

**THE MECHANISM OF ACTION OF CAPSAICIN ON SENSORY
C-TYPE NEURONES AND THEIR AXONS IN VITRO.**

A thesis submitted for the degree of
Doctor of Philosophy
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Abstract.

The mechanism of action of the sensory neurotoxin, capsaicin, on visceral afferent fibres and ganglion cells has been studied using electrophysiological and histological techniques.

Extracellular in vitro recording from adult vagus nerves revealed a depolarization and a reduced C-spike amplitude. These probably reflect effects on unmyelinated sensory fibres, since no such action was detected in fibre trunks lacking sensory fibres, such as preganglionic sympathetic nerves and ventral spinal roots. Ion substitution experiments indicated that the capsaicin-induced depolarization is mediated by a mechanism that involves sodium (Na^+), calcium (Ca^{2+}) and, to a lesser extent chloride, (Cl^-) ions.

In vitro intracellular recordings from sensory neurone perikarya, showed that capsaicin depolarizes 70% of the C-type neurones located within the rat nodose ganglion. The capsaicin-induced depolarization was primarily mediated by an increase in membrane conductance to Na^+ and Ca^{2+} . An additional membrane conductance increase to potassium (K^+) was also induced. However, this depended on an influx of calcium via the primary conductance mechanism.

Histological experiments using light and electron-microscopic techniques indicated that capsaicin can induce substantial cytotoxic damage to a subpopulation of nodose sensory neurones and vagus nerve unmyelinated fibres. Moreover, the cytotoxic effects could be induced by short applications (< 10 mins) and low concentrations (1-10 μM) of capsaicin. The entry of calcium ions into the cells appeared to play a major role in the cytotoxic process, as the replacement of extracellular calcium with magnesium minimised the cytotoxic damage. The failure of calcium channel-blockers to reduce the calcium-dependent neurotoxic effect indicated that calcium entry through

capsaicin-activated channels, rather than voltage-gated calcium channels, initiates the cytotoxicity.

It is suggested that capsaicin opens cationic channels and that calcium entry through these channels might not only modify cell excitability but also prime the neurotoxic process which can lead to cell death.

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Dedication.

To Mum and Dad,
for all their love, guidance and patience

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*In memory of my brother Kevin who passed away
in November 1990.*

Forward

The experiments described within this thesis were conducted during the period 1984 to 1987 on a part-time basis. The introduction to the work represents the available published data during this period, although additional post-1987 studies have been included, occasionally, for clarity and verification. The more recent relevant published data is evaluated fully within the discussion.

The author recognises the valuable assistance of Mr. D. McCarthy and Mr. R. Davey (Electronmicroscopy Unit, The School of Pharmacy) who prepared the tissues for histological analysis and Dr C. Stansfeld who aided in the latter stages of the intracellular recording study.

An account of some of this work has already been communicated to the Physiological Society (Marsh, 1985) and published (Marsh et al., 1987).

Introduction

Amongst the many species of the plant family *Solanaceae* are found *Atropa*, *Datura*, *Hyoscyamus* and *Nicotiana*, all of which contain considerable amounts of pharmacologically active plant alkaloids, which makes these plants extremely poisonous. This family of plants also extends to the genus *Capsicum*. Over thirty species of capsicum have been described, the majority found in south and central America; however probably the best known is the cultivated variety, *Capsicum annuum*, which yields the fruit known as chilli or red peppers. These plants have evolved a protective mechanism for their fruit that not only gives it insecticidal and antiseptic properties but also an ability to produce intense irritation of the mucous membranes when consumed, which most species of animal find extremely distasteful.

Modern horticultural methods have developed consumable milder forms but these fruit are most used when dried and ground, as a spiced food additive, in the form of 'Cayenne' or 'Red pepper'.

An alcohol extraction from *Capsicum annuum* produces five fat-soluble compounds. A vanillylamide derivative of diclenic acid 'Capsaicin' (Fig. R21) has been shown to be the most potent of these compounds at producing the 'hot' sensation of initially warmth and then pain (Monsereenusorn et al., 1982). The effects of topically applied capsaicin to both the mouth and the skin has been attributed to highly selective excitation of afferent receptors of the polymodal 'mechano-heat' class (Kenins, (1982); ^{FOSTER & RAMAGE, (1981)} Lynn, (1990)). Polymodal nociceptors (PMNs) are the cutaneous sensors for potentially dangerous stimuli, responding to noxious stimuli such as intense heat, mechanical pressure and chemical stimuli. These PMNs have been shown to be the cutaneous nerve terminals of unmyelinated afferent C-type axons and can contribute between 50 and 90% of all afferent C-fibre input to the central nervous system (Lynn, 1984).

Chemosensitive PMNs respond not only to capsaicin but also to other pain

producing substances such as bradykinin, potassium, histamine and acetylcholine. All of these substances initially induce an axon-reflex localised inflammation ('flare') and a cutaneous sensitization to noxious stimuli, possibly by the release of endogenous inflammatory substances such as bradykinin, prostaglandins, substance P or somatostatin (Maggi and Meli, 1988).

The role of peptides in capsaicin induced desensitization.

Capsaicin treatment produces a long-term desensitization not only to itself but also to most types of chemical noxious stimuli (Jancso, N., 1968). Moreover this capsaicin-induced 'desensitization', when induced in neonatal rats, becomes irreversible and 'life-long'. The plasma extravasation induced by capsaicin appears to be neurogenic in origin as it is absent in chronically denervated skin, and strong evidence suggests that the neurally-released peptides such as substance P and somatostatin might be the mediators (Lembeck and Gamse, 1982). However the 'life-long' desensitization afforded to neonatally-treated animals does not appear to be due to a localized disruption of the ^{peripheral} sensory mechanisms or a depletion of the mediator substances, as significant reductions have been found not only in the number of unmyelinated fibres in cutaneous nerves but also in the B-type sensory neurone perikaria of the dorsal root ganglia (Jancso et al., 1977; Jancso and Kiraly, 1981). These are the neuronal cell bodies of unmyelinated afferent fibres and have been shown to contain substance P, vasoactive intestinal polypeptide and cholecystokinin (Jancso et al., 1981; Gamse et al., 1980; Nagy et al., 1980).

The neurotoxic effects of capsaicin are extended to the cervical spinal cord (Jancso et al., 1977), where extensive axonal degeneration is induced, particularly in the substantia gelatinosa region.

The effects on the dorsal root ganglion A-cells and their afferent myelinated fibres are minimal (Lawson and Nickels, 1980; Scadding, 1980) but some reduction in the small diameter (< 3µM) myelinated fibre content of lumbar dorsal roots could be

induced using higher concentrations of capsaicin (25-75 mg / Kg) than that estimated to be the ED₅₀ (12.5 mg / Kg) for C-fibre destruction. However these results are complicated by the physiological consequences of systemic capsaicin treatment, so that anoxia induced by bradycardia, hypotension and apnoea (Coleridge et al., 1964) might result in A-cell damage (see Fitzgerald (1983) for critical review).

The ability of capsaicin to induce peptide release is not exclusive to the cutaneous peripheral nervous system as capsaicin has also been shown to both release and deplete substance P from many other regions (Buck and Burks, 1986 for extensive review). Extensive experimentation has been carried out on the rat spinal cord where radioimmunoassay and immunohistochemical techniques have shown that capsaicin induces a calcium dependent *invitro* release of assayable substance P from the spinal cord (Gamse et al., 1979; Theriault et al., 1979), which appears to be released from the upper dorsal horn only (Gamse et al., 1981).

A single intrathecal injection of capsaicin substantially reduces the substance P content of the lumbar spinal cord and induces prolonged increases in the thresholds for thermal and chemical stimuli (Yaksh et al., 1979). The reduction in substance P levels is associated with a simultaneous decrease in the activity of the nociceptive afferent neuronal marker, fluoride-resistant acid phosphatase (FRAP) (Jancso and Knyihar, 1975) but both opioid binding and glutamic acid decarboxylase activity were unaffected.

These results implied a degree of selectivity for substance P-containing unmyelinated or small myelinated afferents projecting from the spinal sensory ganglia to the substantia gelatinosa area of the superficial dorsal horn (Jessel, 1982) and suggested that substance P might play an important role in nociceptive afferent transmission (Henry, 1982). The ability of capsaicin to deplete substance P is selective for sensory neurones as no changes could be found in the content of the intrinsic substance P-containing neurones of the brain or gastrointestinal tract (Nagy et al., 1980; Furness et al., 1982; Buck et al., 1981; Holzer et al., 1982).

Subsequent data has, however, shown that the effects of capsaicin are not selective for substance P containing neurones, as large reductions in several other peptides such as calcitonin gene-related peptide, vasoactive intestinal peptide, and somatostatin have all been described (Holzer, 1988); and as suggested by Lynn (1990), it might be that all peptide containing afferent neurones supplying somatic and visceral tissue could be capsaicin-sensitive, or that the method of administration and dose applied might determine the final selectivity profile (Buck and Burks, 1983).

Localized application of capsaicin to sciatic nerve inhibits the intra-axonal transport of substance P (Gamse et al., 1982; Taylor et al., 1984) and depletes the skin areas which it innervates, while the transport of noradrenaline within sympathetic fibres and acetylcholinesterase in motor fibres is unaffected (Gamse et al., 1982)

THE ANALGESIC ACTIONS OF CAPSAICIN.

The apparent selective effect of capsaicin on nociceptive unmyelinated neurones has been utilized extensively in investigating the physiological consequences of both exciting and inhibiting nociceptive pain pathways. These complex and wide ranging effects are covered in many reviews (Fitzgerald, 1983; Buck and Burks, 1986; Russell and Burchiel, 1984) and therefore a retrospective evaluation of these experiments will be undertaken within the discussion section.

Experiments using adult animals that had been treated neonatally with capsaicin to reduce nociceptive C-fibre input to the C.N.S. have shown analgesic effects to noxious chemical (Faulkner and Growcett, 1980; Hayes et al., 1980; Jancso et al., 1977) and mechanical stimuli (Cevero and McRitchie, 1981; Hayes et al., 1980; Faulkner and Growcett, 1980). Although changes in thermosensitivity have been shown (Holzer et al., 1979; Nagy et al., 1980; Janso and Jancso-Gabor, 1980), the neonatal doses required (30-50 mg/Kg) are higher than that needed to induce analgesia to chemosensitive and mechanosensitive pain (5-15mg/Kg) (Jancso G, 1982) and the effects of capsaicin on noxious thermal stimuli still remain a contentious issue

(Fitzgerald, 1983).

Subcutaneous cumulative daily injections of capsaicin (50-400mg / Kg) to adult rats abolished the capsaicin-induced skin irritation after just two days of the inoculation protocol. When assessed after 5 days the nociceptive pressure thresholds of the hind paws were raised by 48% whilst the nociceptive heat thresholds, using the tail immersion or hotplate tests, were 'unchanged or slightly reduced' (Hayes and Tyers, 1980). Radioimmunoassay of substance P content of both the dorsal horn and hind paw skin in the same set of experiments showed reduced substance P levels in capsaicin-treated animals and so they therefore conclude (in the absence of any change in thermo-sensitivity) that heat and non-heat nociception are mediated by different pathways

Cevero and McRitchie (1981), utilizing the hot plate test were also unable to show any change in the thermal nociceptive threshold whilst mechanical thresholds (paw pressure tests) were elevated. However they found that noxious thermal stimuli to the abdomen, which normally cause a decrease in gastric motility, were ineffective in capsaicin-treated animals. This seems to show that the thermo-somato-visceral reflexes but not the thermal nociceptive threshold evaluations by the hot plate tests, are capsaicin-sensitive; however a reappraisal of the validity of the tail immersion and hot plate test, to selectively excite thermo-nociceptive afferents, might lead to a different conclusion.

Intrathecal injection of capsaicin to the spinal cord of the rat has confirmed the ability of capsaicin to modify perception of nociceptive chemical and thermal stimuli, whilst light touch and mechanical stimuli are unimpaired (Yaksh et al., 1980)

CAPSAICIN AND THE NEUROTOXIC PROCESS.

Jancso et al., (1977) showed that the degeneration of B-type sensory neurones by subcutaneous injection to newborn rats was preceded by severe ultrastructural alterations that consisted of swelling and disorganisation of mitochondrial cristae and dilatation of the cisternae of the rough endoplasmic reticulum and the perinuclear

membrane.

These ultrastructural changes have been confirmed in adult animals where a correlation has been shown between those cells showing mitochondrial swelling and those containing immunohistochemically-labelled substance P and somatostatin. Moreover the time-course of ultrastructural damage followed quite closely that for prolonged desensitization (Chiba et al., 1986).

The ability of capsaicin to induce mitochondrial swelling is not specific to spinal ganglion B-type neurones as localised application of capsaicin to the rat cornea (Szolcsanyi et al., 1975) and sciatic nerve results in axonal oedema and mitochondrial swelling although this may not always result in axonal necrosis (Ainsworth et al., 1981).

The initial ultrastructural changes have been shown to develop, particularly in neonatal rats, into degeneration and glial cell engulfment of both spinal ganglion neurones and the unmyelinated axons and boutons of the dorsal horn (Nagy et al., 1980). Subarachnoid injections of capsaicin to the spinal cord of young rats also results in the degeneration of the primary afferent unmyelinated terminals found within laminae IIb (substantia gelatinosa) (Palermo et al., 1981). This route of administration also results in morphine-sensitive release of substance P and depletion of cholecystinin and somatostatin (Yaksh et al., 1980).

The role of calcium in the neurotoxic process.

The capsaicin-induced degeneration of primary sensory neurones in neonatal rats has been shown to be associated with the appearance of histochemically-detectable calcium ions (Jancso et al., 1978). The assumption was that the calcium had come primarily from the neurotoxically-impaired swollen mitochondria but a possible trans-cell-membrane flux of calcium ions was not excluded.

(Also see section on ion flux measurements).

MEMBRANE EFFECTS OF CAPSAICIN.

In the past decade several groups have undertaken electrophysiological studies in order to find a membrane response that might link the complex sensory neuro-excitatory and neurotoxic actions of capsaicin.

Extracellular recording studies.

Ault and Evans (1980) showed that capsaicin could depolarize the dorsal root nerve bundles of the neonatal rat, in a dose-dependent manner, and that this effect was selective for afferent axons, since both ventral roots fibres and sympathetic ganglia were not depolarized by capsaicin concentrations as high as $25\mu\text{M}$.

Petche et al., (1983) applied 1% capsaicin to the coccygeal nerve and found a 75% reduction in the amplitude of the C-fibre compound action potential whilst the A-fibre compound action potential was unaffected.

The ability of capsaicin to depolarize whole nerve bundles has been confirmed in sciatic nerves taken from adult rats, where dose-dependent depolarizations showed acute tachyphylaxis (Hayes et al., 1984). The depolarizations produced by capsaicin on the sciatic nerve were not mimicked by substance P, bradykinin, histamine, muscarine or 5-hydroxytryptamine. Moreover the capsaicin-induced depolarization was not inhibited by the voltage-sensitive sodium channel blocker tetrodotoxin (TTX).

Localized brief application of capsaicin directly on to the cutaneous saphenous nerve, produces an immediate block of conduction of the unmyelinated C-fibres (Pini, 1983; Baranowski, 1986) and a substantial reduction in the unmyelinated fibre content of the nerve when examined 3-12 months later (Pini et al., 1990).

Intracellular recording studies.

An invitro study by Williams and Zieglansberger (1982) on adult dorsal root ganglion neurones, using high resistance 'sharp' electrodes (2M K acetate), showed that 70% of identified C-cells (conduction velocity $< 5 \text{ m.s}^{-1}$) ie. those with unmyelinated peripheral axons and 58% of A-cells (conduction velocity $> 10 \text{ m.s}^{-1}$) depolarized in response to capsaicin and that this depolarization was associated with an increase in membrane conductance.

In a similar study, Heyman and Rang (1985) confirmed the ability of capsaicin to depolarize a population of C-type neurones by increasing membrane conductance but found that the A-cells were insensitive.

In a study of cultured sensory neurones taken from the newborn rat, Baccaglini and Hogan (1983) showed that 70% of trigeminal cells and 80% of dorsal root ganglia neurones were sensitive to low concentrations of capsaicin ($< 1\mu\text{M}$), which induced a rapid depolarization (3-30mV) and often repetitive action potential firing. Using an antiserum for substance P, they also showed that only 43% of neurones of the trigeminal ganglion cells stained SP-positive, indicating that there is not a complete correlation between existence of substance P within the neurone and capsaicin sensitivity.

Whole-cell voltage clamp experiments on cultured adult and neonatal rat dorsal root ganglion neurones (Bevan and Forbes, 1988) have confirmed the ability of capsaicin to induce a large inward current at hyperpolarized membrane potentials.

Analysis of the current /voltage curves in these experiments predicted a capsaicin current reversal potential close to 0mV, indicating that the conductance mechanism involved a non-selective cation conductance.

Ion flux measurements.

Wood et al. (1988) pursued the membrane effects of capsaicin by undertaking an extensive quantitative study on the ability of capsaicin to induce transmembrane ion fluxes.

They concluded that capsaicin could induce the uptake and accumulation of radioisotopes for calcium ($^{45}\text{Ca}^{2+}$), sodium ($^{22}\text{Na}^{+}$ and guanidine ^{14}C) and could release $^{86}\text{Rb}^{+}$ (a potassium ion flux indicator). No changes in $^{36}\text{Cl}^{-}$ ion fluxes could be found. In the same study, they utilized the monoclonal antibody RT97, which has been shown to recognize a specific neurofilament epitope found in sensory A-cells of the dorsal root ganglion (Wood and Anderton, 1981; Lawson et al., 1984) and found that virtually all neurones that were RT97+ are capsaicin insensitive.

The effect of capsaicin on voltage-activated conductances.

Potassium conductances.

Dubois, (1982) investigated the action of capsaicin on the repolarizing potassium currents (I_{kf1} and I_{kf2}) for the action potential at frog node of Ranvier. Capsaicin (1 - $10\mu\text{M}$) induced a selective reversible inhibition of the I_{kf2} current that appeared to be mediated by an open channel non-voltage dependent mechanism.

The action of capsaicin on potassium conductances was pursued by Taylor et al., 1984 (cultured dorsal root ganglion neurones) and Erdelyi and Such, 1984 (Helix snail neurones). Both groups showed that capsaicin could reversibly inhibit a transient potassium current (I_A) but surprisingly neither noted any significant capsaicin-induced increase in membrane conductance, although the concentrations of capsaicin required (1-300 μM) were much higher than those normally required to induce axonal or neuronal depolarization (0.01-1 μM).

Sodium conductances.

Taylor et al., (1984) showed that capsaicin $10\mu\text{M}$ and $33\mu\text{M}$ could reversibly reduce the peak sodium current amplitude in rat dorsal root ganglion neurones by 50% and 80% respectively, with little effect on the activation or inactivation kinetics. Moreover the inhibition of the sodium current by capsaicin was not subject to desensitization. This effect was also seen by Bevan and Forbes, (1988) and Peterson et al, (1987) on

both the tetrodotoxin-sensitive and insensitive components of sodium inward current found in guinea pig and chicken sensory neurones.

Calcium conductances.

A modification of calcium conductances has been proposed as a possible mechanism mediating the desensitization process (Bevan and Szolcsanyi, 1990, for review) and recent experimental evidence suggests that specific long-term changes to calcium conductances can be induced by capsaicin.

In guinea pig sensory neurones high concentrations of capsaicin ($> 30 \mu\text{M}$) produce a complex reduction in the amplitude of the whole cell total calcium current which appears to be mediated by changes in the activation /inactivation process (Peterson et al., 1989).

Experiments carried out on rat cultured dorsal root ganglion cells have shown that cells which show an initial inward current and increase in membrane conductance in response to capsaicin, have substantially-reduced whole cell calcium currents (Robertson et al., 1989). This effect of capsaicin was dependent on the presence of calcium ions in the external medium, as no inhibition was noted when barium was substituted for calcium as the divalent cation charge carrier through both the voltage activated calcium channels and the capsaicin channels (Bevan and Forbes, 1988). The inhibition of the calcium current by these means has been interpreted as being induced by a process involving the calcium dependent inactivation of calcium channels.

Capsaicin channels.

Capsaicin-activated channels have recently been identified in outside-out membrane patches from dorsal root ganglion C-cells (Forbes and Bevan, 1988). These channels are of high conductance 25-30pS at -80mV and show rectification on depolarization (79pS at +40mV). The non-linearity of the conductance/voltage curve for the capsaicin channel is thought to be mediated by a partial block of the channel at hyperpolarized potentials by calcium ions. This partial block of the channel by calcium

can be reduced by depolarization or removing the calcium ions from the external medium.

The objectives of the project and the use of vagus nerve and nodose ganglion preparation.

The aim of this project was to find a possible unifying mechanism by which capsaicin could induce such profound and complex actions on sensory physiology, utilizing conventional electrophysiological and histological techniques. Experiments were preferentially undertaken on the vagus nerve and nodose ganglion preparation. This preparation is predominantly sensory and has been shown to contain capsaicin-sensitive unmyelinated nerve axons (see below).

The vagus nerve.

The vagus nerve arises from the medulla oblongata brain region and emerges from the central nervous system at the base of the skull, through the jugular foramen. The vagus nerve then passes parallel to the spinal cord towards the abdomen with auricular, pharyngeal, laryngeal, pulmonary and oesophageal branches.

The cervical vagus nerve is multitasking, taking afferent (sensory) information to and efferent (motor) stimuli from the central nervous system. It contains 29,000 nerve fibres, of which only 5000 are myelinated and 66% of these, sensory. The remaining fibres (24,000) are unmyelinated and 87.5% sensory (Evans and Murray, 1954; Paintal, 1973).

The small diameter of the unmyelinated fibres ($< 1\mu\text{m}$) within the vagus nerve, hampers the use of conventional intracellular recording methods. However extracellular, non-invasive, recording methods have given a valuable insight into the electrophysiological and pharmacological properties of these axons.

Electrophysiology of unmyelinated fibres.

The resting ionic membrane permeability of mammalian unmyelinated fibres was investigated by Armett & Ritchie, (1963) using the double sucrose gap technique (Stampfli, 1954). It was found that the resting sodium permeability (P_{Na}) of the unmyelinated fibres of the vagus nerve ($P_{Na}/P_K = 0.25$) was much higher than that found for either the hypogastric nerve ($P_{Na}/P_K = 0.1$), frog muscle fibres ($P_{Na}/P_K = 0.01$, Hodgkin and Horowics, 1959) or squid giant axon ($P_{Na}/P_K = 0.04$, Hodgkin and Katz, 1949). They concluded that the differences in the resting permeability to sodium ions of vagus nerve (predominantly sensory) and hypogastric nerve (predominantly sympathetic efferent) unmyelinated axons, could account for the characteristic differences in after-potentials recorded extracellularly upon electrical stimulation. It is suggested that the positive after-potential recorded from sympathetic unmyelinated axons results from the extracellular accumulation of potassium ions released during the repolarizing phase of the action potential (Greengard & Straub, 1958). The high P_{Na}/P_K ratio found in the sensory unmyelinated fibres of the vagus nerve, limits the depolarizing influence of extracellular potassium ions. The negative after-potential produced, is due to the hyperpolarizing influence of the voltage-sensitive increase in potassium conductance activated during the rising phase of the action potential.

The high resting sodium conductance might be expected to result in substantial resting transmembrane sodium ion flux and intraxonal accumulation. An ouabain-sensitive electrogenic sodium pump, that maintains the transmembrane sodium gradient, in vagus nerve, has been described by Rang and Ritchie (1968). The activity of this pump was particularly pronounced after repetitive activity, and is chiefly responsible for the very slow (> 5 min) hyperpolarization following high frequency (20 Hz / 5 sec) stimulation of the vagus nerve.

The terminology of Erlanger and Gasser (1930) used to define axonal subgroups, was based on conduction velocity measurements from whole nerve bundles. Group A. consists of the fastest conducting, large diameter myelinated somatic afferents

and efferents. This group is divided into subgroups: AI (10-20 μm , 50-100 $\text{m}\cdot\text{s}^{-1}$), AII (5-15 μm , 20-70 $\text{m}\cdot\text{s}^{-1}$) and AIII (1-7 μm , 5-30 $\text{m}\cdot\text{s}^{-1}$). Group B. contains the myelinated preganglionic fibres of the autonomic nervous system whilst Group C. is composed of the small diameter unmyelinated visceral and somatic afferent fibres and the postganglionic autonomic efferents (0.2-1.0 μm , 0.2-1.0 $\text{m}\cdot\text{s}^{-1}$).

