local anaesthetic effect of CQ.

Furthermore, the later finding that the local anaesthetic procaine (discussed below) which is also used as a CICR inhibitor blocked St I function at concentrations that had little or no effect on St II function, supports the preceding argument. It therefore appears highly probable that CQ was interfering with CICR in the St I receptor.

Conclusion: It may be concluded therefore that the Merkel cell appears to be the primary target of CQ action in the vibrissal St I mechanoreceptor. The underlying mechanism(s) of the observed CQ action appear likely to involve interference with intracellular calcium

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mobilisation through CICR. CQ's lysosomotropic action may contribute to the observed effects probably by concentrating the drug within the Merkel cells, but is unlikely to be the major factor. Whatever the mechanism(s) involved, the CQ (and the qualitatively similar QUIN) data have provided the first qualitative pharmacological difference between Merkel cell endings and another slowly adapting nerve terminal.

5.5 EVIDENCE FOR CICR IN MERKEL CELL ENDINGS

i.   *Caffeine: the 'gold standard':* Caffeine, the so called 'gold standard' for demonstrating activation of the RYR and activation of CICR (see Ehrlich & Bezprozvanny, 1994), was found to have a strong excitatory effect on St I receptors. This effect was notably coupled to mechanical stimulation and required calcium influx. The same concentration of caffeine (10mM) consistently inhibited St II receptor responses by about 30%.

The fact that the excitatory effect of caffeine is coupled to mechanical stimulation strongly suggests that caffeine is not simply producing a general increase in calcium influx into the cells or rise in cAMP levels through inhibition of phosphodiesterase (see Kumbarachi & Nastuk, 1982). A marked increase in spontaneous firing would be expected in the former while an increase in responses would be

expected in the latter if cAMP were to play a role in the transduction process. The dose-response curve for caffeine's action on the St I showed that significant effects only became manifested at around 5mM caffeine. This is typical for CICR (e.g. Lee, 1993). Phosphodiesterase inhibition by caffeine occurs at concentrations of 0.1 - 1mM caffeine (Smellie *et al.*, 1979; Sawynok & Yaksh, 1993).

A transient increase in spontaneous firing was observed in both St I and St II (in spite of a concomitant inhibition of stimulated responses) receptors. This is probably a manifestation of caffeine's tendency to increase calcium influx (Bianchi 1962, 1968). It is however unlikely to be a significant factor in the demonstrated caffeine effects. Apart from the fact that the low frequency spontaneous firing was not always observed, it was short-lived, often stopping before discontinuation of caffeine superfusion.

Caffeine action requires calcium influx: Caffeine was able to partially reverse, or at least pause, the progression of Mg^{2+}-induced inhibition of St I receptor function if added before complete inhibition was achieved by Mg^{2+}. Since Mg^{2+} is a well known competitive inhibitor of calcium influx, and known to inhibit SA I receptor responses (Pacitti & Findlater, 1988; Baumann *et al.* 1993), this suggests that some calcium influx was required for caffeine to exert its effect on St I receptors. This is expected in a CICR system. It is noteworthy that Mg^{2+} also blocks the RYR in its own right (see Meissner, 1994; Pozzan

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et al., 1994 for recent reviews). In an intact preparation such as the one used in this study, Mg^{2+} would be expected to first competitively block Ca^{2+} influx before exerting any intracellular CICR blocking action. This might indeed explain the reported greater sensitivity of Ft I (frog Merkel nerve endings) to Mg^{2+} blockade than Ft II slowly adapting mechanoreceptors (Yamashita *et al.*, 1986).