Axonal receptors.

Acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) are transmitter substances that can not only activate chemoreceptive sensory nerve terminals but also have direct effects on the associated axons and perikarya.

5-HT applied to peripheral terminals in man, either subcutaneously or to the forearm blister base, induces intense irritation and pain (Keele and Armstrong, 1964). This pain can be inhibited by the selective 5-HT₃ antagonist ICS 205-930 (Donatsch et al, 1984a). The 5-HT-induced depolarization of the vagus nerve and nodose ganglion has also been found to be ICS 205-930-sensitive (Donatsch et al, 1984b; Round and Wallis, 1985) and it could be, as suggested by Wallis,(1981), that these 5-HT receptors might be an ubiquitous membrane component in a population of C-cells.

The nerve endings of sensory unmyelinated fibres can be directly excited by acetylcholine, whilst the terminals of the myelinated fibres are, in general, less sensitive (Douglas and Ritchie, 1962; Paintal, 1967). The vagus nerve and nodose ganglion have also been found to be depolarized by ACh and other cholinergic agonists that interact with nicotinic receptors (Wallis et al, 1982). The depolarization produced in the vagus nerve is associated with a reduction in the amplitude and slowing of the conduction velocity of the extracellularly recorded C-potential, generated from conducting unmyelinated nerve fibres (Armett and Ritchie, 1960).

Capsaicin-sensitive vagal afferent fibres.

Vagal sensory receptors and their reflex actions have been extensively evaluated by Paintal (1973) and it is clear from the accumulated data that the sensory unmyelinated fibres of the vagus play a major role in visceral co-ordination/pain perception and the

associated reflex actions.

1. Cardio-respiratory effects.

The effects of capsaicin, applied perineurally to the vagus nerve, on cat cardiovascular and respiratory function, has been described (Jancso and Such, 1983). Application of capsaicin to the vagus nerve resulted in a decrease in the mean arterial blood pressure (hypotension) and a reduction in heart rate (bradycardia). The effects of perineurally applied capsaicin on respiratory function proved to be highly variable; however initially a brief transient decrease in respiratory rate was more often observed which could then be followed by a maintained increase (39%) or decrease (56%). These results together with an interaction study to systemically applied capsaicin, phenyldiguanidine and veratridine suggested that the cardio-respiratory vagal afferent fibres mediating the common triad response of bradycardia, hypotension and apnoea, are from different chemo-sensitive axonal populations and that capsaicin is primarily acting via unmyelinated pulmonary and carotid baroreceptors (Coleridge et al, 1964).

2. Hepatic effects.

Histochemically labelled horse radish peroxidase (HRP) retrograde transport (Carobi et al, 1985b) and substance P measurements (Holzer et al, 1982) indicate that many of sensory fibres that innervate the rat liver are vagal in origin. A proportion of these fibres are sensitive to neonatal capsaicin treatment (Carobi et al, 1985a). These fibres are believed to play a role in nociceptive sensory perception, although an additional functional role related to the peripheral release of substance P within the liver and hepatic duct, cannot be discounted (Carobi & Magni, 1985b).

3. Gastrointestinal effects.

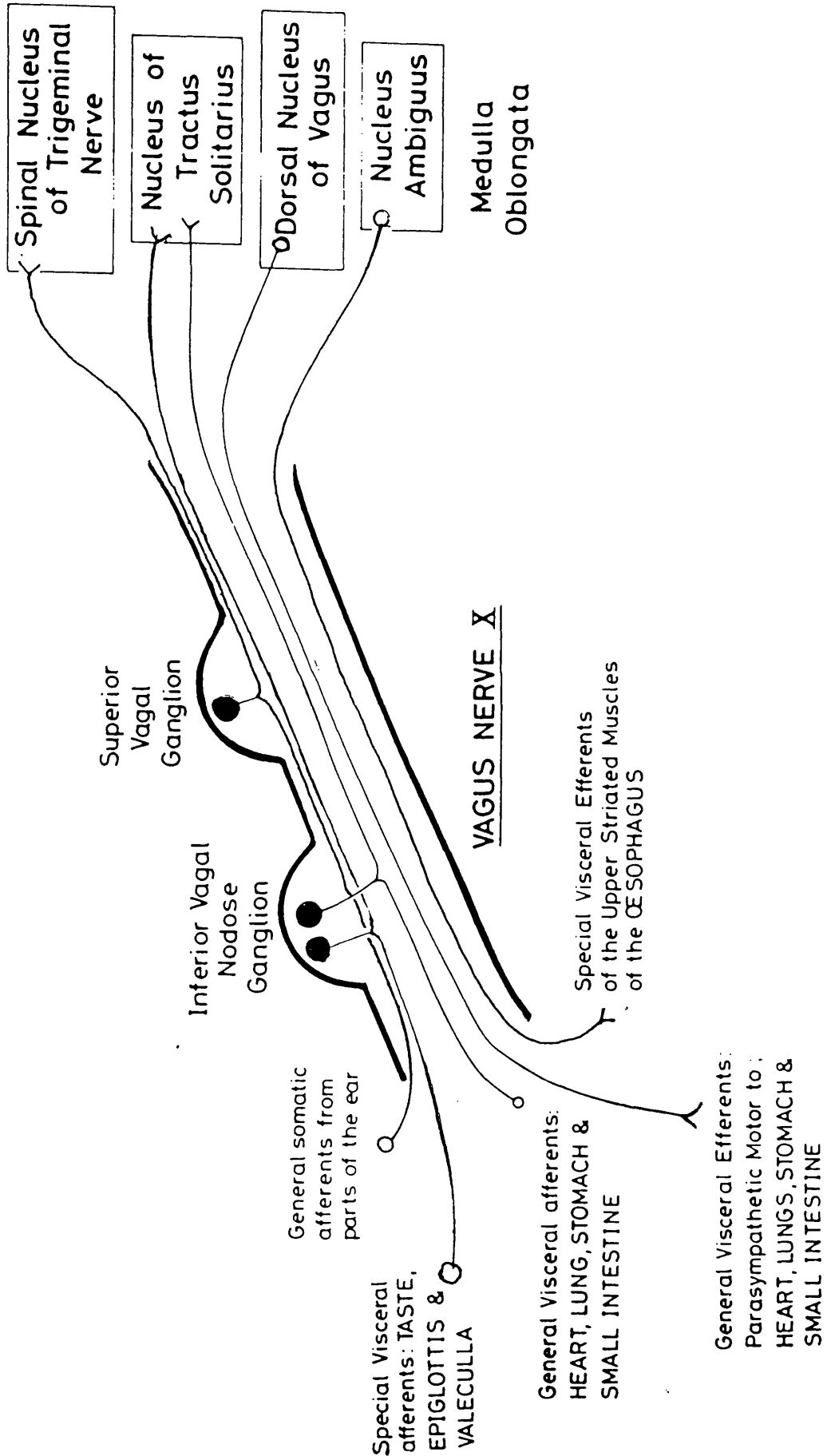
Capsaicin-sensitive vagal nerve fibres are widely distributed throughout the gastrointestinal tract (Maggi & Meli, 1988. for review) and it is thought that the chemo-nociceptive afferents present play a major role in the integration of both intra and extramural reflexes (Melone, 1986).

The nodose ganglion.

The sensory nerve cell soma of the vagus nerve lay within the nodose and jugular ganglia, which are located superficially on the vagus nerve at the level of the carotid artery bifurcation. The cell bodies found within the nodose ganglion have uniform diameters (15-25 μ m) and the size difference between the A-cells (myelinated peripheral axons) and C-cells (unmyelinated peripheral axons) seen in dorsal root ganglia (Lieberman, 1976) is not so apparent.

The pharmacological and electrophysiological properties of rabbit nodose ganglion neurones have been described by Stansfeld and Wallis (1985). Using axonal conduction velocity measurements they characterized two main neuronal groups, A-cells and C-cells. A-cells had short duration (1.16 ms), rapidly rising (385 V/s) action potentials and had a mean axonal conduction velocity of 16.4 m.s⁻¹. These cells were depolarized by GABA but were unaffected by 5-HT or the acetylcholine nicotinic receptor agonist DMPP. C-cells had action potentials of a longer duration (2.51 ms), were slower to rise (255 V/s) and tended to show a pronounced afterhyperpolarization. 80% of the C-cells were depolarized by 5-HT, 75% by DMPP and all of the cells tested responded to GABA. These results show that there are both electrophysiological and pharmacological differences in the two main neuronal groups of the nodose ganglion.

Fig.11.
Efferent and afferent pathways of the vagus nerve.



CHAPTER 2.

METHODS SECTION.

Dissection method.

Adult Wistar rats (250-400g) were anaesthetised by intraperitoneal injection of urethane (1.5g / Kg) and fixed to a cork dissecting board, ventral surface uppermost. The skin overlying the throat region was cut away, displaying the underlying salivary glands, lymph nodes and sternohyoideus muscle, which were removed by blunt dissection and cautery. The sternomastoideus and digasticus muscles were then also removed and a traceotomy tube fitted.

The remainder of the dissection was carried out under a binocular microscope (Carl Zeiss, Jena). The vagus nerve and cervical preganglionic sympathetic nerve were separated from the carotid artery and a suture tied around the artery to allow it to be retracted. The cervical sympathetic trunk and associated superior cervical ganglion was removed and a fine suture (Arbrasilk 5/0) tied around the vagus nerve at the lower cervical region. The vagus nerve was then dissected free of the surrounding tissue and retractors fitted in the region of the hyoid. This allowed the nodose ganglion to be seen and then removed together with 1.5 cm of cervical vagus nerve trunk.

The extracellular recording methods.

Freshly dissected nerves were placed in chilled Krebs' solution and the epineuronal sheath was removed, with the aid of a Carl Zeiss binocular microscope and fine watchmakers forceps. The nerve bundles were then ligated and transferred to a three chambered "Perspex" bath (Fig.M1) compartmentalised by 1mm "Perspex" partitions. Each partition consisted of an upper and lower section in which a slot had been cut to allow the nerve to pass freely between compartments without crushing.

Nerves were sealed into position using silicon grease, which also prevented the free diffusion of drugs between compartments.

A Watson-Marlow (DHRE-22) peristaltic pump continuously perfused the central recording chamber (b) with Krebs' solution (20-25°C) at 4 ml. min⁻¹ (bath fluid

exchange time \approx 10 sec). Drug applications were made by transferring the pump input tubing from the aerated physiological medium to one that contained a known concentration of drug. The chamber effluent was removed to waste via vacuum. An air trap incorporated into the vacuum tubing eliminated aerial interference.

Although chambers (a) and (c) were not normally perfused they contained Krebs' solution which was bubbled with 95% O₂ / 5% CO₂ to prevent anoxia.

Procaine (1mM) was normally added to chamber (c) to convert the evoked compound action potential to the monopolar form.

A differential amplifier (input impedance 40M Ω) was connected to the recording bath by non-polarizing Ag/AgCl electrodes embedded in 5% agar/saline. A stability of less than 0.1mV / hr DC drift allowed continuous high-gain recording of DC potentials.

Preparations were electrically stimulated from within chamber (a) via 0.25mm diameter platinum wires (inter-electrode distance 5mm). A Grass SD9 stimulator provided the stimulus frequency and variable width voltage source as well as oscilloscope and peak height detection synchronization.

A peak height detector (PHD), devised by C. Courtice (1977), was used to continuously display the differential recordings from chambers (b) and (c) of spike amplitude and polarization. This electronic device incorporates both a sample and hold circuit with adjustable delay and hold-time and a variable gain differential amplifier. In effect it samples the amplitude of the largest positive-going voltage deflection and then holds that value superimposed on any standing potential generated at the input of the differential amplifier. The output, displayed on a Servoscribe 220 chart recorder, is therefore a continuous record of changes in membrane potential and evoked spike amplitude. The peak height detector also supplied an amplified oscilloscope output.

The extracellularly recorded dose-response curves and intracellularly recorded slope conductance curves were analysed using the interactive curve-fitting and linear-regression procedures of SIGMA-PLOT 3.1 (Jandel Scientific, 1986).

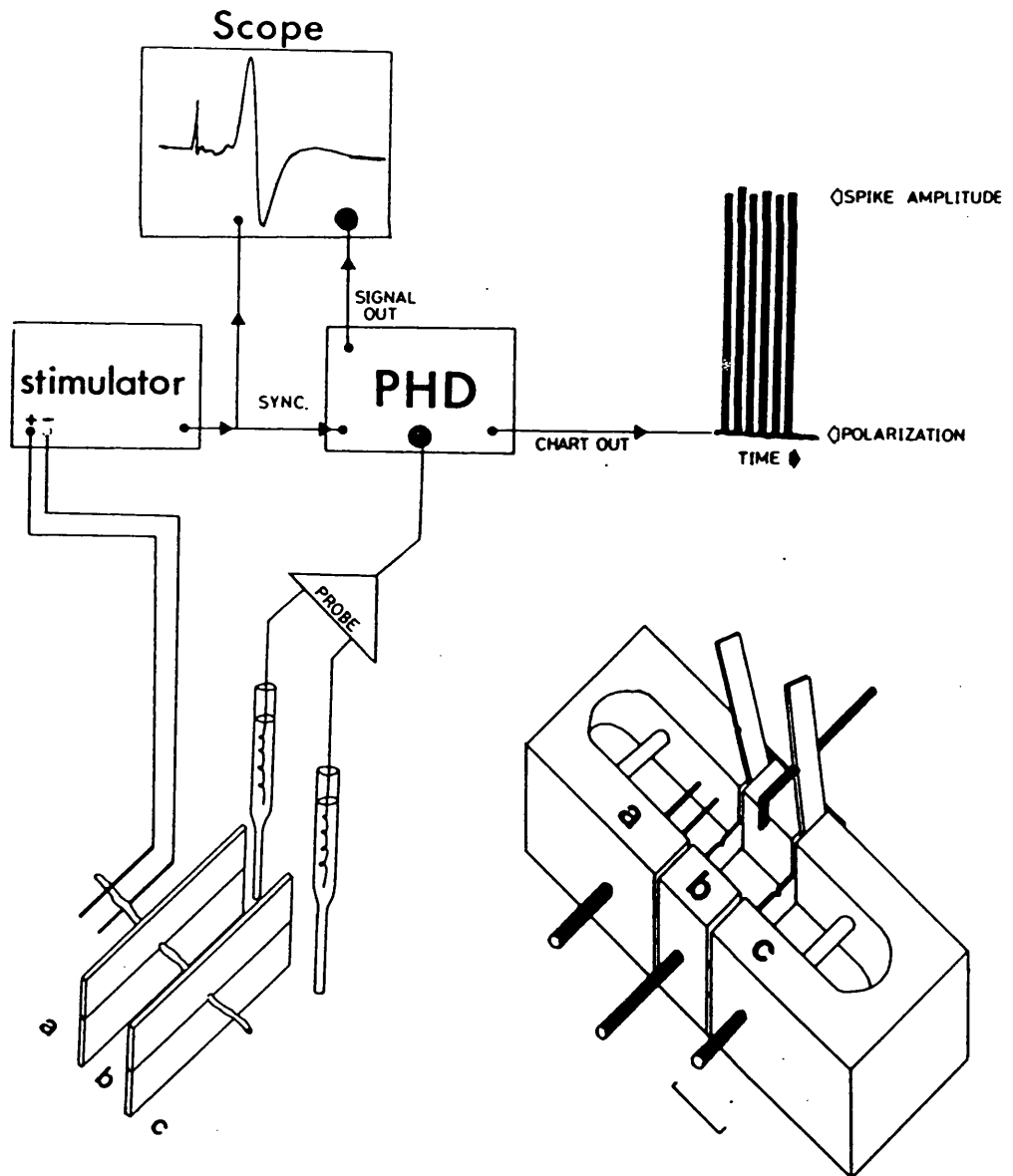
Fig.M1.

The extracellular recording method.

Differential recordings of compound action potential amplitude (spike amplitude) and drug-induced changes in membrane potential (polarization) were made between two sections of nerve separated by 'Perspex' partitions.

The peak height detector (PHD) allowed the continuous monitoring of these two parameters on a single chart record (See text for complete explanation).

Scale bar: 0.5 cm



Intracellular recording methods.

The intracellular recording bath was a two component structure machined from transparent "Perspex" (Fig.M2). The base structure consisted of a central well where the recording bath insert would normally sit, and two side chambers, the stimulus chamber in which two platinum wire stimulating electrodes (S.E.) were placed (inter-electrode distance 5mm), and a reference chamber where the earth reference electrode and thermistor probe (T.P.) for temperature regulation were located.

The earth reference electrode (E.R.) was a silver/silver chloride pellet (1.5mm Diameter) obtained from Clark Electromedical Co.

The recording chamber consisted of a removable "Perspex" insert which had been lathe-turned to fit precisely into the base structure well. A sealing ring (S.R.) prevented overflow leakage. The base of the insert was lined with Dow Corning "Sylgard" silicone plastic to a depth of 5mm allowing the preparation to be firmly fixed using fine entomological pins.

The recording bath was continuously perfused at $5\text{ml}\cdot\text{min}^{-1}$ under gravity feed from 50ml reservoirs located 10cm above the base structure.

For changes in the perfusing medium or drug applications a multichannel tap was constructed to allow the selection of one of the three continuously-aerated superfusing reservoirs. The fluid depth of 3mm was maintained by adjusting the recording bath insert and the stainless steel outflow tube located within the reference chamber.

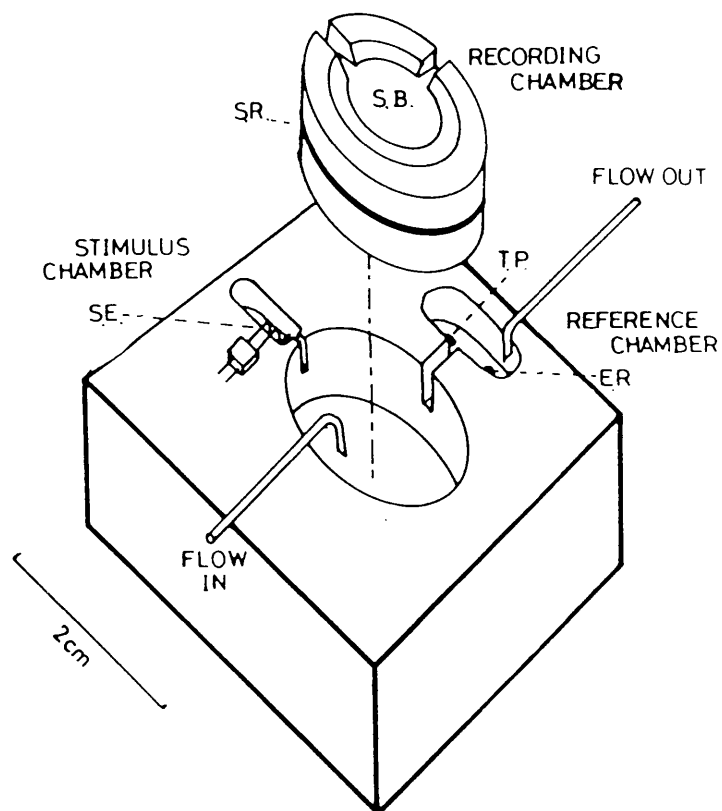
A Watson-Marlow 502S peristaltic pump recirculated the superfusing medium to the reservoir of origin or to waste.

Temperature was regulated by a heat exchange unit devised by J.V. Halliwell and C. Courtice at the School of Pharmacy. A stainless steel disc, 1cm thick, 5cm diameter, had a spiralled channel spark-eroded into its surface to a depth

Fig.M2.**The intracellular recording bath.**

S.E. = Stimulating electrodes, S.R. = Sealing ring, T.P. = Thermistor probe, S.B. = Sylgard base, E.R. = Earth reference.

Full description given in text.



of 1mm. The disc was then capped using 5mm "Perspex" and two stainless steel tubes passed through the cap to make contact with the start and end of the eroded channel. Heat generated from a 50W, 12V light bulb located below the disc, was absorbed by the blackened undersurface, which in turn warmed the superfusing medium which passed through the spiralled channel. A thermistor probe located within the reference chamber of the recording bath provided the feed-back monitoring device which modified the output voltage controlling the bulb intensity. Bath temperature was normally maintained at 30 ± 0.5 °C.

The desheathed nodose ganglion and vagus nerve were placed centrally in the recording bath insert and orientated to allow the vagus nerve to pass through the inter-chamber slot into the stimulus chamber. The ganglion was then firmly fixed to the "Sylgard" base using 5 or 6 entomological pins, the inter-compartment slot filled with silicone grease and the stimulus chamber filled with Krebs' solution.

A Carl Zeiss sliding manipulator (30-G-500) in conjunction with a micro-adjustable tool holder was used to position the electrode above the ganglion, under binocular vision. The electrode was then lowered vertically towards the ganglion, using the fine control until there was an apparent increase in electrode tip resistance, which was continuously monitored on an oscilloscope, using 0.2nA, 30msec constant current pulses.

At this point a transient capacity over-compensation was generated via a footswitch. This generated a high frequency oscillation of the electrode tip ("ZAP"), which would normally be sufficient to induce the electrode to penetrate the neuronal cell body.

The lack of suitable optical and micromanipulator facilities meant that it was not possible to use the two electrode intracellular recording method regarded as preferable in recording from neuronal perikaria. Experiments were done using the single electrode bridge-balance and switch clamp techniques (Finkel and Redman, 1984).

Bridge-balance current clamp.

Fig.M3. shows the bridge-balance circuitry used in the present study when using the current clamp technique.

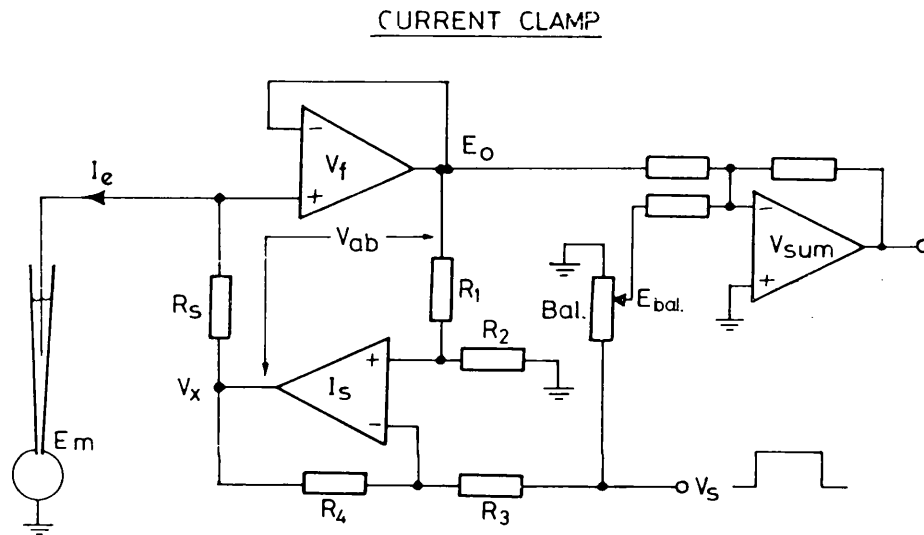
A proportion of the current command voltage (V_S) is selected via a potentiometer (BAL) and added to the inverting side of a summing amplifier (V_{sum}), thus subtracting a square wave pulse from the voltage output, that is proportional to the voltage supply input (V_S). The output from the bridge-balance potentiometer (E_{bal}) was normally set before cell penetration to nullify the voltage drop across the electrode tip generated during the passing of current. The relative position of the bridge-balance potentiometer was used to measure the electrode resistance.

The current intensity was controlled by the current source amplifier (I_S) in response to the analogue control voltage (V_S). The current intensity is monitored and controlled via a feedback from the unity gain voltage following amplifier (V_f). The voltage generated at V_x is therefore equal to the sum of the voltage follower output (E_0) and the analogue voltage source (V_S) and current intensity is therefore proportional to the voltage drop V_{ab} divided by the large series resistance R_S .

Fig.M3.

The bridge-balance circuit used in the present study when using the current clamp technique.

Description in text.



$$R_1 = R_2 = R_3 = R_4 = R$$

$$V_x = \left[1 + \frac{R}{R} \right] \left[\frac{E_o}{2} \right] + \left[\frac{R}{R} \right] V_s$$

$$V_x = E_o + V_s$$

$$\therefore I_e = \frac{V_s}{R_s}$$

$$\text{also } I_e = \frac{V_{ab}}{R_s}$$

Single electrode switch clamp.

The single electrode switch clamp technique allows both voltage recording and current passing capability from the same electrode on a time share basis (Wilson and Goldner, 1975).

The technique utilizes the different electrical charging characteristics of the microelectrode and the cell membrane during the passage of a transient constant current pulse (Fig.M4).

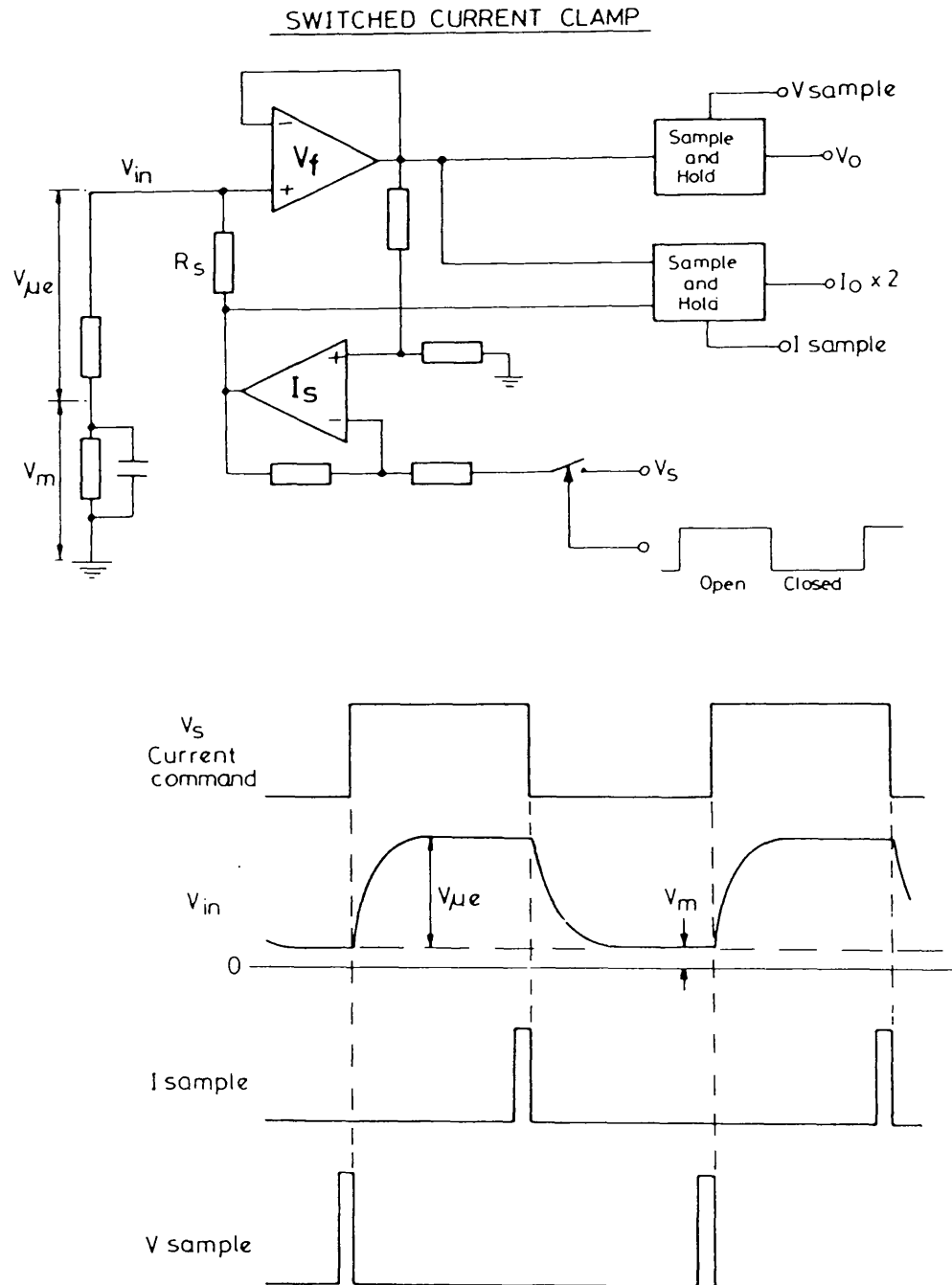
A current passed through an electrode and into a cell produces a voltage proportional to the sum of the microelectrode tip ($V_{\mu e}$) and membrane (V_m) resistances. The electrode has minimal capacitance and therefore a short time constant which enables it to rapidly achieve the proportional voltage whilst the membrane voltage change is initially minimised by the charge-retaining characteristics of the much larger cellular membrane capacitance.

On the termination of a current pulse, the voltage produced across the microelectrode tip rapidly decays, leaving the residual membrane voltage response. A sample and hold circuit (see footnote) activated at this point, produces a continuous sampled voltage output (V_{sample}), updated each cycle. A current monitoring sample and hold circuit produces an equivalent continuous current sample output (I_{sample}), logically activated before the termination of the current passing mode.

Fig.M4.

The switched current clamp circuit.

See text for description.



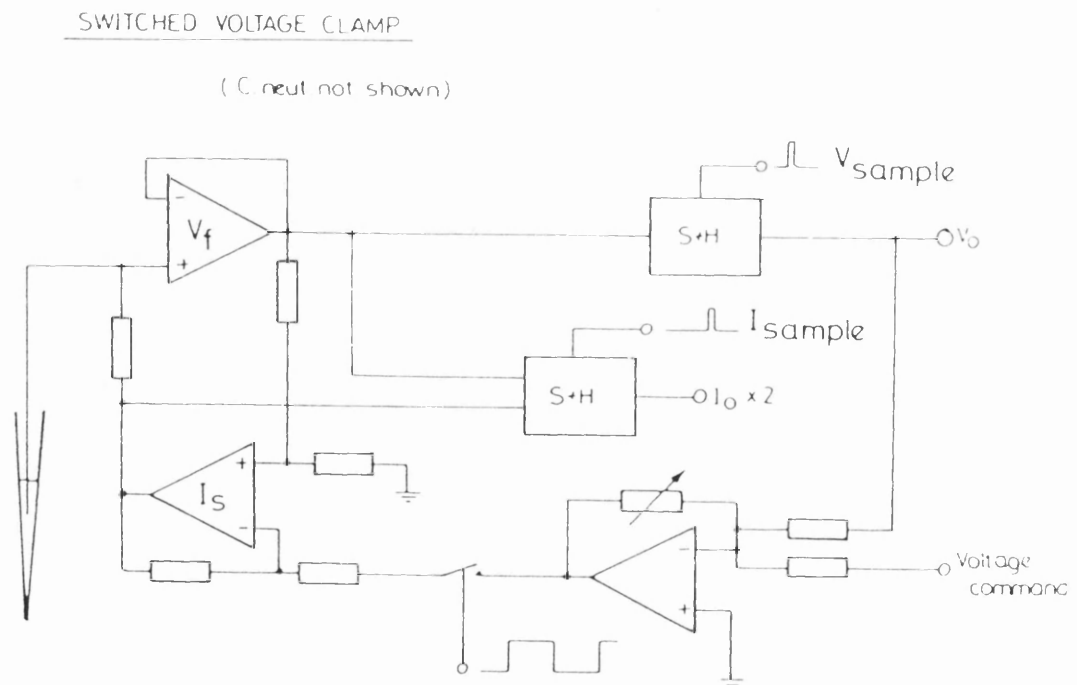
For voltage clamping an additional circuit was incorporated (Fig.M5) which allowed one to step the membrane voltage from its holding potential to a predetermined value and monitor the current produced. The circuit compares the sampled voltage (V_{sample}) with that of a variable command voltage (V_{command}) and a feedback current is then passed into the cell at each cycle until the difference between these two values is minimised.

The recording circuit has a 50% duty cycle with a variable switching frequency. This frequency is normally optimised by capacity neutralization. This assures that the voltage generated at the electrode tip during current injection fully decays before the sample and hold circuit is activated to measure membrane current or voltage.

Switching frequencies of 2.5 to 4kHz were often obtained using this technique.

Footnote; A sample and hold circuit is a field-effect transistor-based integrated circuit, which, in response to a T.T.L. logic pulse, rapidly samples its input and holds a steady equivalent voltage on an output until reset by another T.T.L. pulse.

Fig.M5.
The switched voltage clamp circuit.



Capacity neutralization.

Although short ($< 1\text{ms}$), the time constant of the recording electrodes (generated by a combination of the electrode tip resistance and stray capacitances not associated with the cell membrane) can distort the recorded waveform. To minimise the effects of stray capacitance, an additional variable gain amplifier and feedback capacitor (a differentiator) was incorporated. This enabled an equal and opposite transient voltage to be fed into the voltage following amplifier, achieving capacity neutralization.

Over capacity neutralization leads to gross oscillation of the recording amplifier and electrode tip, which is the basis of the "ZAP" method of cell penetration.

Correct capacity neutralization is particularly important when using the switch clamp technique, as it controls the rate of repolarization following the current injection mode and when correctly adjusted nullifies the use of bridge-balance and allows one to optimise the switching frequency.

Intracellular recording electrodes.

Glass microelectrodes were manufactured on an Ensor moving coil horizontal microelectrode puller, using 1.2mm filamented glass (Clark Electromedical Instruments.). Short shank ($< 1\text{cm}$) microelectrodes were generally used, which minimised flexing of the electrode tip when penetrating the collagen-toughened surface of the ganglion.

Electrodes were selected for use based on their characteristics measured under bridge-balance current clamp. Electrodes which, when filled with 4M potassium acetate or potassium chloride, had resistances greater than $40\text{M}\Omega$ or showed non-ohmic behaviour in response to constant current injection were rejected.

Histological methods.

The freshly dissected nodose ganglia and attached vagus nerves were incubated in oxygenated Krebs' solution or a modified Krebs' solution at 28°C, containing capsaicin or an equivalent amount of ethanol solvent. Incubations were normally 30 mins in duration but were varied from 5 to 60 mins before fixation. Ganglia were then transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C, for 3 hours, buffer-washed and post-fixed in 1% osmium tetroxide before dehydrating. The embedding medium was "Araldite" epoxy resin with a propylene oxide link reagent. Sections were cut on a Reichert ultramicrotome. Thick sections for light microscopy were stained with toluidine blue. Thin sections were stained with saturated alcoholic uranyl acetate and 0.01% aqueous lead citrate before examination under a Phillips 201 transmission electron microscope.

Drugs and Chemicals.

Capsaicin (8-methyl-n-vanillyl-6-nonenamide (Sigma Chem. Co.) was dissolved in ethanol to provide a 10mM stock solution. Muscimol (Fluka), carbachol (Sigma Chem. Co), 5-hydroxytryptamine (Sigma Chem. Co.), hexamethonium (Sigma Chem. Co.) and metoclopramide (Dr. M. Tyers, Glaxo group research) all readily dissolved in the physiological media. All other compounds mentioned were obtained from commercial sources. Inorganic salts were normally obtained from British Drug House (BDH) and were of 'Analar' grade. The concentrations of the ionic constituents used in the various physiological bathing media are shown in Table 1.

TABLE 1

THE IONIC CONSTITUENTS OF KREBS AND MODIFIED KREBS PHYSIOLOGICAL SOLUTIONS

SOLUTION	IONS (mM)	Na ⁺	K ⁺	Ca ⁺⁺	Mg ⁺⁺	Cl ⁻	SO ⁴	PO ⁴	ISETHIONATE	HCO ⁻³	TRIS	GLUCOSAMINE	AERATING GAS	pH
NORMAL KREBS		143	5.9	2.5	1.2	128	1.2	1.2	-	25	-	-	95% O ₂ 5% CO ₂	7.4
SODIUM FREE (TRIS)		-	5.9	2.5	1.2	133.7	1.2	1.2	-	-	143	-	O ₂	7.4
SODIUM FREE (GLUCOSAMINE)		-	5.9	2.5	1.2	124	1.2	1.2	-	-	25	118	O ₂	7.4
LOW CHLORIDE (ISETHIONATE)		143	5.9	2.5	1.2	10.9	1.2	1.2	118	25	-	-	95% O ₂ 5% CO ₂	7.4
CALCIUM FREE		143	5.9	-	3.7	128	1.2	1.2	-	25	-	-	95% O ₂ 5% CO ₂	7.4

Each of the above solutions were aerated at room temperature for at least one hour and Glucose (11mM) added before use.

CHAPTER 3.**EXTRACELLULAR RECORDING RESULTS**

Depolarization of the vagus nerve.

Capsaicin applied within the superfusate to the rat vagus nerve bundle produced a dose dependent depolarization (threshold concentration $0.03\mu\text{M}$. Fig.R1). The depolarization developed sigmoidally with time, reaching a steady-state within three to five minutes. On removing capsaicin the depolarization slowly decayed exponentially.

The time constant for decay increased with capsaicin concentration and, with high doses ($> 3\mu\text{M}$), a wash period of up to 2 hours was often required before full repolarization of the nerve bundle.

Continuous perfusion (> 5 min) with a high concentration of capsaicin often resulted in fade of the depolarization, and doses repeated at short intervals produced acute tachyphylaxis (Fig.R2).

To overcome these problems and enable the construction of a dose-response relationship, only a limited number of capsaicin concentrations were applied to each preparation. The dose-response curves were constructed using a strict experimental protocol: reproducible control responses to $0.3\mu\text{M}$ capsaicin were obtained (at 45 minute intervals); these were then followed by a lower concentration ($0.03\mu\text{M}$ or $0.1\mu\text{M}$) and then finally a higher concentration ($1\mu\text{M}$ - $10\mu\text{M}$) before concluding the experiment.

Fig.R1.

Capsaicin depolarizes the rat vagus nerve with concurrent changes in the evoked electroneurograph.

Results from a single experiment where consecutive increases in capsaicin concentration were applied to the same preparation at the time intervals shown on the left-hand side of the figure.

The traces on the left-hand side of the figure are peak height detector records of membrane polarization and amplitude of the C-spike.

The traces on the right-hand side of the figure are oscilloscope records of the supramaximally stimulated electroneurograph taken during the control period before applying capsaicin and at the peak of the capsaicin-induced depolarization.

Fig.R1.

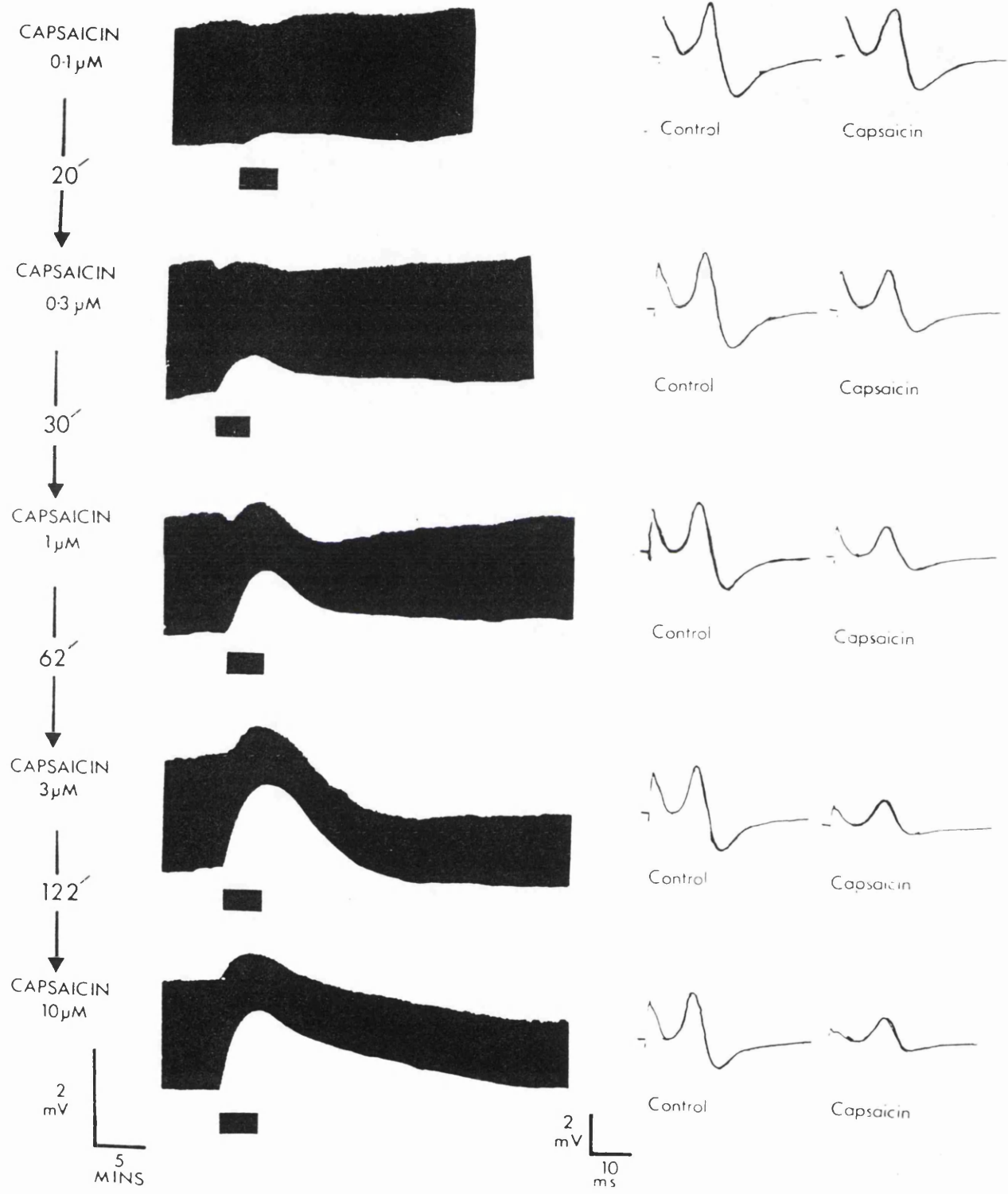
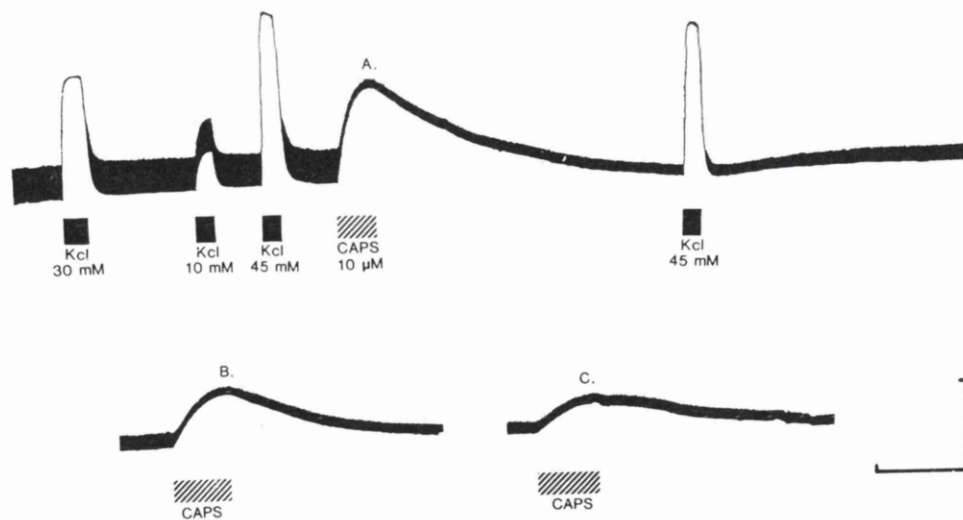


Fig.R2.

The maximal capsaicin-induced depolarization (A) is compared to the depolarization induced by raising extracellular potassium ions. The capsaicin induced depolarization is reduced in amplitude on subsequent applications (B & C at 60 minute intervals), indicative of tachyphylaxis, whilst the depolarization to potassium ions proves to be reproducible.

Scale: 2mV / 10 mins.



Quantitation: The analysis of the dose / depolarization curve.

The log concentration / depolarization curve induced by capsaicin was analysed by conventional pharmacological techniques to test for a possible receptor-coupled event.

Fig.R3. shows a comparison of the data obtained in the present study with two theoretical curves. The theoretical curves were constructed from the hyperbolic function shown in Eqn.2A; the sigmoidal derivative generated when the log agonist concentration is plotted on the abscissa is displayed.

$$\text{Eqn. 2A.} \quad V = \frac{V_{\max} X^n}{X^n + ED_{50}^n}$$

Where $ED_{50} = 0.6 \mu\text{M}$

$V_{\max} = 2 \text{ mV}$

$n =$ molecular ratio of ligand to receptor.

$V =$ response depolarization (mV)

$X =$ Agonist concentration.

The capsaicin dose-response curve closely resembles the theoretical curve when $n=1$. This was confirmed using the Hill-plot (Fig.R4). This double logarithmic plot linearised the capsaicin dose-response curve and allowed a least-squares regression analysis procedure to calculate the slope of the line and hence a value for n . The implication of this data is that capsaicin molecules might interact with a receptor site on a one-to-one basis to trigger the depolarizing event.

Fig.R3.

Capsaicin-induced depolarizations of the rat vagus nerve. Abscissa: logarithm of capsaicin concentration (M); Ordinate: depolarization (mV). Points show mean depolarization amplitude; bars indicate s.e.m; number of observations at each concentration is indicated.

The solid line fitted to the data was calculated by an iterative curve-fitting procedure allowing free estimates of V_{max} , ED_{50} and slope. The theoretical curves are solutions to Eqn.2A where $V_{max} = 1.962\text{mV}$, $ED_{50} = 0.58\mu\text{M}$ and $n =$ molecular ratio of ligand per receptor are also shown. Note how the data more closely fits the curve if $n = 1$.

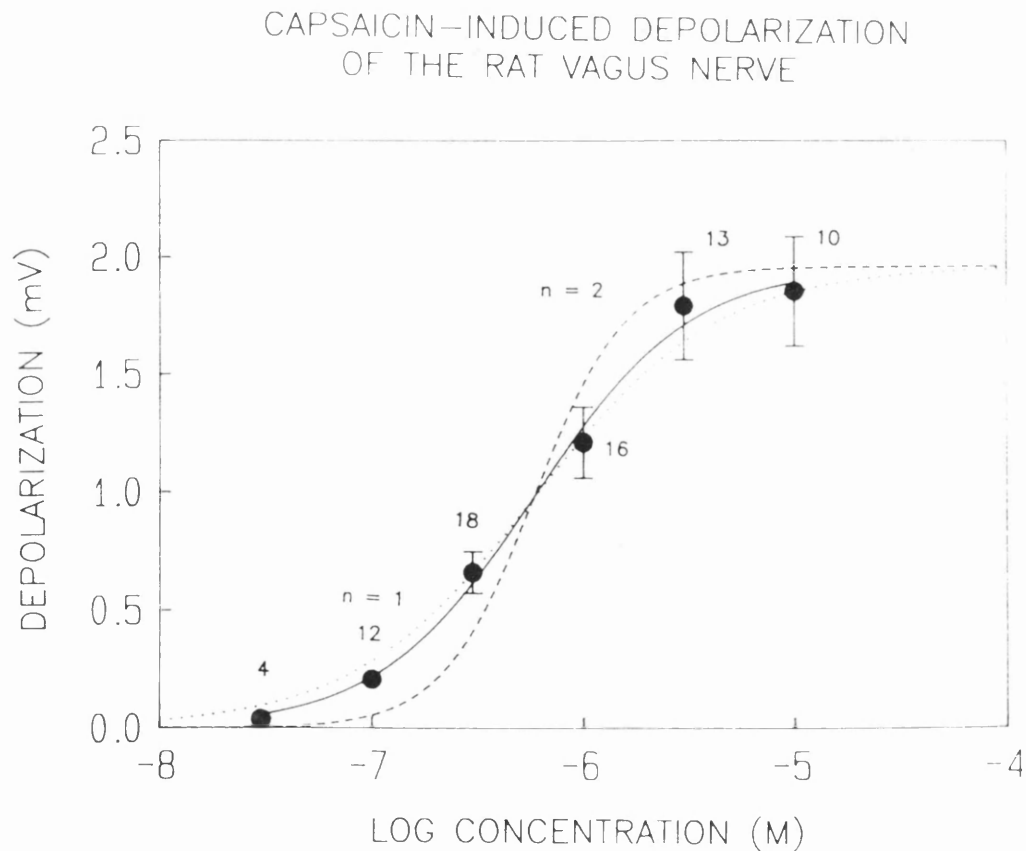
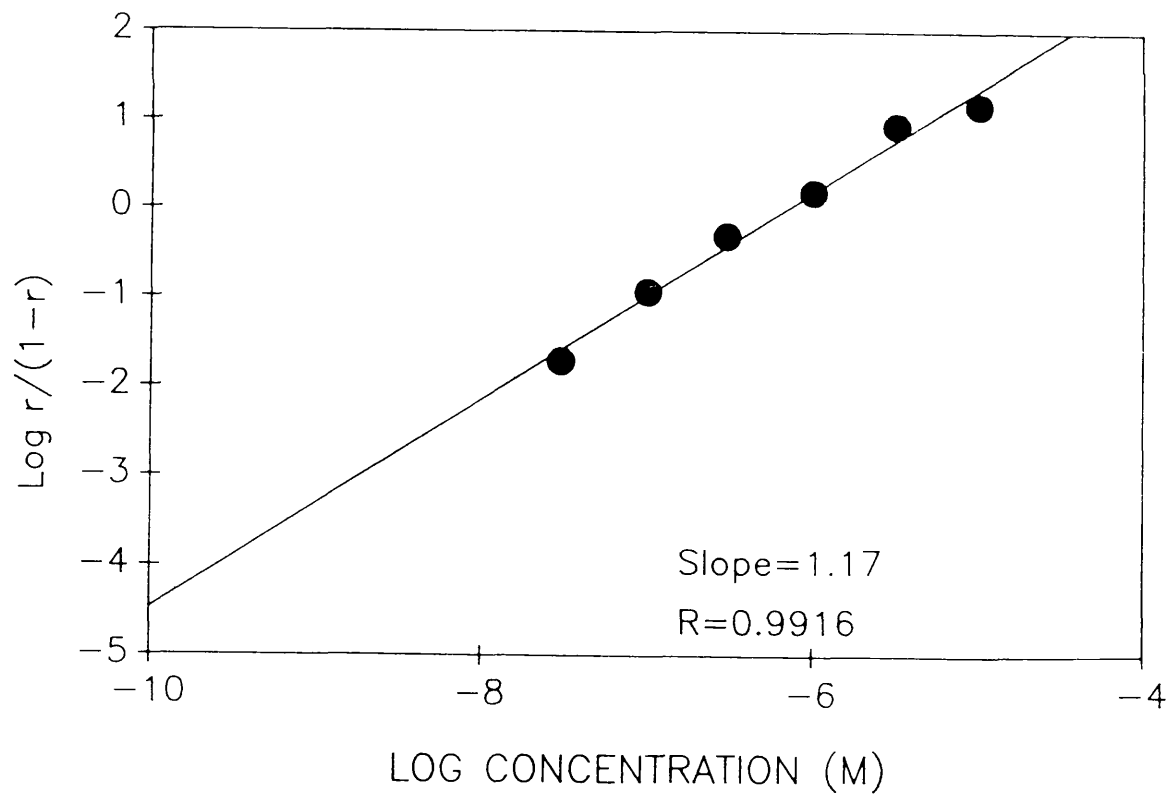


Fig.R4.