Caffeine vs St II: The inhibitory effect of caffeine on the St II receptor is difficult to explain unless the receptor's transduction process is assumed to involve mobilisation of intracellular calcium as well. Caffeine has been shown to inhibit intracellular calcium mobilisation through the IP_3 second messenger pathway in *Xenopus* oocytes (Parker & Ivorra, 1991), cerebellar microsomes (Brown *et al.*, 1992), and in permeabilized smooth muscle cells (Hirose *et al.*, 1993). The possibility that an IP_3 system operates in St II mechano-electric transduction is thus suggested by this data. It is noted that 10mM caffeine was sufficient to cause almost complete inhibition of IP_3 action (Ehrlich & Bezprozvanny, 1994) while only about 30% inhibition was observed in St II responses in the present study. This may simply be because a whole tissue preparation was used in this study or that the IP_3 system (if it were to be shown to exist) is not the sole mechanism of such Ca^{2+} mobilisation in the St II. It is difficult to investigate this any further since substances that affect mobilisation of Ca^{2+} through IP_3 need to be injected intracellularly (see literature review); this is at

present precluded by the small size of the nerve endings. Moreover, the Merkel cell endings are the primary interest of the present study.

ii.   *Effect of Ryanodine:* Ryanodine, the plant alkaloid after which the receptor for CICR was named, binds virtually irreversibly to the ryanodine receptor and activates intracellular calcium release (Ehrlich & Bezprozvanny, 1994, Meissner, 1994 for reviews). Ryanodine was found to cause a sustained, though not dramatic, increase in St I receptor responses, without significantly affecting spontaneous firing. The same, or higher concentrations of ryanodine had no effect on St II receptors which could be subsequently shown to be blocked by caffeine.

When caffeine was superfused after about 1 hour post ryanodine (e.g. Figure 3.43a) on an St I receptor, there was a further rise in responses which recovered to pre-ryanodine levels on washing out caffeine. Caffeine thus appears to have displaced the tightly bound ryanodine from the receptor.

The effect of ryanodine is supportive of the caffeine findings, thus strengthening the case for a CICR process in the mechano-electric transduction process of St I but not St II receptors.

iii.   *Procaine inhibits CICR:* Further evidence in support of a CICR process in St I was obtained using a specific CICR inhibitor, procaine (Galione *et al.*, 1991; Khoyi *et al.*, 1993; Lee, 1993; Huang, 1995).

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Procaine dose-dependently inhibited St I function at micromolar concentrations that had little or no effect on St II receptor responses.

Procaine is better known for its local anaesthetic activity. As noted earlier, the St I and St II receptors have myelinated afferent fibres of similar diameter (see literature review). It is thus unlikely that procaine can be blocking impulse transmission in the nerve as a local anaesthetic would, but is exerting its specific CICR blocking action in the St I receptor while sparing the St II, which apparently lacks a similar CICR process. The finding that procaine directly prevented the effect of caffeine when they were superfused together is in strong support of this.

The CICR blocking action of procaine in other tissues requires milimolar concentrations (as high as 10mM, e.g. Khoyi et al., 1993; Huang, 1995). In St I receptors superfusion of 50 - 100μM was sufficient to block responses. Higher concentrations (300 - 500μM) apparently blocked nerve transmission as well since St II function was blocked. Like in the CQ experiments, recovery of St II responses after inhibition by procaine was much faster and rather abrupt. Procaine, like CQ is a weak base (pK$_a$ 8.9) which can be avidly taken up and concentrated by Merkel cells. It is quite likely that superfusion of 50 - 100μM procaine is enough to block CICR because the Merkel cells concentrate the drug, thus achieving the milimolar concentrations required to block CICR. The slow recovery of St I responses after

procaine treatment would seem to support this.

iv.   *Heparin for CICR?* Heparin was originally used in an attempt to see if the possible IP_3 system in St II endings suggested by the caffeine experiments could be blocked by this competitive inhibitor of IP_3-mediated calcium release (Ghosh *et al.*, 1988; Taylor & Richardson, 1991). While heparin was found to have no effect on St II responsiveness, St I receptors were dose-dependently excited by heparin. Unlike caffeine, heparin did not affect spontaneous firing in either receptor. Because heparin has been shown to activate CICR in SR vesicles (Bezprozvanny *et al.*, 1993; Ritov *et al.*, 1985), this appeared to be in line with the other data supporting the presence of CICR in St I. Heparin is however a highly charged molecule which does not easily cross intact membranes (see Knaus *et al.*, 1990; Lacinova *et al.*, 1993). This suggests that the action of heparin is extracellular and probably unrelated to CICR, at least not directly. Furthermore, when pentosan polysulphate (PPS), another polyanion was used, it had no effect on the function of either receptor type, suggesting that the observed effect is specific to heparin.

Heparin vs high-threshold (L-type) Ca2+ channels: Heparin has been found to bind to the high-threshold (L-type) calcium channel which it activates, at relatively high concentrations (Knaus *et al.*, 1990, 1992). The same group of workers also showed that extracellularly

applied heparin increased current through L-type Ca^{2+} channels in whole cell patch clamped guinea pig cardiac myocytes (Knaus *et al.*, 1990, but see Lacinova *et al.*, 1993). This appears to be the most likely explanation for the action of heparin on the Merkel cell receptors. Merkel cells have been shown to have high-threshold L-type, calcium channels (Yamashita *et al.*, 1992). The lack of effect of heparin on the St II suggests that Ca^{2+} influx in this receptor occurs by a different channel. As far as CICR is concerned, it would appear that heparin is acting indirectly by increasing the initial calcium influx.

Conclusion: In conclusion, there appears to be sufficient evidence in support of the presence of a calcium-induced calcium release mechanism in the mechano-electric transduction process of the Merkel cell receptors of rat vibrissal follicles.

5.6 MECHANO-ELECTRIC TRANSDUCTION IN MERKEL CELL RECEPTORS

The findings of the present study are strongly supportive of a mechanosensory role for Merkel cells. Not only were the effects of CQ/QUIN, caffeine, ryanodine, procaine and heparin mostly specific for St I receptors, the effects of CQ and caffeine on the St II were actually the reverse of their effects on the St I. It is difficult to see how there can be so much difference between the St II terminal and the apposing neurites of the St I, if the neurite were the transducer proper.

Iggo and Findlater (1984) proposed a model of mechano-electric transduction in the cutaneous SA I receptor, based on the assumption that the Merkel cell is the transducer. They proposed that mechanical distortion of filamentous rods of Merkel cell led to alteration of membrane permeability of the epidermal surface of Merkel cell, resulting in Ca^{2+} entry and subsequent mobilisation of the dense cored granules and release of the putative transmitter substance. The transmitter was proposed to alter the membrane permeability of the nerve plate and lead to the development of a generator potential with subsequent initiation of an impulse in the myelinated afferent unit.

Following the description of 3 types of ionic channels in isolated Merkel cells by Yamashita and co-workers (1992), they postulated that mechanical deformation of Merkel cells activated putative

mechano-sensitive channels which then generated evoked depolarising potential leading to activation of the L-type Ca^{2+} channel and resultant calcium influx. The calcium influx was subsequently responsible for the release of neurotransmitter. The two K^+ channels they found (I_A and I_{KD}) were proposed to stabilise the membrane potential and lengthen the ISIs during sustained discharge.

In the following section, an attempt is made to give a sequential account of the series of events in the Merkel cell receptor that culminate in the generation of action potentials in response to mechanical stimulation. The findings of the present data are incorporated into the proposed model which also attempts to explain most of the electrophysiological data on Merkel cell receptors.

i.   Mechanical deformation:

The impingement of a mechanical stimulus activates mechanically gated ion channels in the Merkel cell. These channels are most probably located or concentrated at the cytoplasmic extensions characteristic of Merkel cells. In cochlear hair cells the putative mechano-sensitive (MS) channels have been shown using antibodies to the Amiloride-sensitive epithelial Na^+ channel to be located at the stereocilia (Hackney et al., 1992). This investigation was prompted by the observation that amiloride blocked the MS channels in hair cells (Jørgensen and Ohmori, 1988; Rüsch et al., 1994). At the high concentrations required to block MS channels, amiloride also blocks