Hill-plot of data shown in Fig.R3. Ordinates: $\log(r/1-r)$ where r = fraction of maximal response, Absissa: \log concentration of capsaicin (M). The slope and the correlation coefficient (R) were determined by least-squares regression analysis.



The assumption of this type of analysis is that the amplitude of the depolarization is directly proportional to the number of receptors occupied and that the maximal depolarization is achieved when all the receptors are agonist-coupled. However the amplitude of the depolarization produced by an agonist which increases membrane conductance is determined not only by the proportion of receptors occupied but also by the difference between the resting membrane potential and the reversal potential of the conductance being induced (the driving force). Ginsborg (1967) derived Eqn.2B. to explain the relationship between receptor occupancy and depolarization.

$$\text{Eqn. 2B.} \quad \Delta V = (e-E) \frac{g}{G + g}$$

$e = \text{reversal potential}$
 $E = \text{membrane potential}$
 $G = \text{resting conductance}$
 $g = \text{agonist induced conductance}$

A reconstruction of Ginsborg's equation is shown in Fig.R5. The data assumes that the maximal agonist-induced conductance (g_{Max}) = 2 * G (resting membrane conductance). The K_a ($g/g_{\text{max}} = 0.5$) used to calculate the concentration-conductance curve is that estimated from the capsaicin dose-depolarization curve ($0.6\mu\text{M}$).

Depolarization is compared to the proportionate conductance increase generated from Eqn.2C. derived from a simple Langmuir adsorption isotherm.

$$\text{Eqn. 2C.} \quad \frac{g}{g_{\text{Max}}} = \frac{X}{X + K_a}$$

It can be seen from these curves that the depolarization produced for a given change in conductance is greater at lower agonist concentrations when the driving force is of greater amplitude. The depolarization curve under these conditions showing a lower ED_{50} for the agonist. Further theoretical considerations are shown in Fig.R6

where it can be seen how both the maximally achievable depolarization and ED_{50} for a dose-response curve is related to not only the agonist dissociation equilibrium constant (K_a) and therefore the number of receptors occupied but also the ratio of the maximal agonist-induced conductance to the resting conductance (g_{Max}/G).

The maximal depolarization can be defined by the following equation.

$$\text{Eqn. 2D.} \quad V_{max} = (e-E) * \frac{g_{Max}}{G + g_{Max}}$$

The effects of maximal conductance ratio (g_{Max} / G) on the recorded ED_{50} values is shown in Fig.R7. Note the left-ward shifts in the dose response curves as the conductance ratio increases. This apparent increase in affinity of the agonist is defined in Eqn.2E.

$$\text{Eqn. 2E.} \quad ED_{50} = K_a \frac{g}{G + g_{Max}}$$

(See Appendix 1. for derivation from equations 2A & 2B.)

To summarize: depolarization is a hyperbolic function of receptor occupancy; the maximal amplitude is determined by conductance ratio (g_{Max}/G) and the driving force ($e-E$). ED_{50} values of the agonist-induced depolarization are determined by the agonist-induced conductance ratio and the agonist dissociation constant (K_a).

Fig.R5.

The relationship between agonist induced increase in membrane conductance and depolarization. The ED_{50} value for conductance increase is that determined from the capsaicin data for depolarization (0.6 μ M). The curves were calculated from equations 2A and 2B by setting $g_{Max}/G = 2$.

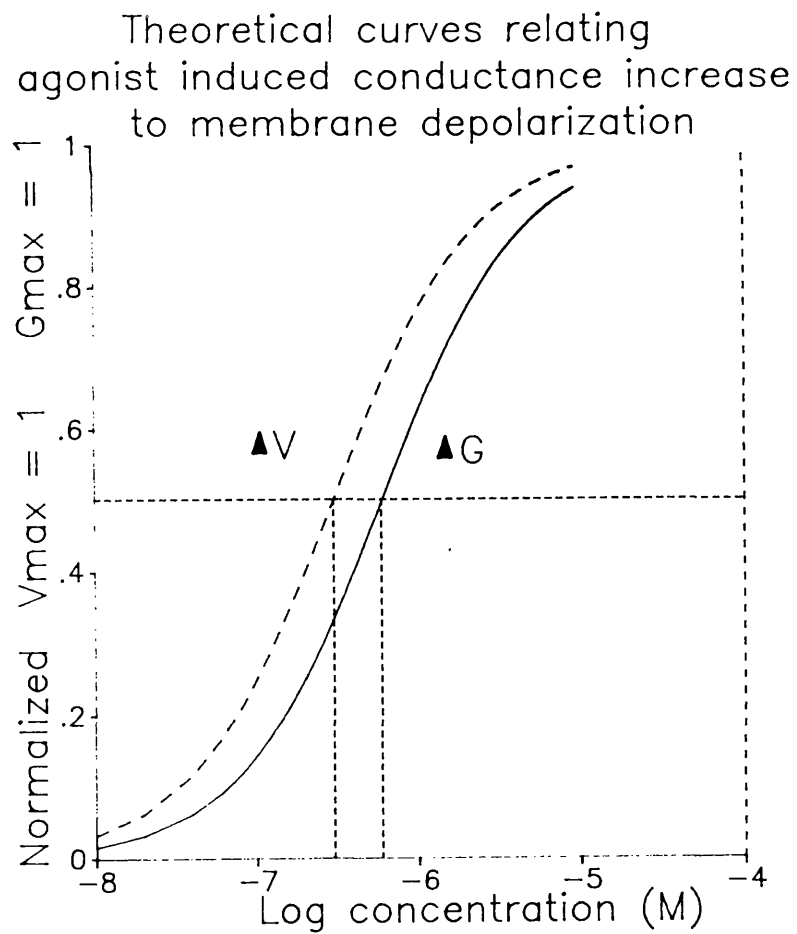


Fig.R6.

Theoretical curves generated using Eqn.2B.

Note how the maximal amplitude of the drug-induced depolarization decreases as the agonist induced conductance ratio decreases. For comparison a proposed agonist binding curve calculated from Eqn.2A, is shown by the dashed line (-----).

Maximal depolarization is achieved when the drug-induced depolarization (ΔV) = The driving force ($e-E$).

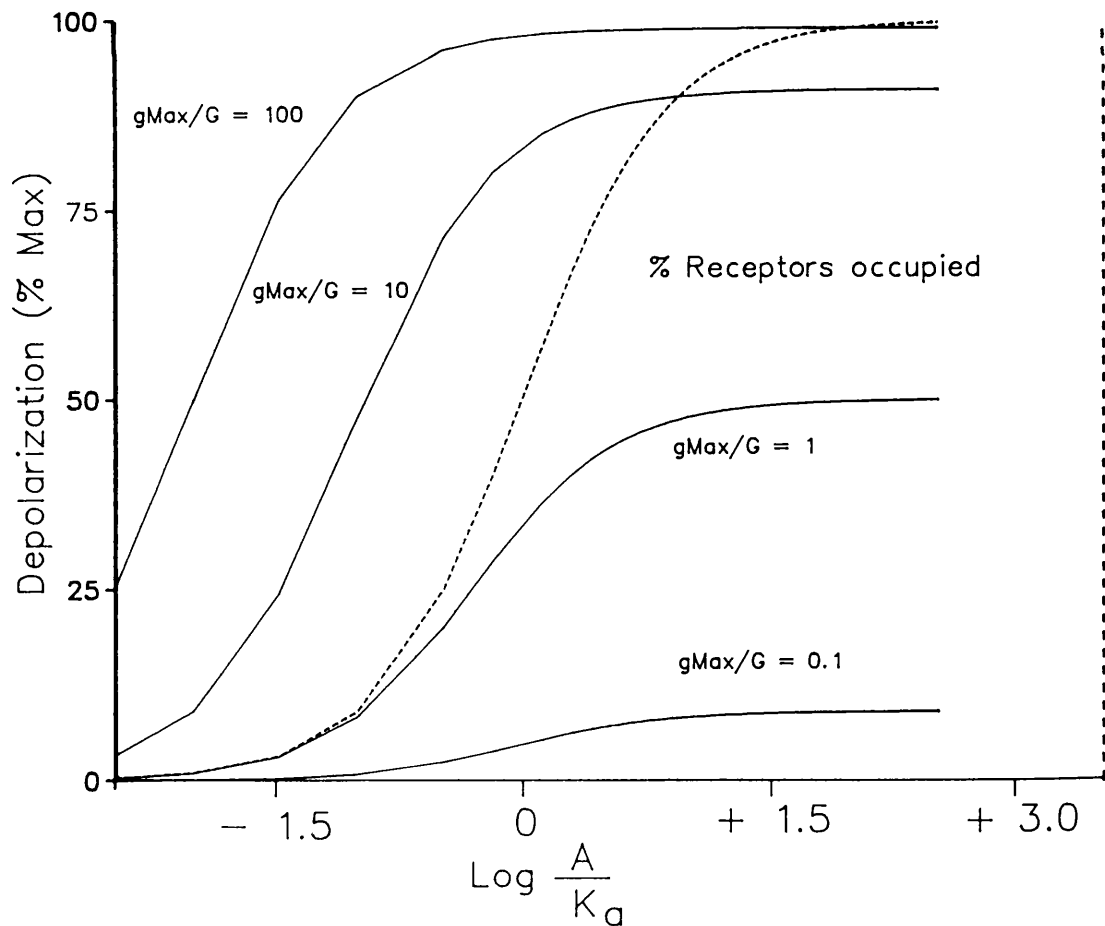
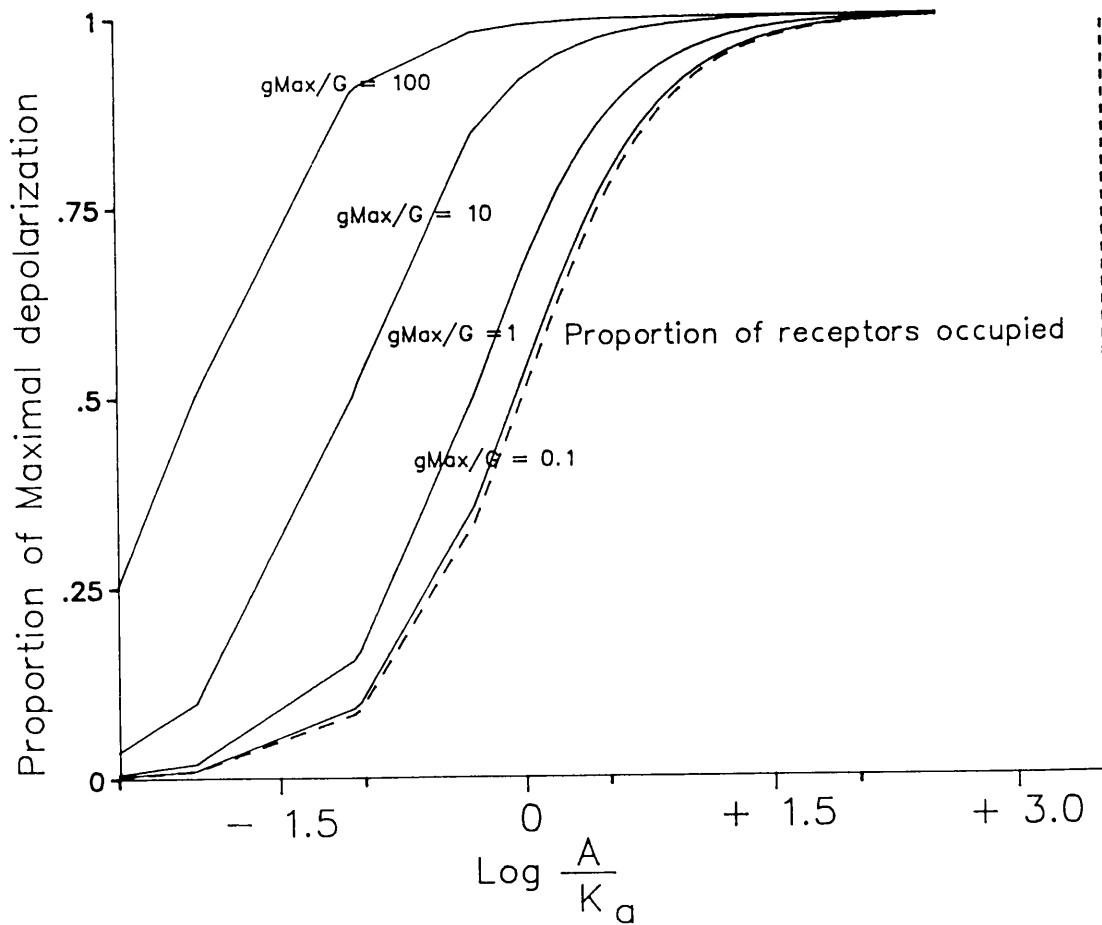


Fig.R7.

The normalization of theoretical curves shown in Fig.R6. Each curve has been plotted as a function of its own maximum, enabling a comparison of ED₅₀ values at each agonist-induced conductance ratio.



Effects of capsaicin on the compound action potentials.

On stimulating the vagus nerve a complex electroneurograph is produced. The waveform structure of the electroneurograph is determined by the strength and duration of the electrical stimulus applied (Fig.R8).

At low stimulus intensities, a fast-conducting group of fibres is excited ($A\alpha$ -spike); these are large-diameter (5 - 15 μm) myelinated axons (see Histological Results). As the stimulus strength is increased a slower component is seen ($A\delta$ -spike) which also originates from myelinated axons but of smaller diameter (1 - 5 μm) and therefore lower conduction velocity.

The vagus nerve contains an abundance of high threshold sensory and motor unmyelinated nerve fibres which require prolonged high-intensity stimulus parameters to initiate conduction (Fig.R8). These fibres have a diameter of less than $1\mu\text{m}$ and tend to conduct very slowly ($< 1 \text{ m}\cdot\text{s}^{-1}$). However, because of the large number of axons involved in this group, they produce the predominant component (C-spike) of the electroneurograph recorded from a supramaximally stimulated rat vagus nerve (Fig.R8).

The different sensitivity of myelinated fibres and unmyelinated axons to applied electrical stimulation allowed dual-pulse experiments to be conducted, where high-amplitude stimulus pulses (10 to 30 volts, 1msec duration) which induce a maximal C-spike were alternated with low-amplitude stimuli (1 to 10 volts, 0.2msec duration) sufficient to induce a maximal A-spike (Fig.R9). The A-spike under these conditions is recorded uncontaminated by the large stimulus artifact induced by the stimulating parameters required to excite the slower-conducting unmyelinated fibres.

Fig.R8.

Oscilloscope records of the effects of increasing stimulus voltage on the recorded electroneurograph.

A. Using a stimulus width of 0.2 msec a maximal $A\alpha$ -spike can be generated at 3V whilst the slower conducting $A\delta$ component requires a significantly larger voltage before a maximum is achieved.

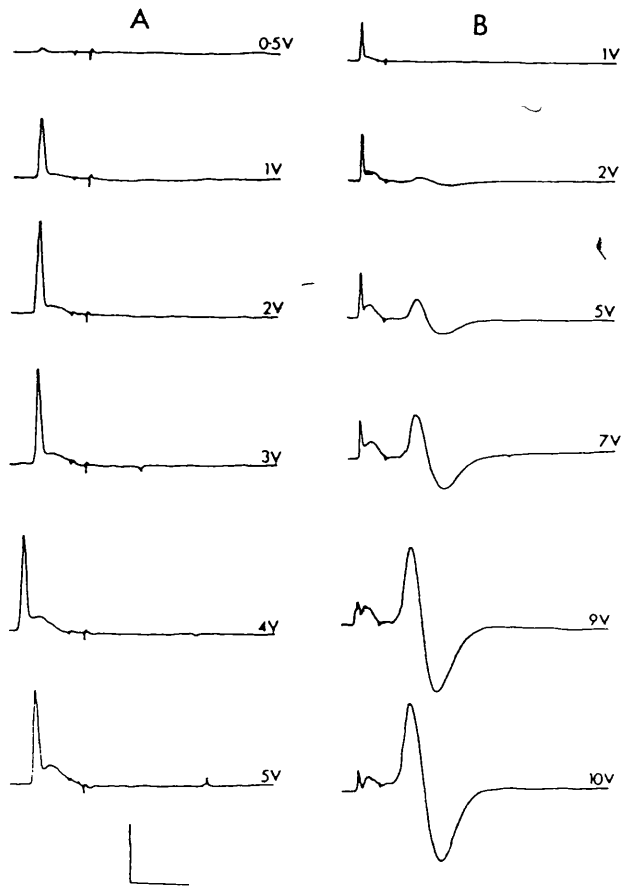
Scale: 0.5mV, 5msec

B. Using a stimulus width of 1msec, a slower component can be induced. This result^S from the excitation of the high threshold unmyelinated fibres, the C-spike. Note that the voltages required to achieve a maximal C-spike elevation results in gross distortion of the $A\alpha$ -spike component.

Scale: 1mV, 10msec.

C. Strength-duration curve for the electrical excitation of A & C-spikes in the rat vagus nerve (taken from a different experiment to that shown in A & B), showing the low excitability of the high threshold unmyelinated C-fibres.

Fig.R8.



C.

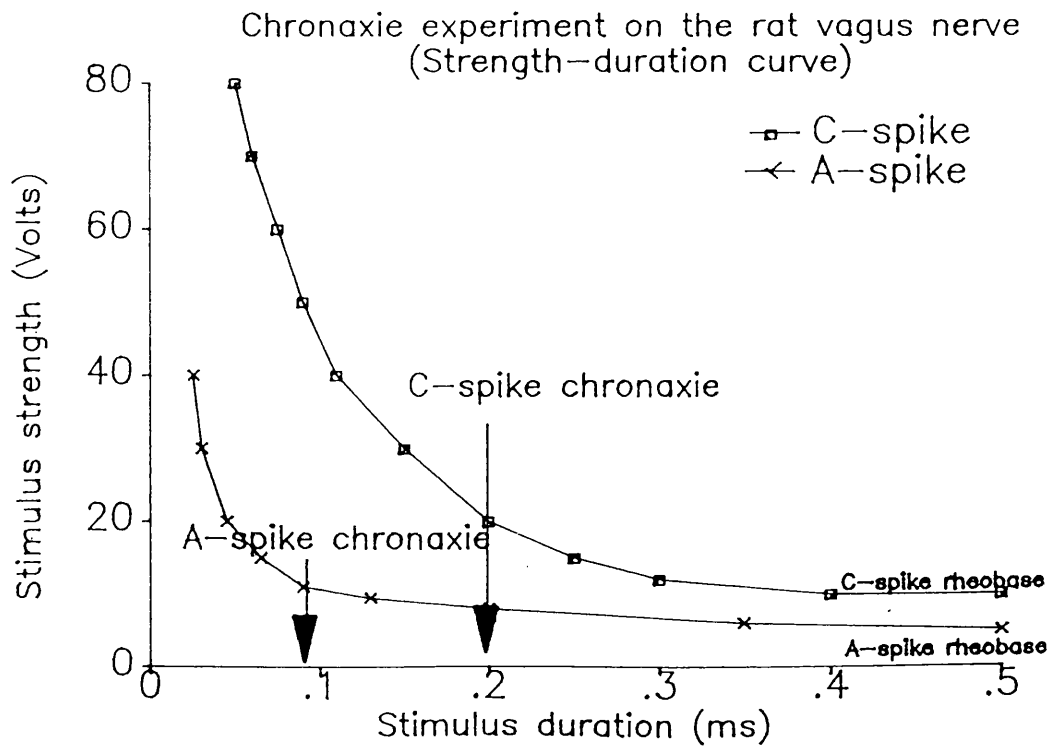
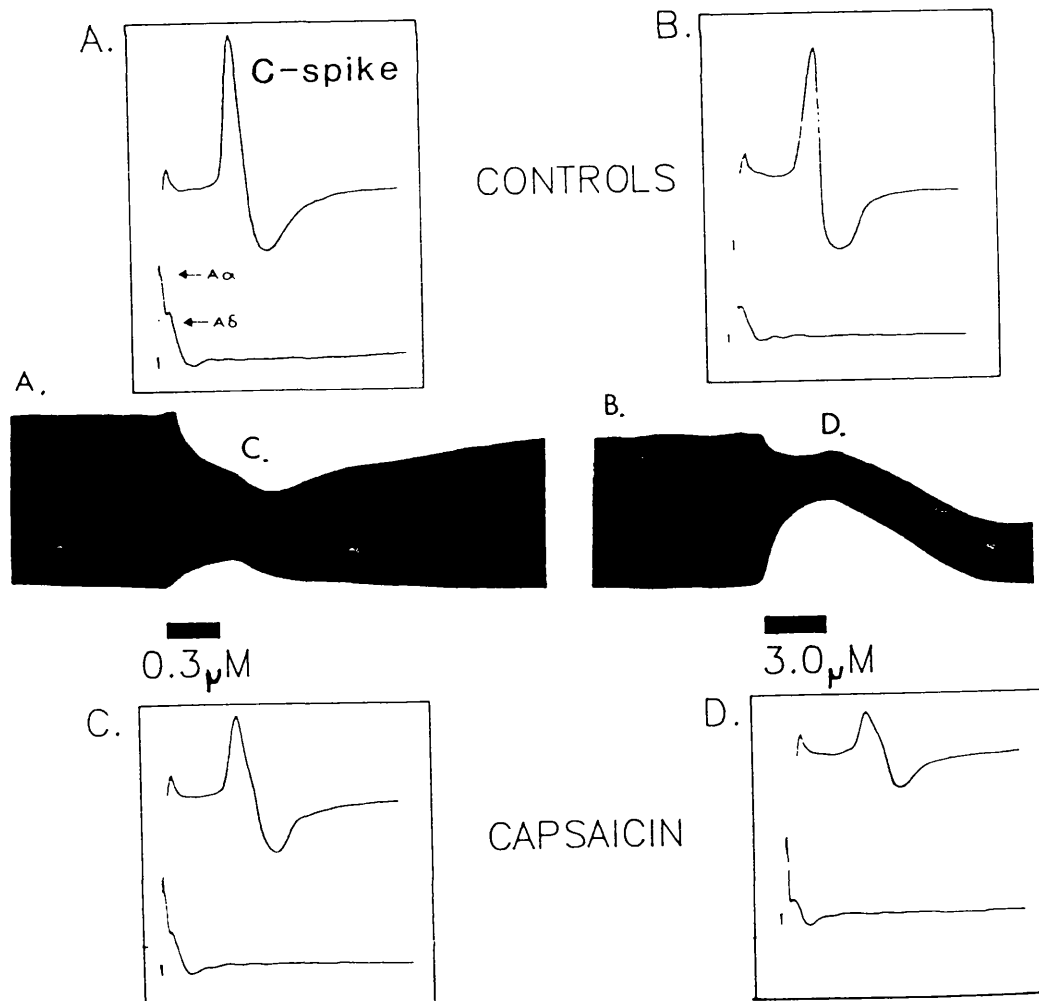


Fig.R9.