Ca^{2+}/Na^+ and Na^+/H^+ exchangers (see Kleyman & Cragoe, 1988), T- and L-type Ca^{2+} channels (Tang et al., 1988; Garcia et al., 1990). This makes it difficult to interpret results when amiloride is used to study function in intact functioning preparations as used in this study.

ii. Ionic influx:

MS channels are known to be relatively non-selective in terms of their permeability. Hair cell and oocyte MS channels exclude anions but are weakly selective among cations, allowing the passage of Na^+, K^+ and Ca^{2+} (see Hamill et al., 1992 for review). It is likely therefore that opening the MS channel of Merkel cells would admit both Na^+ and Ca^{2+} into the cell. This would produce some degree of depolarisation in the cell thus activating the high threshold membrane Ca^{2+} channel (as proposed by Yamashita et al., 1992).

The question of calcium and Merkel cells has been a rather vexing one. Though it has been shown that Ca^{2+} influx is required in the transduction process of Merkel cell receptors (e.g. Yamashita et al, 1986; Paciti & Findlater, 1988; Baumann et al, 1993), the specific organic blockers of voltage-gated Ca^{2+} channels like verapamil and ω-conotoxin have had unconvincing actions on the function of the receptors. Rather high concentrations are required for appreciable inhibition. In the isolated skin-nerve preparation, Baumann et al., (1993) found that the 100µM concentration of verapamil required to block responses was also capable of blocking nerve transmission in

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the preparation. Furthermore the L-type Ca^{2+} channel opener Bay-K 8644 had only a salutary effect on receptor function. The blocking effect of the competitive inorganic Ca^{2+} channel blockers like Mn and Mg have however remained unequivocal. It may indeed be that the high threshold Ca^{2+} channel found in Merkel cells are not as sensitive to the conventional blockers as suggested by the rather high concentrations required to block it by Yamashita *et al* (1992). Other voltage-activated Ca^{2+} channels e.g. P-type (see Regan *et al*, 1991; Spedding & Paoletti, 1992) may also play a role.

The present study found that externally applied heparin had a strong stimulatory effect on St I but not St II receptors. This supports the presence of L-type Ca^{2+} channels in the St I since heparin has been shown to bind to and increase currents through this channel (Knaus *et al.*, 1990). Lacinova *et al* (1993) however found that heparin decreased I_{Ca} in cardiac myocytes, an effect that was not shared by other polyanions. The effect of heparin on St I agrees more with the previous finding by Knaus *et al.*, (1990) but also found (like Lacinova *et al.*, 1993), that the effect was not shared by another polyanion, pentosan polysulfate.

Two phases of Ca^{2+} entry: It seems reasonable to speculate from the foregoing that Ca^{2+} influx into Merkel cells occurs in two phases by two mechanisms - one through the opening of the MS channel and the other through the voltage dependent Ca^{2+} channel. This could, at least in part, explain the relative resistance of the

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receptor function to the organic blockers which are expected to block only one phase of Ca^{2+} influx. Calcium entry through a non-voltage dependent channel may partially compensate for the block of the voltage-activated channel and thus mask the effect of the organic Ca^{2+} blockers on receptor function.

The first phase of Ca^{2+} influx occurs during the activation of the mechanosensitive (MS) channel. It is proposed that this phase of Ca^{2+} influx contributes mainly to the dynamic phase of responses to mechanical stimulation and hence might explain the high dynamic sensitivity of the receptor. It is well known that MS channels inactivate fairly rapidly and are unusually voltage sensitive (Sukharev et al., 1993; Hamill & McBride, 1992). The depolarisation following ionic influx through the MS channel would therefore be expected to also inactivate it. Thus sustained mechanical displacement will not keep the channels open long enough for the typical slowly-adapting response.

The second phase of Ca^{2+} influx, would occur as a result of activation of the high threshold Ca^{2+} channel as suggested by Yamashita et al., (1992). The finding by the present study that heparin was dose-dependently excitatory to St I receptors, turned out to be evidence in support of opening of a voltage-gated Ca^{2+} channel in the Merkel cell receptor. The fact that heparin did not cause spontaneous firing suggests that the agonistic effect of heparin is only manifested when the channel is activated by depolarisation.

This second phase of Ca^{2+} entry is proposed to be responsible

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for the static responses to mechanical stimulation. It is proposed that the Ca^{2+} which enters through this voltage dependent process is amplified by intracellular calcium release through the ryanodine receptor channel.

Recent studies in our laboratory using microfluorimetric techniques have shown that Merkel cells do indeed contain releasable intracellular Ca^{2+} stores (Chan, 1995). The data from the present study provides functional evidence for a CICR process in the receptor; a process which appears to be distinctly absent in the St II nerve terminal. CICR being a regenerative but self-limiting process, it is predictable that sustained mechanical stimulation will lead to adaptation. Gottschaldt *et al.* (1973) found that St I receptors adapted within a few minutes of sustained mechanical displacement while St II receptors kept firing for hours.

It is also predictable that an agonist of CICR like caffeine should manifest its greatest effect on the static phase of responses and that the effect of any effective blocker of the Ca^{2+} channel should largely spare the dynamic responses. When the data from the present experiments were re-analysed by looking at percentage change in responses during the static and dynamic phases of stimulation, it was found that while the dynamic, static and total counts were remarkably in tandem during the control period, they broke ranks when caffeine was applied. The static counts proportionately rose to a much greater extent (sometimes several fold) than the dynamic counts (sometimes

hardly changed). Mg^{2+}, on the other hand affected the counts proportionately, the counts remaining in tandem. Presumably Mg^{2+} is equally affecting both phases of Ca^{2+} entry. It is indeed a frequent observation in our laboratory that the dynamic counts are relatively resistant to blockade, and are usually the last to go, whenever responses were being blocked. This could be explained if the dynamic phase is considered to occur mainly as a result of opening of the MS channels which are relatively difficult to block.

iii.   Intracellular events:

The intracellular events following ionic influx would include mobilisation of intracellular calcium and subsequent release of the putative neurotransmitter and initiation of the process) for adaptation. It is difficult to say whether the initial phase of Ca^{2+} influx through the MS channel is sufficient on its own to induce neurotransmitter release or whether it also requires amplification via CICR. It is probably more likely that the CICR is a later event designed for slow adaptation.

In any case, the increase in Ca^{2+}_i would lead to mobilisation of the dense cored granules and subsequent release at the synaptiform junction between the Merkel cell and the expanded neurite (Iggo & Findlater, 1984; Yamashita *et al.*, 1992). Binding of the neurotransmitter to the appropriate ligand-gated ion channels on the nerve terminal would lead to the generation of the receptor potential

and action potentials when the appropriate threshold is attained.

iv.   Adaptation:

Receptor adaptation probably occurs due to a combination of several processes from Ca^{2+} influx into the Merkel cell, intracellular Ca^{2+} mobilisation (which is self-limiting), including re-uptake of released Ca^{2+} into the stores, to the outward K^+ currents in Merkel cells (Yamashita et al., 1992) and the inherent adaptive properties of the nerve terminal itself. The relative contributions of any of these mechanisms to the process of adaptation in the receptor is uncertain.

According to Yamashita et al., (1992), the K^+ currents activated in Merkel cells by depolarisation may suppress transmitter release by stabilising the membrane potential and lengthening the ISIs during sustained discharge at the afferent fibre. If that were the case, it would be expected that blocking the K^+ channel (e.g. with quinacrine as shown in that study) would be excitatory, inducing both spontaneous firing (due to instability of the membrane potential) and the stimulated responses. Interestingly, this was only observed in St II receptors in the present study. Responses of St I receptors were inhibited instead. It is quite probable though that the likely effect of quinacrine (and chloroquine) on intracellular Ca^{2+} release (see earlier discussion) was overwhelming, especially since the intracellular Ca^{2+} mobilisation is proposed to occur at an earlier stage in the proposed sequence of events.