Dual-pulse experiment on the effects of capsaicin on membrane polarization and C & A-spike amplitude. At the top of the figure are shown the control oscilloscope records of the compound action potentials induced by the dual-pulse protocol (A. & B.).

Below: chart records of capsaicin-induced changes in vagus nerve polarization and C-spike amplitude (timing of oscilloscope records are indicated).

At the bottom, oscilloscope records of the compound action potentials recorded at the peak of the depolarization induced by $0.3\mu\text{M}$ (C.) and $3.0\mu\text{M}$ (D.) capsaicin.



The C-spike proved to be particularly sensitive to capsaicin. Thus, low concentrations (0.03-0.1 μ M) of capsaicin produced substantial reductions in C-spike amplitude when the surface depolarization was small, without any effect on the A-spike (Fig.R10). Concentrations > 0.1 μ M did produce some suppression of the A-spike (Fig.R12).

The C-spike conduction velocity (estimated from the conducting distance and time to peak of the C-spike) could not be accurately determined; however a temporal segregation of the C-spike into two components was noted in several experiments.

The onset time-course of C-spike amplitude reduction closely resembled that of the depolarization. When using concentrations of less than 0.3 μ M, the rate of recovery only slightly exceeded that of repolarization. Higher doses of capsaicin, however, produced a reduction in C-spike amplitude that was still evident after the preparation had apparently fully repolarized and a prolonged period of washout had taken place.

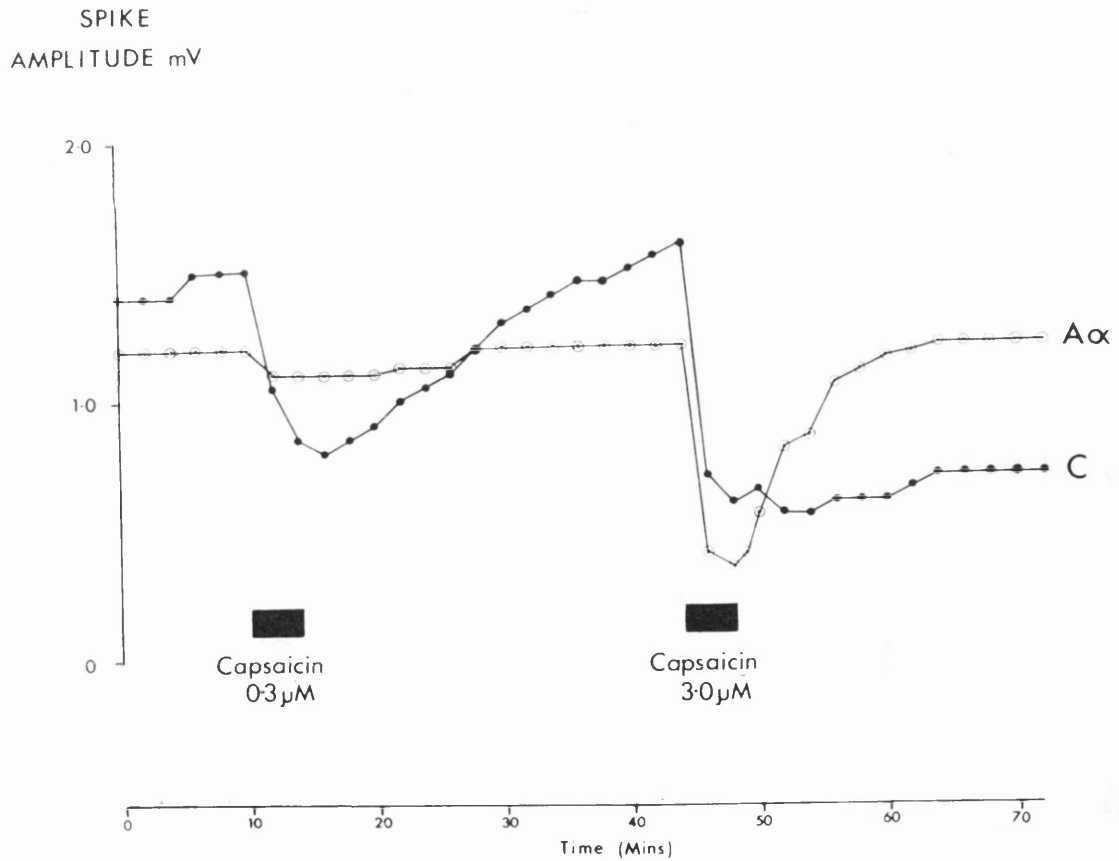
The short conducting distance (< 0.5 cm), meant that the A α and A δ components of the electroneurograph could not be clearly defined and so analysis of the effect of capsaicin on myelinated fibres was carried out only on the faster conducting A α component.

The onset time for reduction of the A-spike amplitude was similar to that for inhibition of the C-spike but the amplitude of the A-spike recovered rapidly and fully on terminating the drug application, while the C-spike was still substantially depressed (Fig.R10).

The results of these experiments into the effectiveness of capsaicin in reducing the amplitude of the A & C spike components of the evoked electroneurograph are displayed graphically in figs R11 & R12 and analysed in the following sections.

Fig.R10.

Capsaicin reduces the amplitude of both A & C-spikes. The small effect on the amplitude of the A-spike, however, rapidly recovers on the termination of the drug application.



Analysis of the capsaicin induced inhibition of the C & A-spike amplitudes.

There are many mechanisms by which capsaicin can inhibit a conducted compound action potential, several of which cannot be resolved using extracellular recording techniques. The complexities of the accumulation of intracellular ions, the voltage sensitivity of sodium channel inactivation and temporal dispersion of the conducting units, limits this analysis to virtually a qualitative study.

The effects of capsaicin on the conducted C & A spike amplitude is shown in Figs.R11 & R12. The raw data was fitted by a non-linear iterative curve fitting procedure allowing estimates of %-inhibition maxima, ED₅₀ and slope. The summary of this analysis is shown in Table 2.

Table 2.

	ED ₅₀ (μ M)	Slope	%INHIB (Max)	DEPOLARIZATION (mV)
DEPOLARIZATION OF VAGUS NERVE	0.58 (.093)	1.17 (0.15)	N/A	1.962 (.093)
INHIBITION OF C-SPIKE AMPLITUDE	0.428 (.275)	0.647 (.122)	82.14 (8.614)	N/A
INHIBITION OF A-SPIKE AMPLITUDE	1.99 (.72)	1.01 (.63)	42.04 (16.65)	N/A

(Numbers shown in brackets = S.E.M)

The slope and shape of the curve describing the inhibition of the C-spike (Fig.R11) is considerably different from that obtained from the depolarizing action of capsaicin on the whole vagus nerve (Fig.R3), so an attempt was made to fit the data for inhibition of C-spike amplitude to a two-site model, assuming two populations with differing sensitivity (Fig.R11).

The data was re-analysed and an iterative fit conducted for a double rectangular hyperbola allowing free estimates of ED_{50} and maxima. Although no clear conclusions could be drawn from the results of the analysis, a two-site fit could be obtained, suggesting a high affinity site ($ED_{50} = 0.096 \pm 0.06 \mu M$) which accounts for 44% of the inhibition of the C-spike and a lower affinity site ($ED_{50} = 2.453 \pm 3.186 \mu M$) which inhibits the C-spike amplitude by a further 37% at the maximal concentration of capsaicin ($10 \mu M$).

Fig.R11.

(A). The effects of capsaicin on the amplitude of the C-spike compound action potential. The data has been fitted by iteration to both single (solid line) and double (dashed line) sigmoidal functions.

The slope, ED₅₀ and maxima for the single sigmoidal curve is shown in Table 2. The correlation coefficient obtained for double sigmoidal analysis ($r=0.993$) was greater than that obtained for the single sigmoidal function ($r=0.963$).

(B). The residuals graph, describing the differences between the actual data and the fitted curves, suggests that the two-site fit model (filled circles) more closely resembles the experimental data.

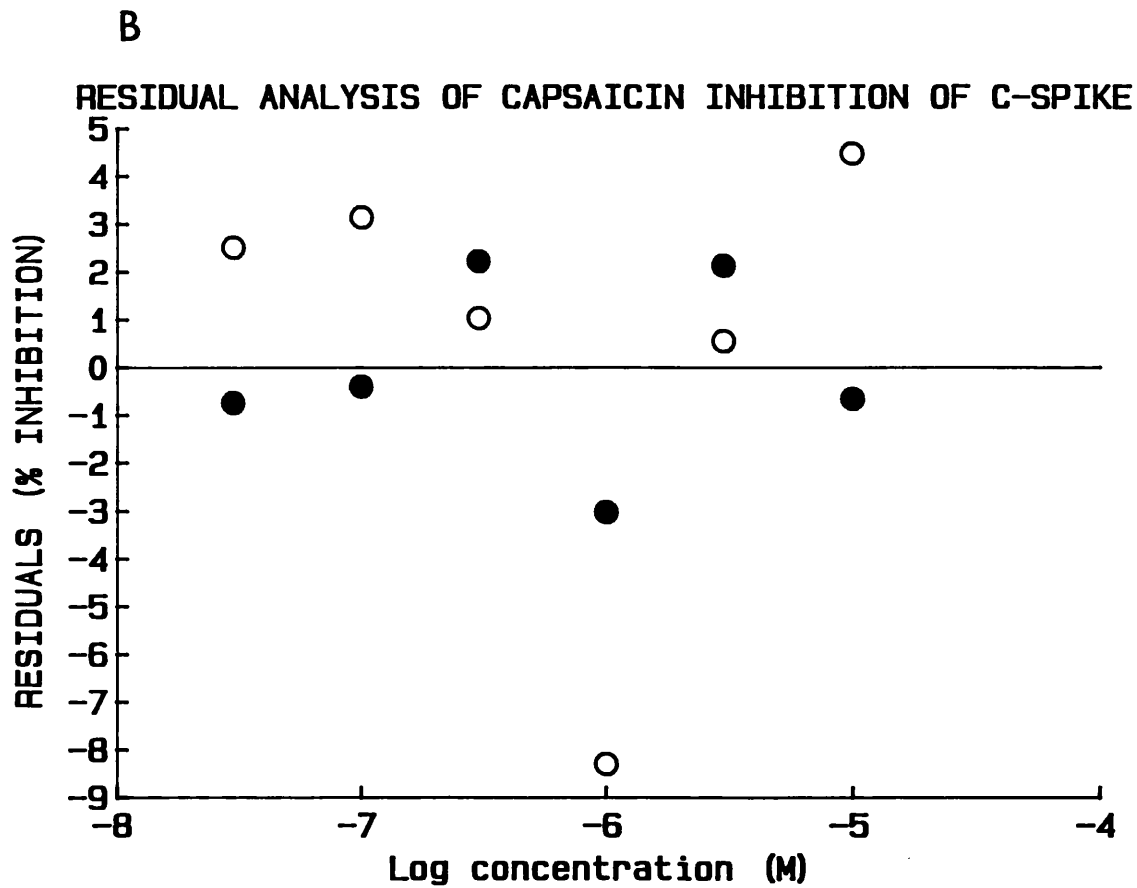
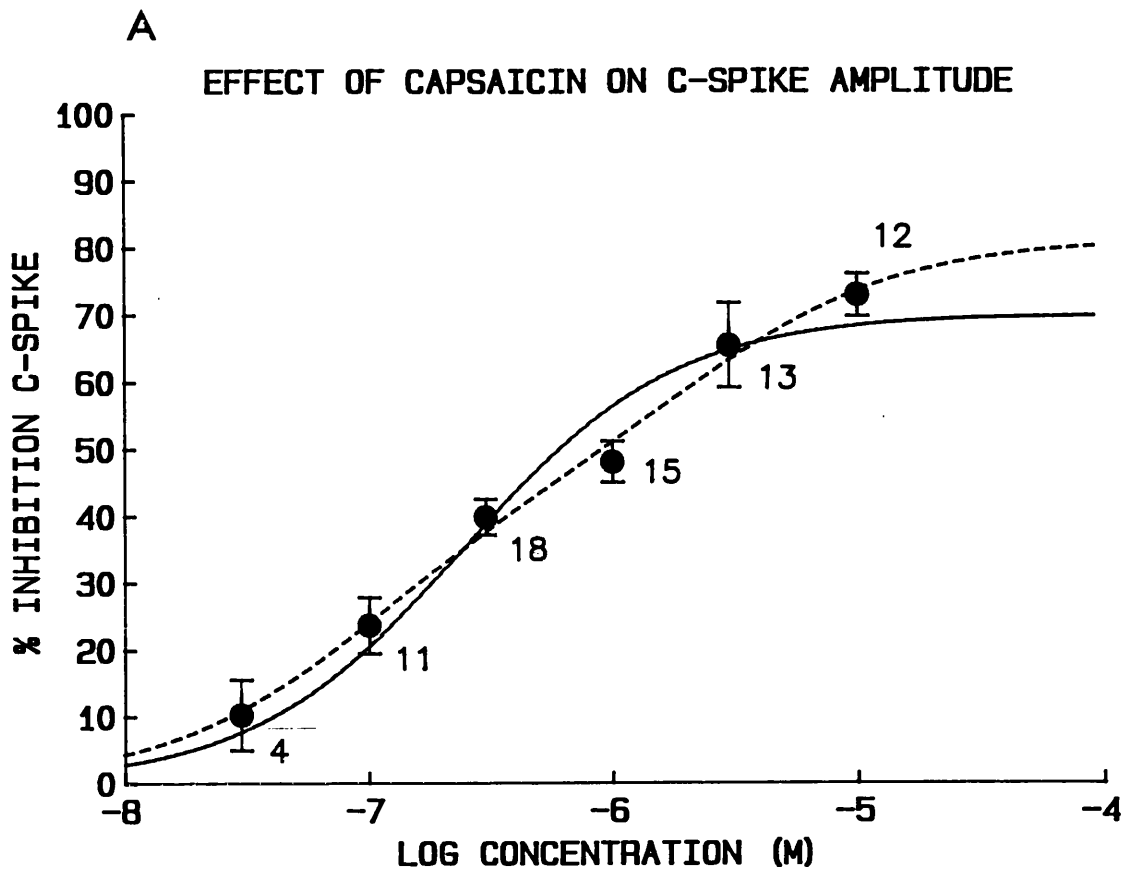
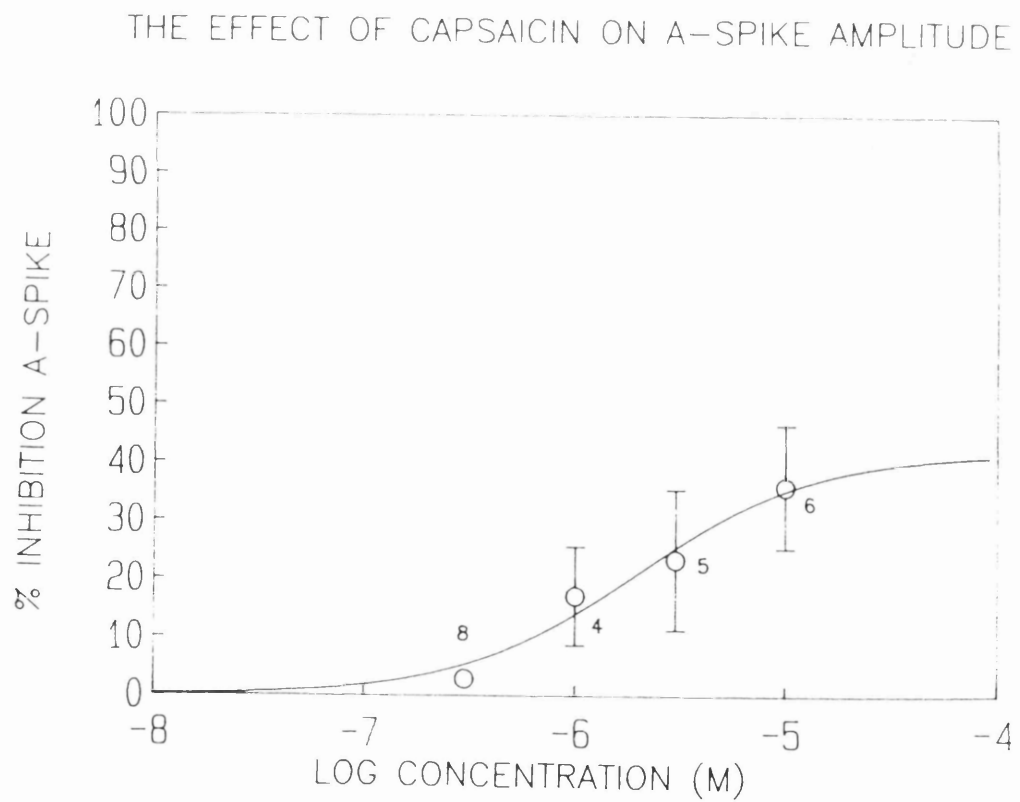


Fig.R12.

The effects of capsaicin on the compound A-spike amplitude. Results of the iterative curve fitting are shown in Table 2.



Ionic substitution experiments.

There are several mechanisms by which capsaicin can induce a reduction in the resting membrane potential; such as:

1. Increase in the membrane permeability to sodium and or calcium ions.
2. Increase in the membrane permeability to chloride ions.
3. Decrease in the resting potassium permeability.
4. Inhibition of electrogenic pump mechanisms.

The conductance mechanism by which capsaicin produces axonal depolarization was investigated by replacing the inorganic ions of the Krebs' solution with ions of lower permeability. The ionic substitution experiments conducted using extracellular recording technique are invariably difficult to interpret but the qualitative assessment undertaken has given some insight into possible ionic mechanisms involved in capsaicin induced depolarization.

Sodium substitution.

Control depolarizing responses to capsaicin ($0.3\mu\text{M}$) were obtained in normal Krebs' solution. The superfusing Krebs' medium was then replaced with a sodium-free (tris(hydroxymethyl)aminomethane or glucosamine-substituted) solution (see Table 1.). On applying sodium-free solution there was an immediate hyperpolarization and abolition of all conducted electroneurograph components. After 15 minutes, capsaicin was reapplied. The capsaicin-induced depolarization recorded in the absence of external sodium ions was substantially smaller than that induced in its presence (Fig.R13).

In 4 experiments the mean reduction in amplitude was $-62 \pm 5 \%$. The inhibitory effect of removing the sodium ions from the external medium was immediate and showed none of the time-dependence shown in experiments that will be described later on substituting chloride with the impermeant anion isethionate. Prolonged superfusion in sodium-free media often impaired the ability of the preparation to recover both the conducted action potentials and its responsiveness to capsaicin when returning to a sodium-containing solution.

The hyperpolarization produced by sodium-free solution may be a consequence of the reduction in the small but significant resting sodium conductance that contributes to the resting membrane potential in unmyelinated nerve fibres (Armett and Ritchie, 1963). The amplitude of this hyperpolarization is a function of the resting sodium/potassium permeability ratio. Hence, if capsaicin increased P_{Na}/P_K , the hyperpolarization produced by Na-free solution should increase in the presence of capsaicin. To test this a series of experiments were conducted using short (2 minute) applications of sodium free media in normal Krebs' solution and then in the presence of $1\mu\text{M}$ capsaicin (Fig.R14). Hyperpolarizations induced by sodium free solutions in the resting state could be reproducibly obtained at short intervals. The hyperpolarizations induced by sodium-free solution were potentiated when superimposed on a capsaicin-induced depolarization. Moreover the increase in the amplitude of this hyperpolarization declined concurrently with the recovery of the depolarization.

In contrast, when the preparation was depolarized with 18mM KCl, application of Na-free solution produced a further depolarization instead of a hyperpolarization (Fig.R15). This sodium-free solution induced depolarization was probably mediated by a change in the junction potential at the electrode / bath solution interface since it was still present when the vagus nerve preparation was absent. These results indicate that the increase in sodium permeability shown during the capsaicin induced depolarization does not result from a voltage dependent phenomenon, but that (in part) an increase in the sodium / potassium permeability ratio underlies the capsaicin-induced depolarization.

Fig.R13.

The effect of sodium-free (Tris) solution on the amplitude of the capsaicin-induced depolarization.

Responses to capsaicin ($0.3\mu\text{M}$) were obtained in normal Krebs' solution and then in the absence of external sodium ions (substituted with Tris). Sodium-free solution hyperpolarized the vagus nerve, inhibited the C-spike compound action potential and reduced the amplitude of the capsaicin-induced depolarization.

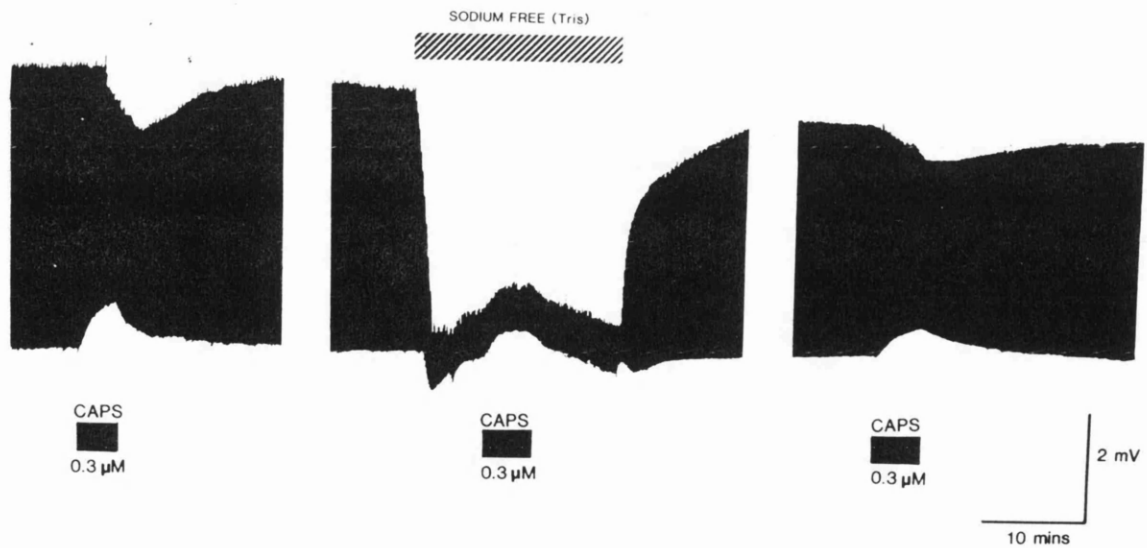


Fig.R14.

The effect of capsaicin on sodium-free-induced hyperpolarization.

Two minute applications of sodium-free (Tris) solution, applied to the rat vagus nerve induces a low amplitude hyperpolarization. The amplitude of the hyperpolarization increases when applied in the presence of $1\mu\text{M}$ capsaicin.

Note how the amplitude of the hyperpolarization decreases during the repolarizing phase of the capsaicin response.