The foregoing proposed steps in the transduction process of Merkel cell receptors is an attempt to integrate existing information and the findings of the present study, and is by no means complete or exhaustive. Direct evidence will still need to be obtained for several of the events proposed, but the proposal might at least help in the search.

5.7 TRANSDUCTION IN ST II NERVE TERMINALS

Although the St II receptor has served mainly as a convenient 'control' receptor which has no cellular attachment, in our study of Merkel cell receptors, the data obtained on the St II endings warrants a few comments on the process of mechano-electric transduction in the St II nerve ending. The process is also unknown in this receptor.

There is little reason to suppose that the mechanism of mechano-electric transduction in the St II differs significantly from what obtains in other mechano-sensitive nerve endings (see literature review). A mechanical stimulus is thought to open mechano-gated ion channels with the attendant generation of the receptor potential. Action potentials are generated subsequent to activation of voltage-gated channels at the action potential generating site of the receptor. Sodium is considered to be the main ion gated by the mechano-gated channel and is responsible for the generator potential. The generator potential is abolished when Na^+ is replaced by an impermeant ion and the Na^+

influx is resistant to TTX in the crayfish stretch receptor (Brown *et al.*, 1978).

It is however known that Ca^{2+} influx is also required for normal responses of this (St II) receptor and in the cutaneous SA II - the organic Ca^{2+} blockers block these receptors. The precise role of Ca^{2+} in this process is unclear, since in principle, Na^+ influx and its subsequent removal by the Na^+-K^+ pump could fit a simple model of mechano-electric transduction in these receptors.

The results of the present study suggest that the process is also fairly complicated in the St II. Caffeine's inhibition of the process suggests the possibility that not only is Ca^{2+} influx required, but a second messenger system for amplifying the Ca^{2+} signal through intracellular release, may be present. It has not been possible to test this further in this study because IP_3 - the proposed second messenger needs to be applied intracellularly. Heparin, the major inhibitor of the system is also impermeable. However, the fact that extracellular heparin had no effect on the St II suggests that high threshold L-type Ca^{2+} channels are not involved in the St II transduction process like they appear to be in the St I.

All that may be said with any degree of certainty is that mechano-electric transduction in the St II terminal may be more complicated than the simplicity of the receptor may suggest.

5.8 WHAT NEXT?

Using the findings of the present study as a backdrop, more direct evidence for Merkel cell mechanotransduction can be obtained at the cellular and molecular level.

Functionally, since CICR has been shown to operate in the intact functioning receptor, single isolated Merkel cells may now be used to directly demonstrate CICR. Such a demonstration will be proof of a mechanotransductive role for Merkel cells. The requirement for Ca^{2+} entry through mechanical stimulation in the transduction process can be bypassed by permeabilising the cells. Permeabilised Merkel cells can then be challenged with the proposed endogenous second messenger of CICR cyclic ADP-ribose (e.g. see Lee *et al.*, 1994). Isolated Merkel cells may be alternatively injected with caged photolysable compounds for demonstration of CICR.

Morphologically, Merkel cells and their nerve terminals and the other nerve endings in the vibrissa may be immunolabelled for the ryanodine and IP_3 receptors. If the ryanodine receptor can be demonstrated in the Merkel cells and not the apposed nerve terminals, it will be proof of a mechanotransductive role for Merkel cells when the electrophysiological data from this study are taken into account.

The isolated vibrissal preparation however remains a vital tool in the exploratory process. In the long run, it will be necessary to

insure that findings at the molecular level can be verified using a sturdy functioning preparation as this.

5.9   CONCLUSION

The present study is the first to provide evidence of functional and pharmacological differences between vibrissal Merkel cell endings and another vibrissal slowly adapting nerve terminal, the St II. These qualitative differences are considered to be due to the possession of Merkel cells by the St I and hence constitute strong (though indirect) evidence that the Merkel cells are the actual mechanotransducers in the receptor. By providing functional evidence for a calcium induced calcium release (CICR) process in Merkel cell receptor function, the study has added a new step in the previously proposed sequence of events involved in mechanotransduction in the Merkel cell receptor.

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