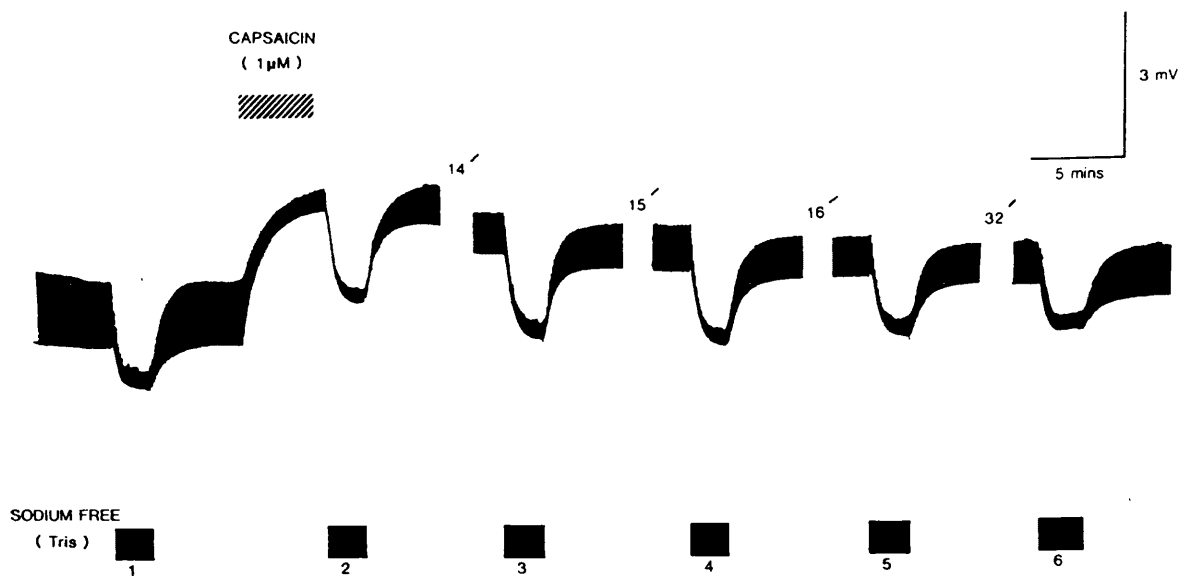
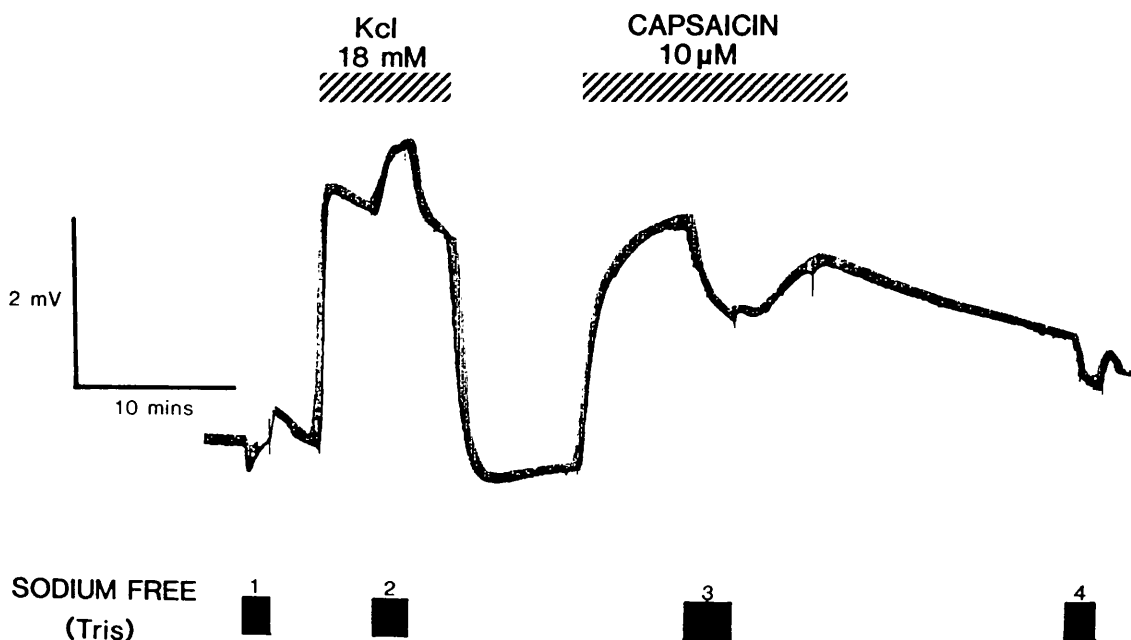


Fig.R15.

The effect of raised extracellular potassium concentration and capsaicin on the amplitude of sodium free-induced hyperpolarization.

In this example sodium free (Tris) solution when applied to the resting nerve, produced a biphasic change in membrane potential. In the presence of raised potassium, sodium-free solution produced an apparent depolarization; however this depolarization could be induced when the vagus nerve was absent from the recording chamber (see Marsh, S.J, (1990)) and therefore would appear to result from a junction potential generated at the Ag/AgCl electrode / bath solution interface.

Capsaicin-induced depolarization increased the amplitude of the hyperpolarization produced on removal of external sodium. The recorded hyperpolarization ^{leads to an} however underestimate the true effectiveness of the removal of sodium ions to reduce the amplitude of the capsaicin-induced depolarization; if one coarsely corrects by extrapolation and subtraction of the junction potential the depolarization is reduced by 69%.



Chloride substitution.

In order to examine the chloride dependence of the capsaicin-induced depolarization experiments were conducted using low-chloride, isethionate-substituted Krebs' solution. Control responses to capsaicin were obtained in normal Krebs' solution and then repeated in low-chloride media.

Superfusing the vagus nerve with low-chloride Krebs' solution produced an initial hyperpolarization which could not be readily distinguished from the large hyperpolarizing junction potential measured in the absence of the vagus nerve. Following the hyperpolarization the preparation slowly depolarized and capsaicin responses during this initial 20 minute period were potentiated ($+16 \pm 6 \%$, $n = 10$) but subsequent depolarizations (after 90 minutes, when one might expect a significant reduction in intracellular chloride concentration: Keynes and Ritchie, 1965) were attenuated below the control responses ($-49 \pm 3\%$ $n=5$; Fig.R16). The chloride-dependent depolarization induced by the Gaba_a receptor agonist, muscimol, has also been shown to be initially potentiated and then abolished over the same time course (Brown and Marsh, 1979).

This suggests, in part, an involvement of outward movement of chloride ions in the capsaicin-induced depolarization. However, in experiments when this protocol was repeated in the absence of extracellular calcium, an initial potentiation but no late suppression in low-chloride solution was evident ($+180 \pm 20 \%$ $n = 4$, after 90 minutes) suggesting that the chloride conductance increase might be secondary to an inward movement of calcium ions (Fig.R17).

Fig.R16.

The effect of reducing external chloride concentration on the amplitude of the capsaicin-induced depolarization.

Applying low chloride (isethionate-substituted) Krebs' solution resulted in a transient hyperpolarization (at arrow) and an increase in the amplitude of the C-spike compound action potential. Capsaicin ($0.3\mu\text{M}$) applied during this initial hyperpolarizing phase produced a depolarization larger than that recorded in control media. Subsequent responses to capsaicin however were attenuated.

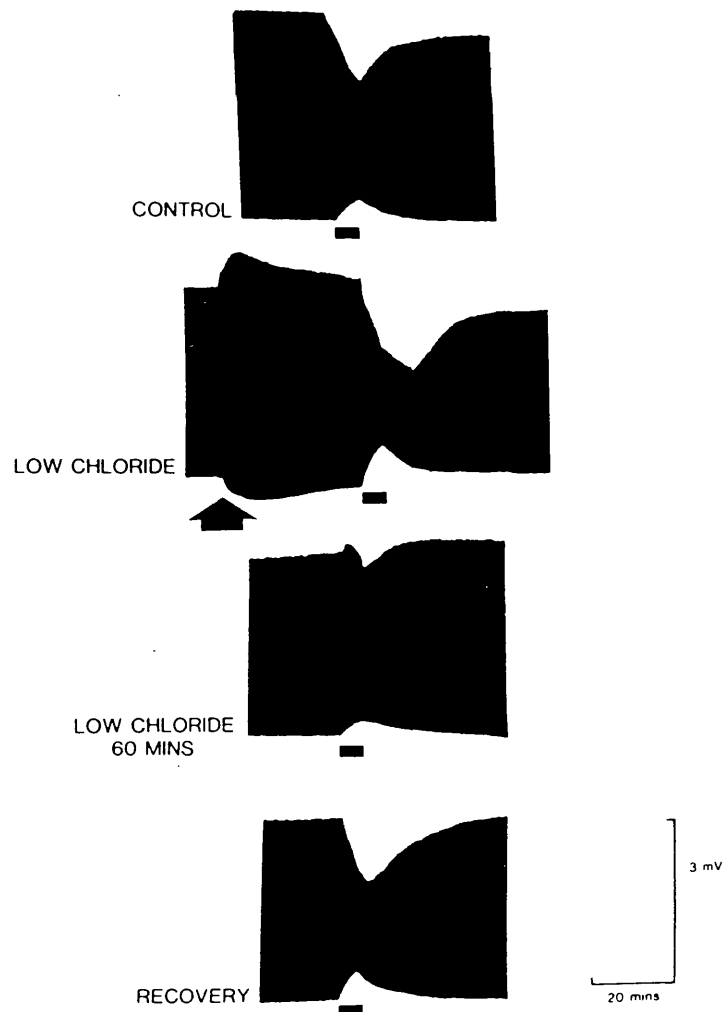
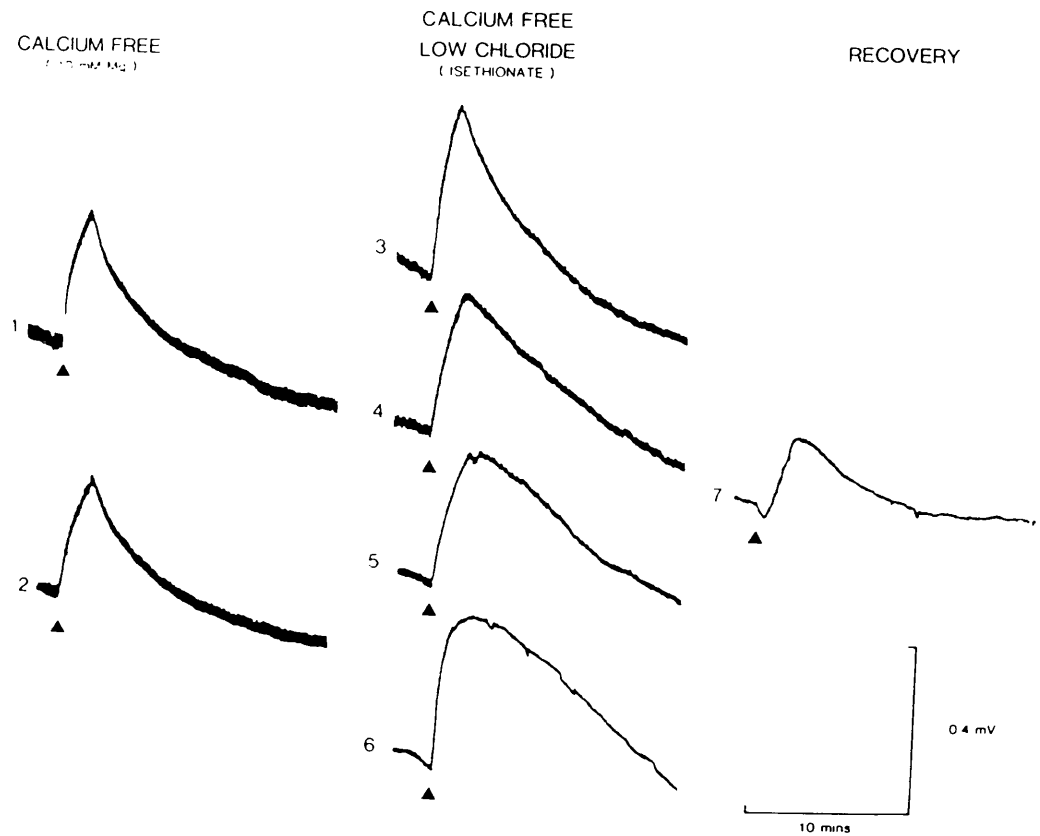


Fig.R17.

Calcium-free solution prevents the late reduction in the amplitude of the capsaicin induced depolarization seen in low-chloride Krebs'.

D.C. recording of surface polarization of the rat vagus nerve. Control responses to $0.03\mu\text{M}$ capsaicin were obtained in calcium free / 10mM magnesium Krebs' solution (1 & 2) and then repeated at 30 minute intervals in calcium-free / low-chloride krebs' solution (3 to 6). Responses to capsaicin were potentiated in the low-chloride / calcium-free Krebs' solution and this potentiation was maintained on long exposure (3 hours).



Divalent cation substitution.

Raising the external calcium concentration from 2.5mM to 10 mM did not significantly affect the amplitude of the capsaicin-induced depolarization. However removing calcium and replacing it with 1mM EGTA or 10mM magnesium chloride grossly potentiated (mean increase 180 ± 36 %, n = 7) and prolonged the depolarization and decrease in C-spike amplitude (Fig.R18).

This effect was mimicked by adding 10mM magnesium to normal Krebs' solution but not by adding cadmium ($200\mu\text{M CdCl}_2$) or cobalt (5mM CoCl_2). The addition of calcium-containing Krebs' solution during a potentiated capsaicin response resulted in a significant increase in the rate of repolarization and a prolonged hyperpolarization (Fig.R19); moreover tachyphylaxis was less evident.

Fig.R18.

Calcium-free / 10mM magnesium Krebs' solution potentiates the depolarizing action of capsaicin. Responses to capsaicin ($0.3\mu\text{M}$) recorded in normal Krebs' solution and after 20 minutes in calcium-free / 10mM magnesium Krebs' solution.

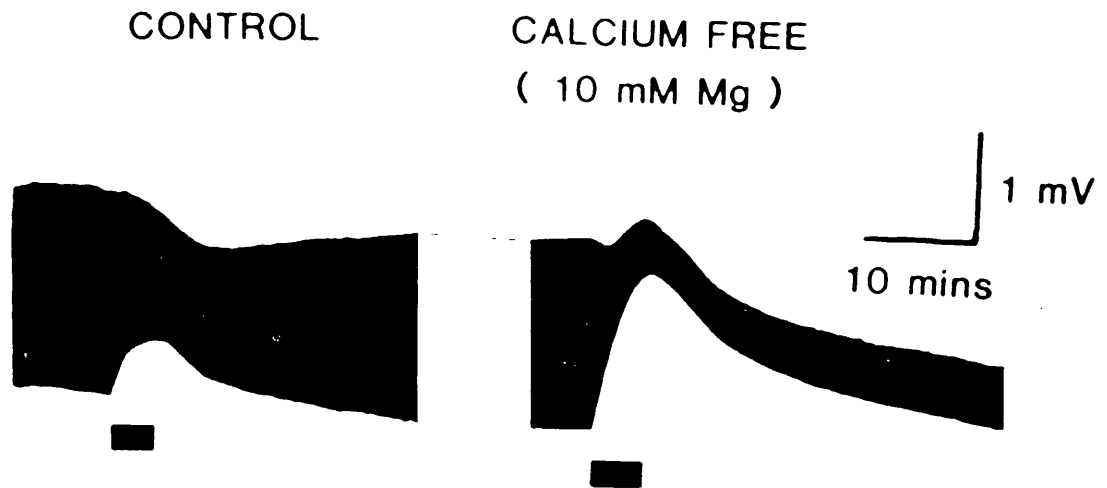
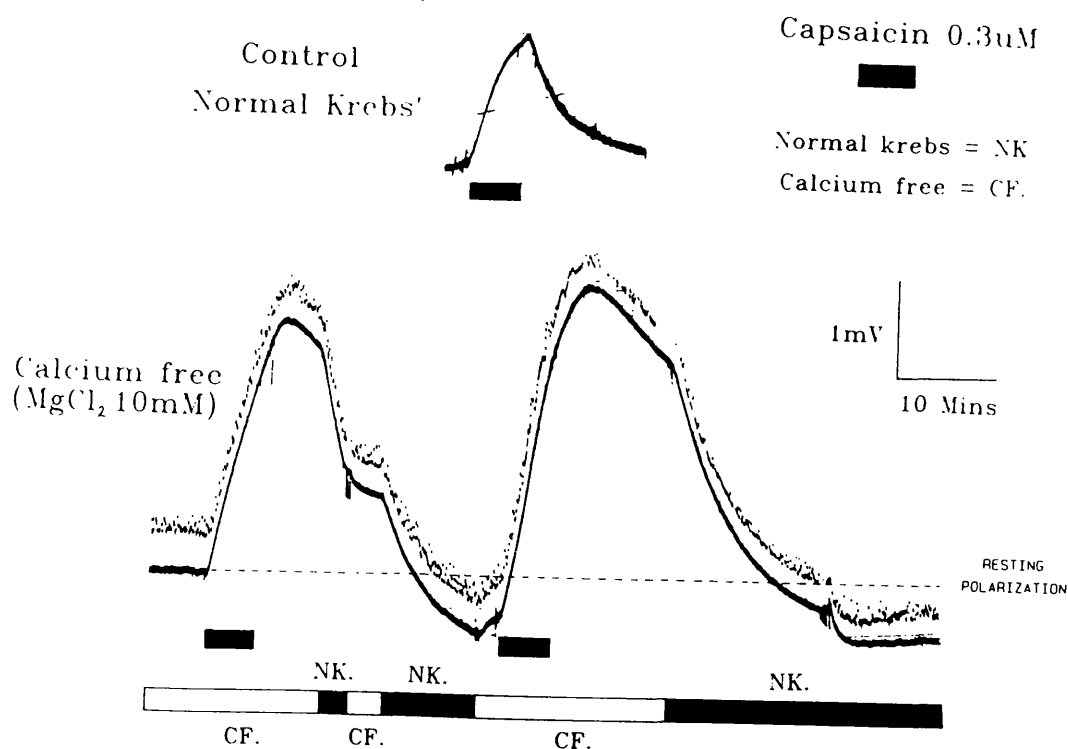


Fig.R19.

Calcium-free (10mM $MgCl_2$) solution potentiates the amplitude and duration of the capsaicin-induced depolarization of the rat vagus nerve.

Control responses to capsaicin ($0.3\mu M$) were obtained in normal Krebs' solution. The preparation was then washed for 20 minutes in calcium-free Krebs' solution (CF) before reapplying capsaicin.

The peak amplitude of the depolarization produced by capsaicin in the absence of extracellular calcium was increased two-fold. Removing capsaicin resulted in a slow repolarization, the rate of which was increased by superfusion with calcium-containing normal Krebs' solution; moreover this high rate of repolarization terminated in a hyperpolarization, an effect not previously seen.



Receptor type: a pharmacological profile.

Vagus nerve axons possess receptors for several transmitters (see introduction). The following experiments were undertaken to test whether capsaicin might act on them, either directly or indirectly by releasing transmitter.

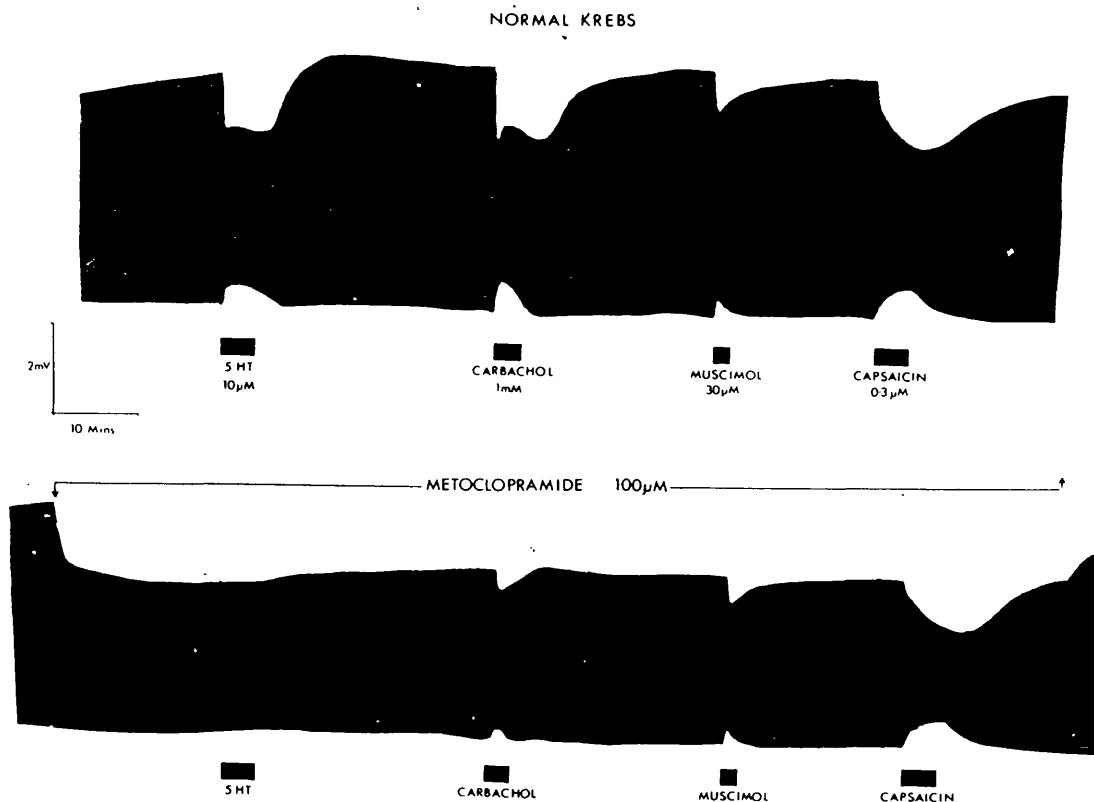
The GABA_A-receptor antagonist bicuculline (3μM, n=3), the acetylcholine nicotinic-channel blocker, hexamethonium (2mM, n=1) and the 5-HT-receptor antagonist metaclopramide (100μM, n=3) (Fig.R20) all blocked or substantially reduced the depolarization induced by the appropriate agonist without affecting the capsaicin-induced depolarization. Glutamate (1mM) and aspartate (1mM) failed to produce a significant depolarization of the vagus nerve and therefore a direct involvement of an excitatory amino acid receptor was dismissed.

Fig.R20.

The effect of metaclopramide on depolarizing responses to various axonal-receptor site agonists.

Control depolarizing responses to 5-HT, carbachol, muscimol and capsaicin were obtained in normal Krebs' solution and the same concentrations reapplied in the presence of metaclopramide ($100\mu\text{M}$).

At this concentration metaclopramide reduced the amplitude of the C-spike without effecting membrane polarization, abolished the 5-HT-induced depolarization, substantially reduced the carbachol-induced depolarization but had no effect on capsaicin and muscimol-induced responses.



Capsaicin structural analogues.

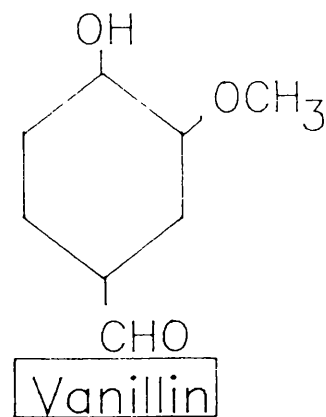
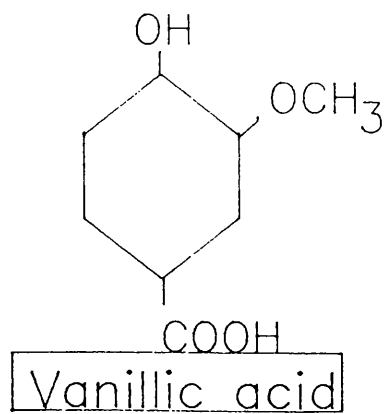
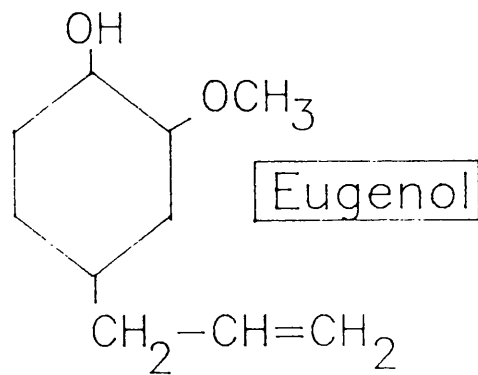
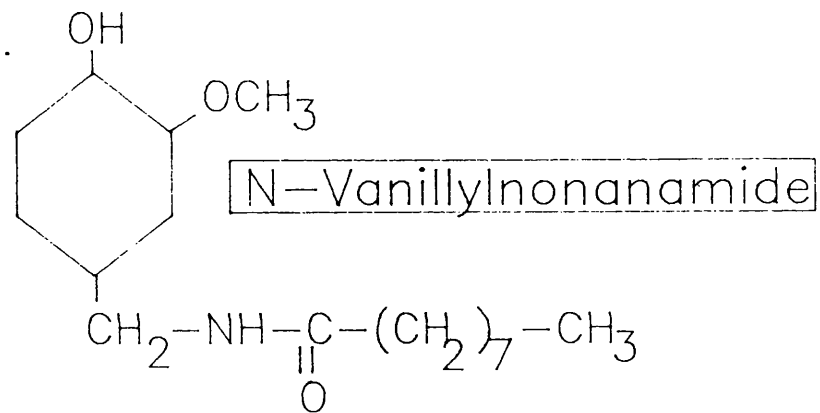
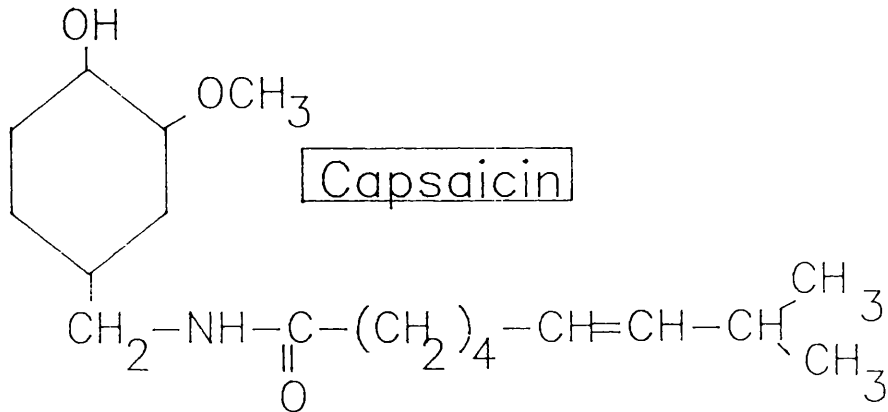
Structural analogues of capsaicin are not readily available commercially and so only a limited study could be conducted. Some analogues studied are shown in Fig.R21.

Nonanoyl vanillylamide (Pelargonic acid vanillylamide) is a capsaicin congener with the ability to mimic the irritant actions of capsaicin when instilled into the rat eye, induce the release of substance P from spinal cord dorsal horn slices (Bucsics and Lembeck, 1987) and cause a selective degeneration of B-type sensory neurones and terminals in new born rats (Jansc6 and Kir6ly, 1981).

Nonanoyl vanillylamide depolarized the rat vagus nerve but was less potent ($ED_{50} = 3\mu\text{M}$) than capsaicin ($ED_{50} = 0.6\mu\text{M}$) a potency ratio similar to that found in the experiments of Bucsics and Lembeck, (1987).

Capsaicin congener experiments previously conducted (Toh, Lee and Kiang, 1955; Bucsics and Lembeck, 1981; Jansc6 and Kir6ly, 1981) have concentrated on manipulations of the alkyl side chain, and revealed an optimum length of 8-10 carbon atoms for pain-producing potency. The aromatic ring structure and the distance linking the acylamide and vanillyl moieties would also appear to be important structural prerequisite for activity. The ability of the aromatic ring structure and derivatives thereof to produce a capsaicin like effect was tested on rat vagus nerves. However vanillin (1mM), vanillic acid (1mM) and eugenol (10mM) all failed to produce a capsaicin like effect.

The chemical structure of capsaicin and some related compounds.



The sensory-axon specific action of capsaicin.

The rat vagus nerve contains an abundance of both sensory afferent and motor efferent unmyelinated nerve fibres, indistinguishable in conduction velocity under the recording conditions used. To test for a sensory specific action, capsaicin was applied to a variety of rat nerve bundle preparations.

1. Rat preganglionic cervical sympathetic nerve.

Capsaicin in concentrations up to $10\mu\text{M}$ failed to depolarize or change the amplitude of the compound action potential (Fig.R22) of the unmyelinated efferent fibres of the cervical sympathetic nerve ($n = 4$). Axonal-receptor-site agonists such as γ -amino-butyric acid (GABA_a -receptors, Brown and Marsh, 1978), 5-hydroxytryptamine (5-HT-receptors, Wallis, 1981) and carbachol (nicotinic-receptors, Arnett and Ritchie, 1960) all depolarize the cervical sympathetic nerve bundle (complete data not shown) and reduced the compound action potential amplitude (see Fig.R22 showing GABA-induced inhibition of C-spike amplitude).

2. Rat sciatic nerve.

The rat sciatic nerve contains both afferent and efferent unmyelinated fibres and has been shown to be depolarized by capsaicin (Hayes et al, 1984). Experiments were conducted to confirm the above result and monitor the effects on the electroneurograph. Capsaicin depolarized the rat sciatic nerve ($n=4$) and reduced the amplitude of the slow C2 component of the electroneurograph (Fig.R23). The amplitude of the maximal depolarization produced by $10\mu\text{M}$ capsaicin ($0.23 \pm .08\text{ mV}$, $n=4$) was far less than that induced on the vagus nerve ($1.9 \pm 0.3\text{ mV}$, $n=10$) but capsaicin inhibited the C2 component ($35 \pm 9\%$, $n=3$) with minimal effects on the C1 component ($5 \pm 6\%$, $n=3$).

No detectable effect was noted on fast conducting A-spike.

3. Rat optic nerve.

The rat optic nerve bundle consists mostly of large diameter sensory myelinated axons and a very small number of unmyelinated fibres. These are thought to be either sympathetic in origin, innervating blood vessel smooth muscle of the eye (Hughes, 1977) or might represent tissue sections taken at the point of the node of ranvier (Forrester and Peters, 1967).

Capsaicin applied at 1 μM to three optic nerve preparations failed to induce a detectable depolarization. However when the capsaicin concentration was raised to 10 μM , two of the optic nerves responded with a small depolarization (0.04mV). Glycine 2mM applied to the same preparations depolarized by 0.15mV. In these experiments no appreciable C-spike component was present and no effect was seen on the fast-conducting sensory A-spike.

4. Rat dorsal and ventral root nerves.

Myelinated and unmyelinated primary afferent fibres projecting to the spinal cord enter via the dorsal roots before ramifying into selective areas of innervation eg. unmyelinated fibres preferentially terminate within laminae 1 and 2 (substantia gelatinosa). Efferent fibres leave the spinal cord by way of the ventral roots, a nerve bundle that consists mainly of myelinated fibres but also a small number of sympathetic unmyelinated axons.

Capsaicin depolarized the dorsal root fibres in a dose-dependent manner (threshold concentration 0.1 μM) reducing the amplitude of the C-spike ($n = 3$). Applied to two ventral root preparations, capsaicin (10 μM) failed to produce any significant depolarization. This confirms the data of Ault and Evans (1980).

Fig.R22.

A comparison of the effects of GABA (γ -aminobutyric acid) and capsaicin on the C-spike amplitudes in rat preganglionic sympathetic and vagus nerves.

1. AC-coupled response showing ability of GABA ($100 \mu\text{M}$) but not capsaicin (1 and $10 \mu\text{M}$) to depolarize and inhibit the C-spike amplitude. Changes in membrane potential were monitored on a separate trace but not shown.
2. The effects of GABA ($100 \mu\text{M}$) and capsaicin (1 and $10 \mu\text{M}$) on the amplitude of the C-spike in rat vagus nerve.

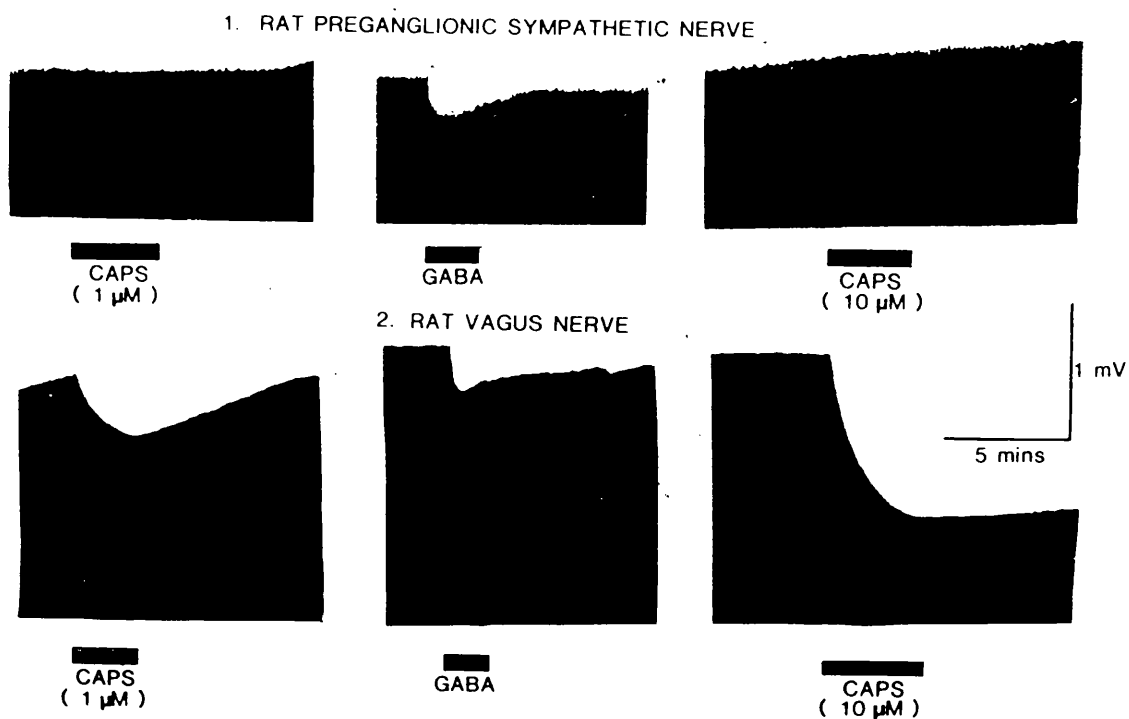


Fig.R23.

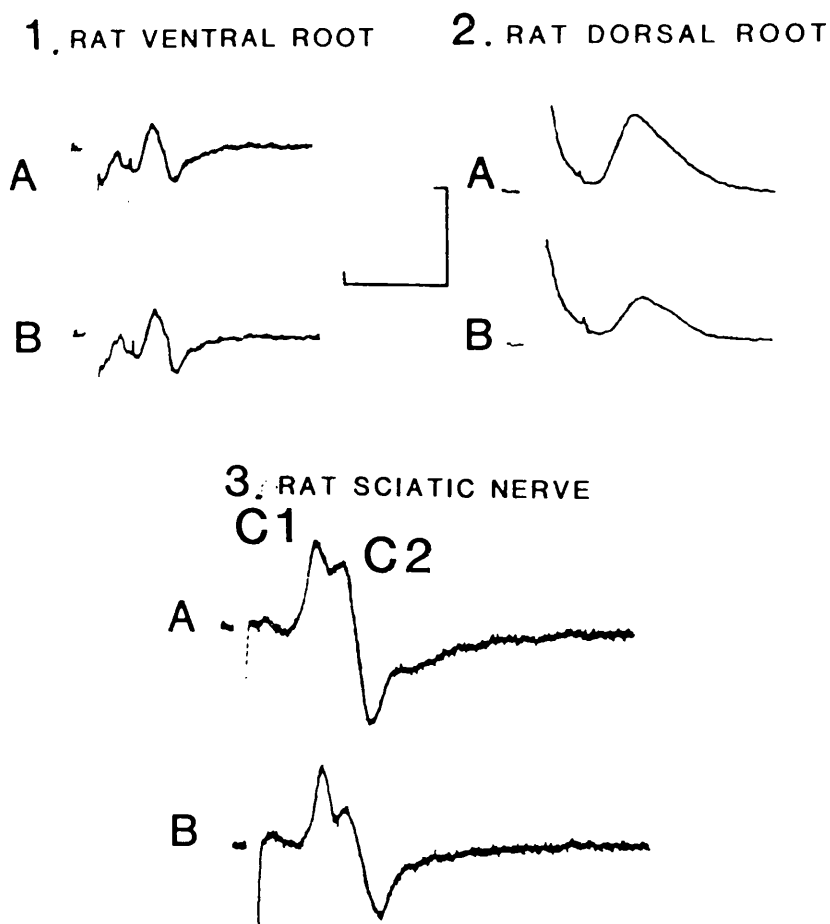
Capsaicin depolarizes dorsal root and sciatic nerve bundles and induces concurrent reductions in the C-spike amplitude.

Below are oscilloscope records of electrically evoked C-spikes for rat ventral root, dorsal root and sciatic nerve.

Capsaicin ($1\mu\text{M}$) depolarized (data not shown) and reduced the amplitude of the C-spike in sciatic nerve and dorsal roots but was ineffective in ventral roots.

The C-spike recorded from sciatic nerve consisted of two components (C1 and C2), the slower of which appeared to be more sensitive to capsaicin.

Scale: 20msec / 0.2mV



Extracellular results discussion.

The *in vitro* extracellular recording experiments described within this thesis have confirmed the ability of capsaicin to depolarize whole nerve bundles (Ault and Evans, 1980; Hayes and Tyers, 1984). However the technique of simultaneously monitoring the evoked electroneurograph, utilized in the present study, suggests that the capsaicin-induced depolarization of the vagus nerve trunk might preferentially emanate from the unmyelinated C-fibres within.

In agreement with this inference, the ability of capsaicin to depolarize whole nerve bundles was restricted to preparations that are known to contain sensory C-fibres e.g, vagus, dorsal root and sciatic nerves, whilst those bundles that have a motor function (ventral roots and preganglionic sympathetic) were capsaicin-insensitive.

The myelinated fibres of the vagus nerve had a low sensitivity to capsaicin ($ED_{50} = > 2\mu M$) but surprisingly, the sensory myelinated fibres of the optic nerve were capsaicin-insensitive. This raises the question of whether the action of capsaicin on the myelinated fibres of the vagus might be a consequence of C-fibre activation. The complex capsaicin-induced conductance process, proposed later in this thesis, envisages a secondary increase in membrane potassium conductance resulting from membrane depolarization and calcium entry. It is suggested that this process might result in the accumulation of extracellular potassium ions and subsequent depolarization of the adjacent capsaicin-insensitive unmyelinated and myelinated fibres.

Analysis of the dose-depolarization and compound action potential dose-inhibition curves has shown that, using extracellular recording techniques, it is only possible to state a functional potency for capsaicin (ED_{50}) not the affinity constant of capsaicin for its receptor site. Within this limitation, effective ED_{50} values for depolarization ($ED_{50} = 0.58\mu M$) and C-spike depression (Site 1, $ED_{50} = 0.095\mu M$) suggests that, within the vagus nerve, high capsaicin sensitivity might be restricted to a sub-population of C-fibres.

The apparent lower affinity site ($ED_{50} = 2.45\mu M$) for the inhibitory effects of

capsaicin on C-spike amplitude, suggested by the two-site fit, shows a similarity to the ED_{50} for reducing the A-spike ($ED_{50} = 1.99 \mu M$) and might therefore represent a non-selective action of capsaicin, perhaps a secondary consequence of capsaicin-sensitive C-fibre activation.

Ionic substitution experiments.

The mechanism by which capsaicin reduces the amplitude of the compound action potential, cannot be resolved using the extracellular recording technique. The accumulation of intracellular and extracellular ions, voltage sensitivity of sodium channel inactivation, temporal dispersion of the conducting units and the capsaicin-induced changes in membrane potential and membrane conductance might all contribute to the observed effect. Nevertheless, some interesting inferences can be extracted from the effects of ion substitution experiments in a qualitative way.

In the present experiments three principal effects were seen. Firstly, the depolarizing response was reduced by removing external sodium ions. Secondly, the depolarization was increased by removing external calcium ions. Thirdly, the response was attenuated on reducing external chloride ions. These three effects could be accommodated by the following sequence of events.

- (a). The primary action of capsaicin is to increase Na and Ca conductance.
- (b). The influx of calcium secondarily activates a Ca-dependent potassium conductance, so attenuating the depolarization.
- (c). The influx of calcium can also activate a calcium-dependent chloride conductance which, in these fibres, might add to the depolarization.

The line of reasoning leading to this interpretation is explained further below.

1. Sodium dependence.

The small diameter of the unmyelinated axons ($< 1 \mu m$) of the vagus nerve does not allow the direct measurement of the axonal membrane potential by conventional intracellular recording techniques.

The partition method used in the present study, grossly underestimates drug induced changes in membrane potential, as the surface recording electrodes are partially 'shorted' by the low resistance extracellular pathway between the electrodes. This short circuit factor (S) estimated to be approximately 0.1 means that less than 10 % of the actual change in membrane potential is recorded (Marsh, 1990). By using the sucrose gap method, which utilizes a deionised sucrose solution to increase the resistance of the extracellular pathway, it is theoretically possible to achieve a short circuit factor close to unity and therefore record, extracellularly, the changes in membrane potential one might get from conventional intracellular recording methods (Stampfli, 1954). This technique was used by Armett and Ritchie (1963) to investigate the role of potassium and sodium ions on the resting membrane potential of unmyelinated nerve fibres. They found that the relationship between the external potassium concentration and membrane potential, did not follow what one might have predicted from a single cation Nernstian dependency. Using the equations described by Hodgkin and Horowicz (1959)*, they proposed that the resting membrane potential (ca. -60mV), although predominantly dependent on the potassium distribution, was subject to a large resting sodium conductance. They estimated that, at rest, the potassium permeability was only four times that for sodium ($P_{Na}/P_K = 0.25$), a permeability ratio far greater than that measured for squid axon ($P_{Na}/P_K = 0.04$, Hodgkin and Katz, 1949) or muscle fibres ($P_{Na}/P_K = 0.01$, Hodgkin and Horowicz, 1959).

* Hodgkin and Horowicz (1959):

$$E = - \frac{RT}{F} \ln \frac{[K]_i + \alpha [Na]_i}{[K]_o + \alpha [Na]_o}$$

E = Membrane potential
 R = Gas constant
 T = Absolute temp
 F = Faraday's constant
 α = Permeability ratio (P_{Na}/P_K)
 $[]_o$ = External concentration
 $[]_i$ = Internal concentration

The results obtained by Armett and Ritchie (1963), explain the pronounced hyperpolarizing effect of sodium-free Krebs' solution on the resting potential of rat vagus nerve seen in the present study. Depolarization of excitable membranes invariably leads to an increased potassium conductance and therefore a decrease in the steady-state permeability ratio (P_{Na}/P_K). One would predict therefore that sodium-free induced hyperpolarization would be reduced at depolarized potentials, as is seen when sodium-free solution is applied in the presence of raised potassium solution. The enhancement of the sodium-free induced hyperpolarization during the capsaicin induced depolarization, can therefore best be explained by a capsaicin-induced increase in sodium conductance.

2. Calcium dependence.

The mechanism by which calcium-free Krebs' solution enhanced the capsaicin-induced depolarization could not be resolved using the extracellular recording technique. This effect could be mediated via several mechanisms: (a) an increase in receptor affinity; (b) increased capsaicin induced unitary conductance; (c) decreased calcium-dependent potassium conductance.

a). Receptor affinity.

In the present study, full dose / depolarization curves were not constructed in calcium-free Krebs' solution so changes in affinity could not be evaluated. The low affinity of capsaicin ($> 100nM$) negates its use in binding experiments. However, a high affinity capsaicin receptor agonist (resiniferatoxin) has recently been described, which is three orders of magnitude more potent than capsaicin (Szallasi and Blumberg, 1989). It is suggested that, with this probe, the calcium dependence of capsaicin receptor binding might be best resolved in future experiments.

b). Conductance.

Experiments using membrane patches from dorsal root ganglion sensory neurones, have described capsaicin activated single channels that show a reduced conductance in the

presence of calcium ions (Forbes and Bevan, 1988), which would result in an increase in the amplitude of the whole cell current induced by capsaicin and the calcium-free enhancement of capsaicin-induced depolarization shown in the present study.

c). Calcium-dependent potassium-conductances.

Following a period of repetitive firing, unmyelinated axons often produce a pronounced after-hyperpolarization (AHP), which can last several minutes. There are several phases to this response, the slowest of which is mediated by an electrogenic sodium pump (Rang and Ritchie, 1968). A fast (50 - 500 ms), calcium-dependent component to the AHP has recently been described (Jirounek et al., 1989), which is mediated by a calcium activated potassium conductance.

Calcium-activated potassium currents are a wide-spread phenomenon that can play a major role in spike repolarization and neuronal excitability (Meech, 1978; MacDermott, 1982; Pennefather et al., 1985; Storm, 1987). One might therefore expect that any calcium entering the cell via the capsaicin conductance mechanism would activate these channels and induce partial repolarization. The implication is that all of the capsaicin-induced depolarizations in normal Krebs' solution are, to some degree, attenuated by a calcium-activated hyperpolarization. Thus the potentiation seen in calcium-free Krebs' might represent a diminution of this repolarizing influence. The failure of voltage-sensitive calcium-channel blockers, such as cadmium, to potentiate the capsaicin-induced depolarization, suggests that the calcium entry is primarily through the capsaicin conductance mechanism, rather than through Ca-channels.

3. Chloride dependence.

The results obtained using isethionate Krebs' are also suggestive of secondary action of capsaicin following channel activation and calcium entry, in that the reduction in capsaicin-induced depolarization, seen on long-term incubation in chloride-free solution, did not occur in the absence of external calcium ions. Calcium-activated chloride conductances have been described in other neuronal tissues (Owen et al., 1984; Mayer, 1985) but a role in unmyelinated fibres has yet to be established.

CHAPTER 4

INTRACELLULAR RECORDING RESULTS.

The identification of neuronal cell types
of the rat nodose ganglion.

Neurons of the rat nodose ganglion were identified as A or C-type neurons by their conduction velocity (C.V.), determined from the conducting distance (C.D.) and by the latency to spike invasion of the neuronal soma following electrical stimulation of the attached vagus nerve. An example of an experiment is shown in Fig.R24, where 31 sensory neurons were impaled and conduction velocities for each neuron determined. The majority of neurons identified (>90%) by these means are clearly C-type and conduct at less than 1 m.s^{-1} .

55 randomly selected C-type neurons had a mean orthodromic conduction velocity of $0.77 \pm 0.02 \text{ m.s}^{-1}$ at 30° C (Fig.26). The conduction velocity distribution of 43 A-type neurons is displayed in Fig.R26. The mean conduction velocity of this group was $13.44 \pm 1.43 \text{ m.s}^{-1}$ (range 3-50 m.s^{-1}). This analysis assumes a single group of data normally distributed about a mean and no attempt was made at fitting the data to a multiple distribution due to the small number of measurements available at higher conduction velocities.

In the experiment shown in Fig.R24, two A-type neurons are identified and sub-categorised as A(δ) (3 m.s^{-1}) and A(α) (15 m.s^{-1}).

Sub-categorisation of A-type cells was in accordance with that suggested by Gasser and Grundfest (1939) (see Introduction) for somatic afferents where myelinated A-fibre conduction velocities within the range 3-15 m.s^{-1} are described as A(δ) and those that conduct at 15 m.s^{-1} or greater as A(α).

Cell type was preferentially identified by orthodromic action potential conduction velocity. However there are several other electrophysiological characteristics such as spike duration and overshoot, by which one is able to distinguish between the two primary cell types. The difference in electrophysiological characteristics are summarized in Table 3 and displayed in Fig.R25.

Fig.R24.

The identification of neuronal subtypes using conduction velocity measurements.

Upper diagram shows an impalement map: the positions of the cells from which the estimates of conduction velocity were taken are labelled. The lower traces are oscilloscope records of somatic action potentials from 3 of the 31 neurons impaled in this single experiment and shows examples of the three main groups described in the text.

Scale: 40mV/10ms

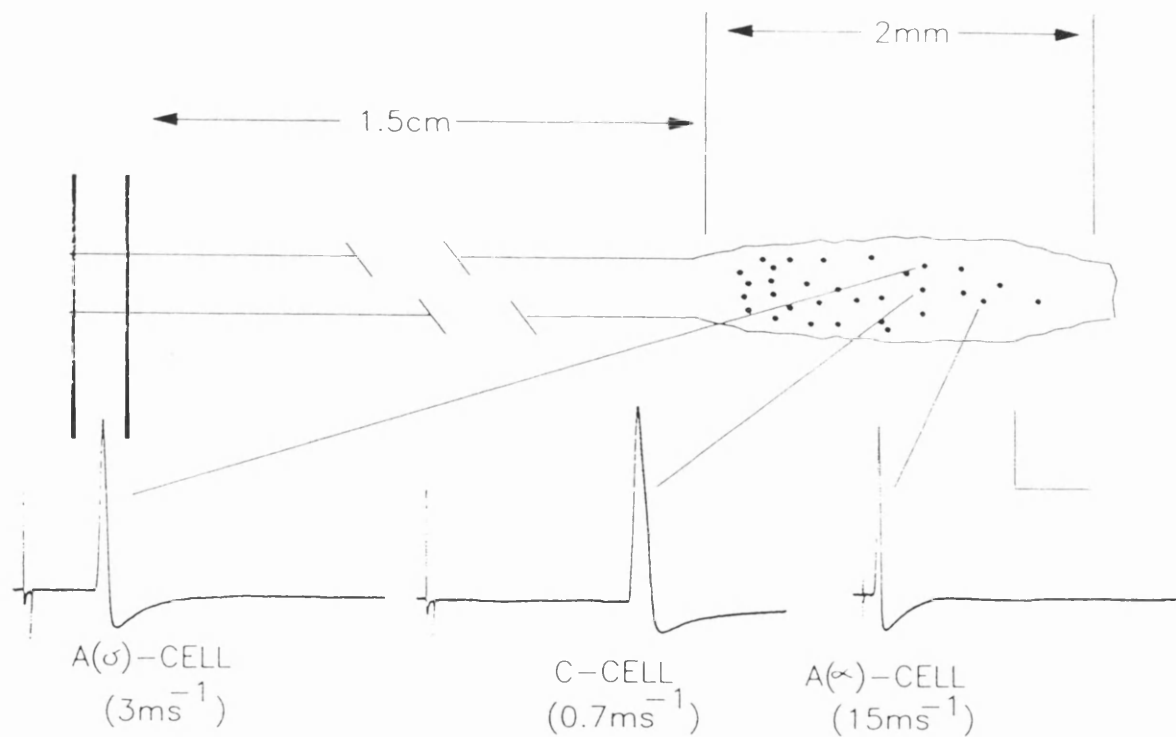


Fig.R25.

Intracellular current clamp recordings from rat nodose A-type and C-type neurons.

Shown are oscilloscope records of membrane voltage responses to constant +ve and -ve current injection.

The upper trace, recorded from an A-cell ($C.V. = 5 \text{ m.sec}^{-1}$), shows the short duration action potential, small after- hyperpolarization and pronounced membrane rectification, indicative of nodose A-cells.

The lower trace shows a typical sensory C-cell ($C.V. 0.8 \text{ m.sec}^{-1}$), which contribute 95% of the neuronal population of adult nodose ganglia.

The action potential overshoot is greater (dotted line = 0 mV) and the spike duration longer than that found in A-cells.

C-cell action potentials are normally followed by prolonged after-hyperpolarizations which can last 20-50 msec.

Current injections;

upper trace $\pm 0.4 \text{ nA}$, 50 msec

lower trace $\pm 0.2 \text{ nA}$, 50 msec

Fig.R25.

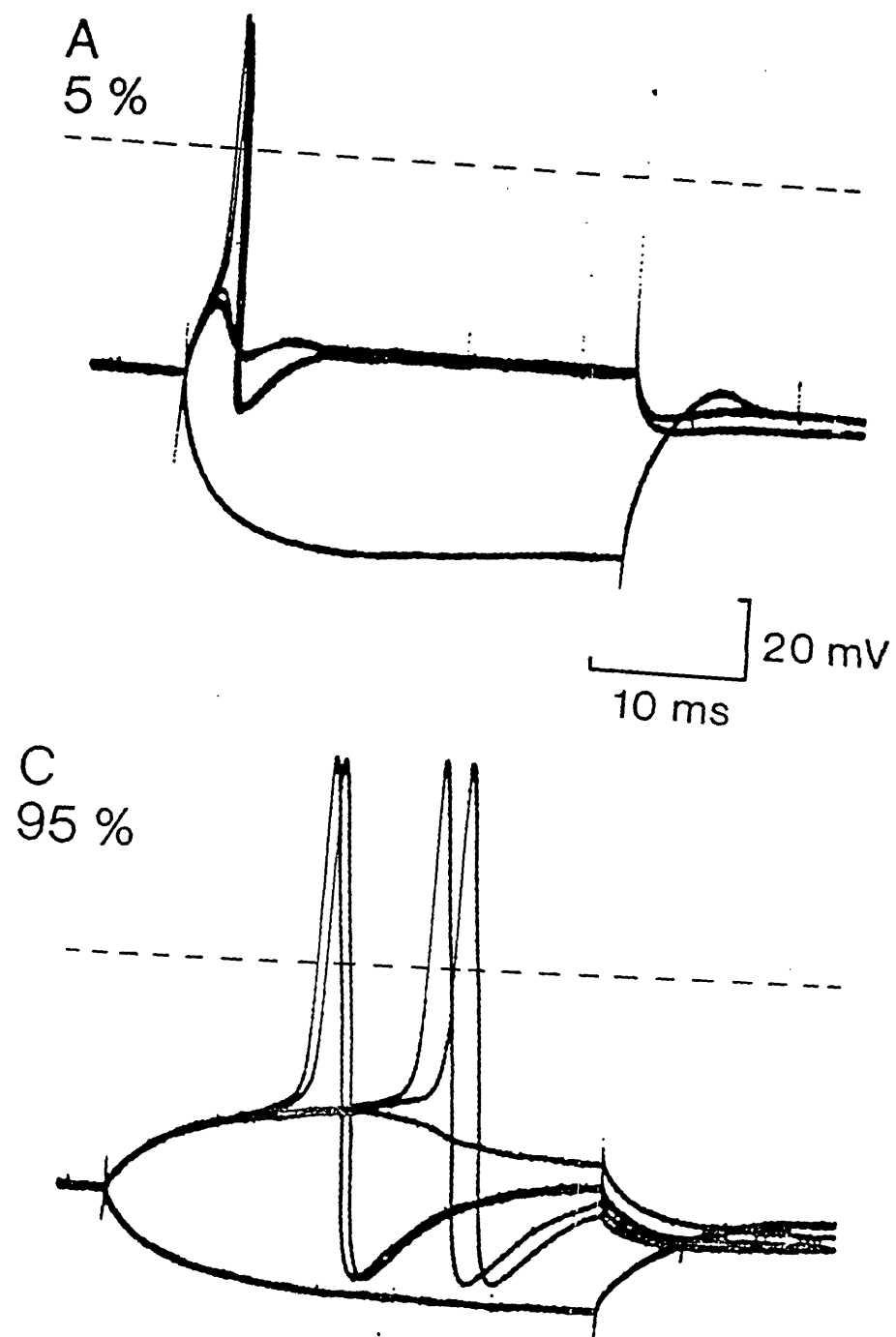


Table 3

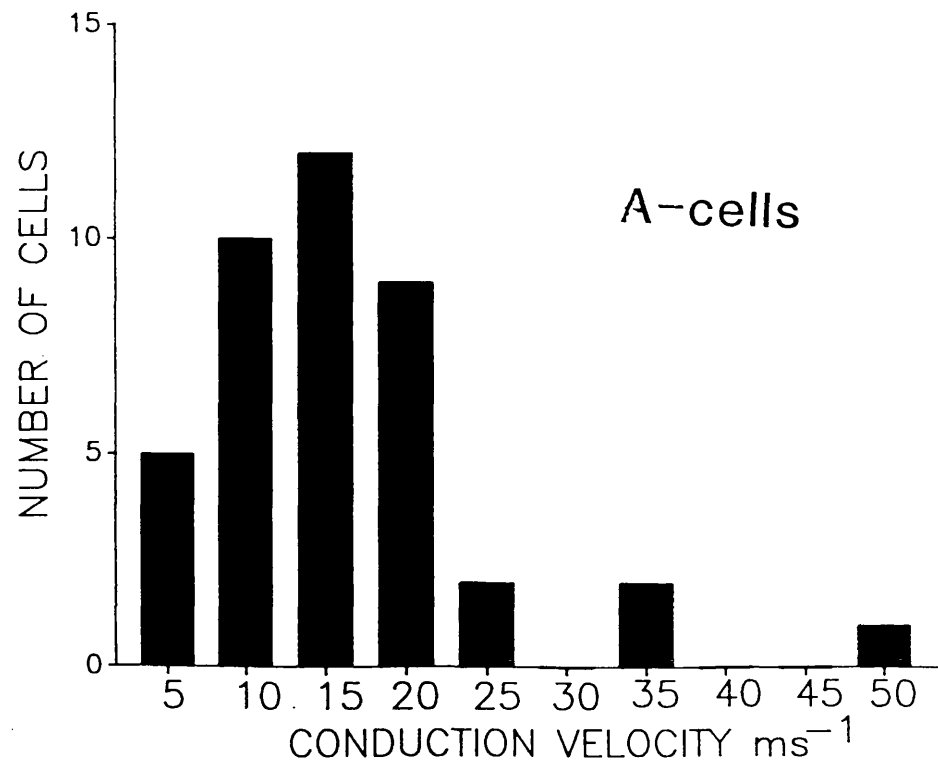
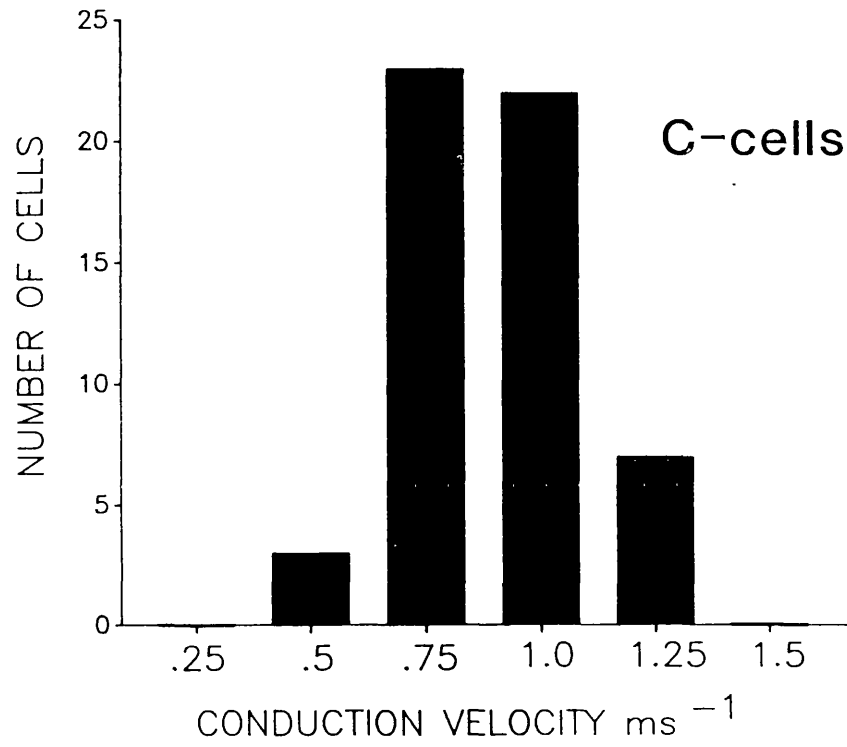
A cells

C cells

Resting potential	$-56 \pm 6 \text{ mV} (16)$	$-54 \pm 1 \text{ mV} (54)$
input impedance	$61 \pm 26 \text{ m}\Omega (44)$	$74 \pm 5 \text{ m}\Omega (54)$
Spike over-shoot	$23 \pm 1.8 \text{ mV} (20)$	$33 \pm 4 \text{ mV} (15)$
Spike duration	1–2 msec	3–6 msec

Fig.R26.

The distribution of C-type and A-type cell conduction velocities in adult rat nodose ganglia at 30 °C.



**The effect of capsaicin on the conduction velocity of orthodromic action potentials
in C-type neurones.**

Capsaicin-induced changes in the conduction velocity of the orthodromic action potential were analysed in 27 capsaicin-sensitive nodose C-type neurones.

25 of the neurones responded with depolarization, an increase in the latency of the somatic invasion and a decrease in the amplitude of the action potential. The reduction in axonal conduction velocity, although highly variable, was dose-dependent in individual cells (Fig.R27).

Capsaicin ($0.3\mu\text{M}$) applied to 10 C-type neurones and their axons, reduced the conduction velocity by $10.3 \pm 1.5 \%$. Capsaicin ($1\mu\text{M}$) blocked conduction in 4 of 17 preparations, and reduced the orthodromic conduction velocity in a further 11 preparations by $17.15 \pm 3.9 \%$. In several cells capsaicin prevented the axonal action potential from fully invading the neuronal soma and a subthreshold proximal process potential was seen, from which estimates of conduction velocity could still be made. In two cells capsaicin depolarized the neuronal soma but no significant effect was seen on orthodromic conduction velocity.

Approximately 70% of the C-type neurones tested proved to be capsaicin sensitive. In 3 slower-conducting A-type neurones, high concentrations of capsaicin ($3\text{--}10\mu\text{M}$), produced a small depolarization ($<10\text{mV}$) and a reduction in conduction velocity. Both these effects rapidly and fully reversed on removal of capsaicin. However the vast majority (83%) of A-type neurones were capsaicin insensitive. No correlation could be found between the amplitude of the somatic capsaicin-induced depolarization, the control conduction velocity and the capsaicin-induced changes in conduction velocity.

The measurements of changes in conduction velocity induced by capsaicin were normally obtained at the peak of the somatic depolarization. However the conduction velocity sometimes continued to decrease after the somatic depolarization had peaked. This probably represents differences in active-site accessibility. Surface nodose

neurones were impaled but the peripheral axons of these cells tend not to follow a continuous superficial position within the vagus nerve (Evans and Murray, 1954) and therefore one might expect a slower rate of action of capsaicin at the axonal site.

Fig.R27.

The effect of capsaicin on C-type neuron conduction velocity.

Capsaicin was applied in cumulative doses to the nodose ganglia and vagus nerve trunk and orthodromic action potentials elicited and recorded at the peak of the somal depolarization. In the presence of $0.3\mu\text{M}$ capsaicin the membrane potential was reduced by 4mV and a significant reduction in conduction velocity was noted. An increase in the capsaicin concentration to $1\mu\text{M}$ produced a much larger depolarization and a further decrease in conduction velocity.

Scale: $40\text{mV} / 10\text{ms}$

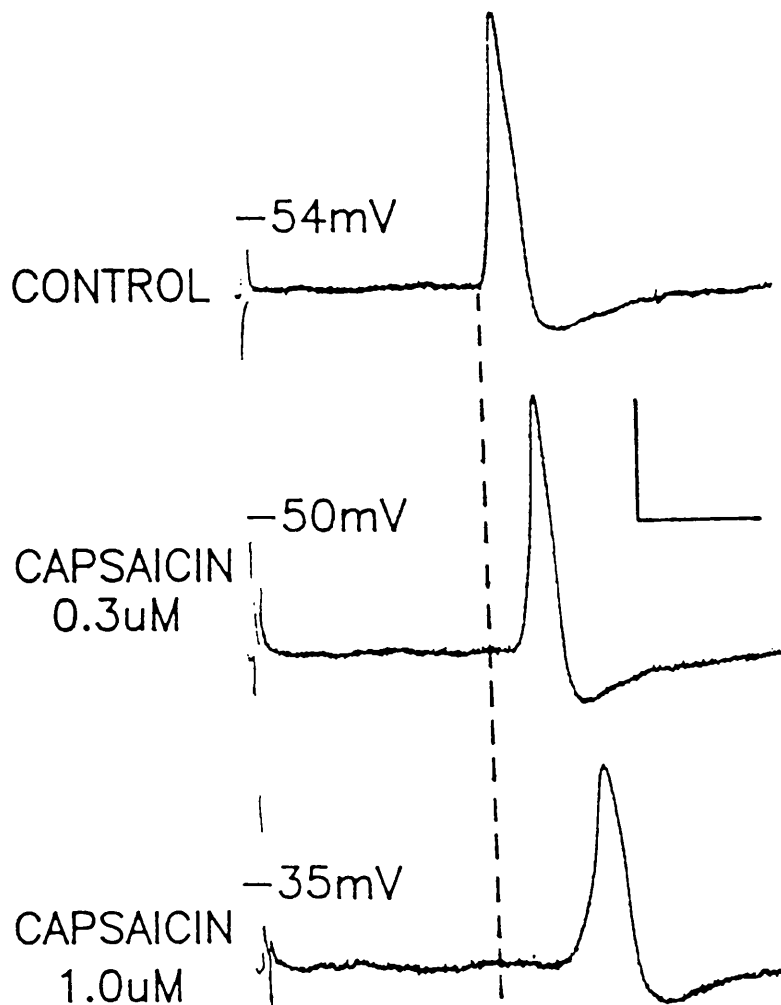
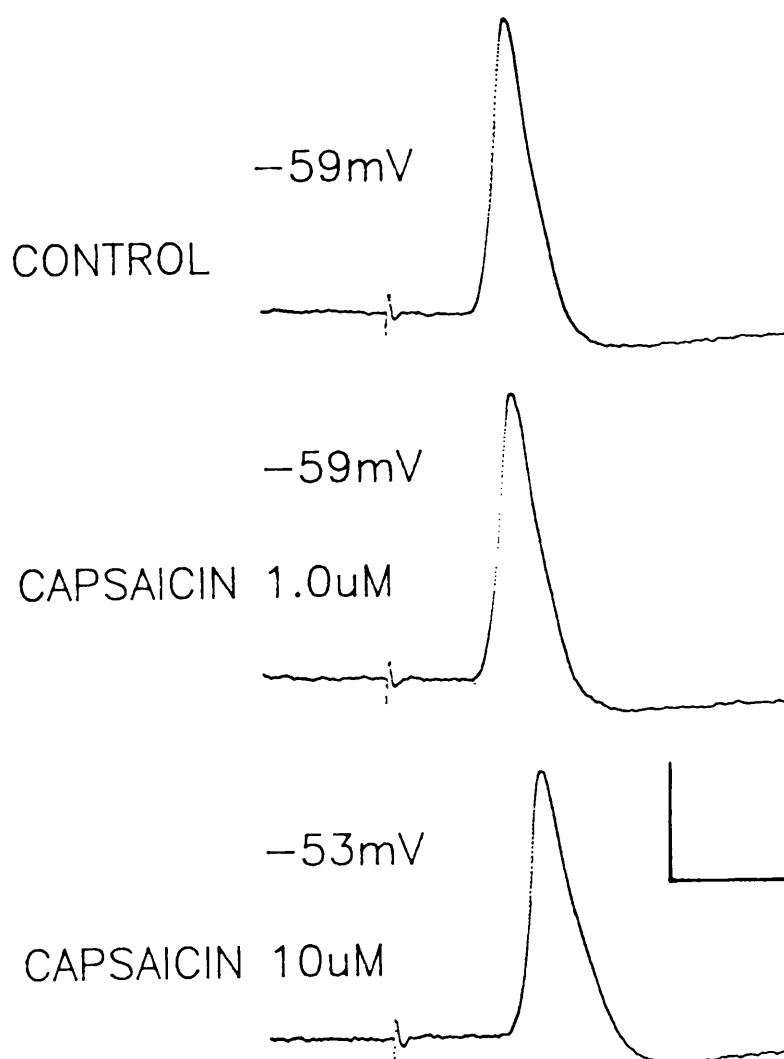


Fig.R28.

The effect of capsaicin on A-type neuron conduction velocity.

Scale: 40mV / 2ms



The effect of capsaicin on sensory neurone
membrane conductance.

The nature of the capsaicin-induced depolarization was investigated using intracellular voltage and current clamp recording techniques.

The effect of capsaicin on sensory neurone somal membrane conductance was analysed in 54 C-cells and 6 A-cells.

30 % of the C-cell neurones of nodose ganglion were insensitive to capsaicin, but no obvious correlation between the basic electrophysiological criteria such as conduction velocity, input impedance or spike amplitude / duration and capsaicin sensitivity could be found.

Capsaicin was applied to 6 A-type neurones under both current and voltage clamp recording conditions. In none of these cells did capsaicin produce a significant change in membrane conductance but a small depolarization ($< 10\text{mV}$) was noted in three cells using high concentrations of capsaicin ($> 3\mu\text{M}$).

Capsaicin was also applied to 3 sympathetic neurones of the rat superior cervical ganglia. Even in concentrations as high as $30\mu\text{M}$, no inward current or change in conductance was noted.

Current clamp recording.

Under current clamp recording mode the majority of the C-cells responded to capsaicin ($0.3 - 1\mu\text{M}$) in a multiphasic manner; an initial fast depolarization ($3-30\text{mV}$) was followed by a prolonged hyperpolarization ($5-20\text{mV}$) and occasionally a very late slow depolarization (Fig.R29).

Capsaicin produced quite pronounced effects on the action potential, decreasing its amplitude, increasing the duration and slowing its rise time (Fig.R29). These effects

were often maintained, in part, after the initial conductance changes had subsided.

By monitoring a hyperpolarizing membrane voltage response to a negative constant current injection during the application of capsaicin, it was clear that membrane resistance had decreased during both the early depolarization and late hyperpolarizing phase. ^{The} C-cell membrane however does show significant rectification on depolarization, so the decrease in resistance produced by capsaicin might in part be due to a voltage-sensitive phenomenon. To exclude the voltage-dependent increase in membrane conductance, cells were repolarized to their initial membrane potential by injecting hyperpolarizing current. Under these conditions the conductance increase was less than that evaluated at the peak of the depolarization but still significant.

Fig.R29.

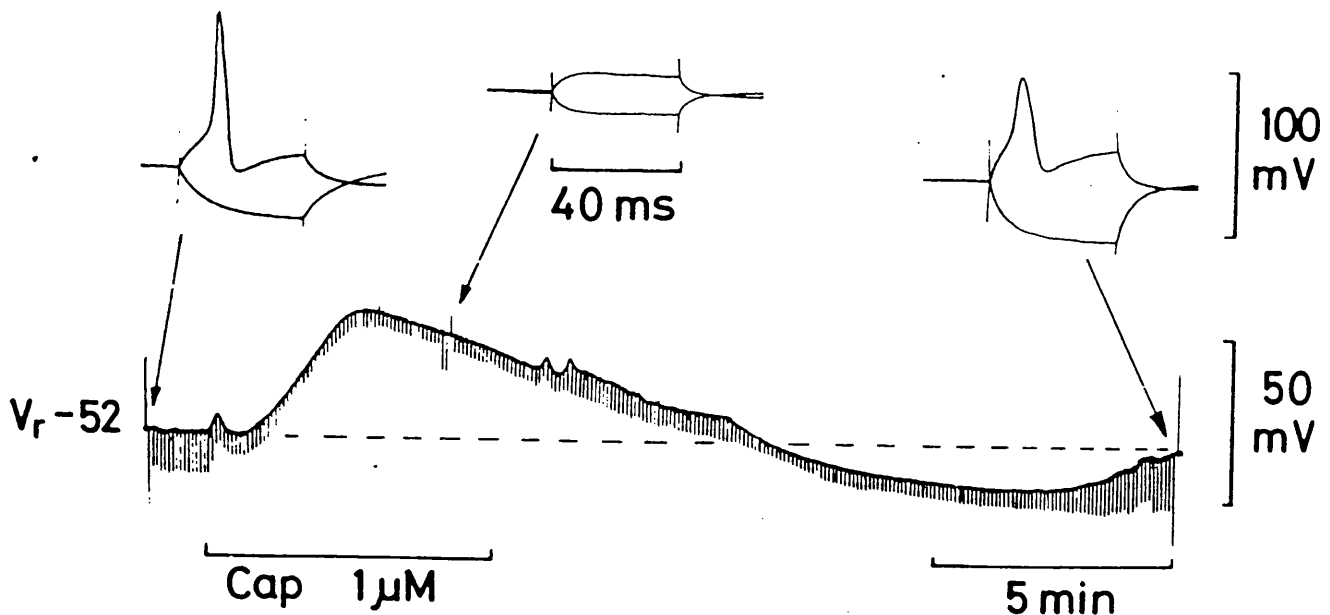
Current clamp recording of the effect of capsaicin on a sensory C-cell.

Downward deflections are membrane voltage responses (electrotonic potentials) to hyperpolarizing constant current (-0.2 nA) injections.

The oscilloscope records shown above the trace are membrane voltage responses to both depolarizing and hyperpolarizing current injection and were obtained at the times indicated by the arrows.

Capsaicin ($1\mu\text{M}$) initially depolarized the neuron, decreasing concurrently the amplitude of the electrotonic potentials and abolishing the action potential. Note how the depolarization fades during the continuous application of capsaicin.

On removing capsaicin a pronounced hyperpolarizing phase is evident which is associated with a further decrease in the amplitude of the electrotonic potentials. A late repolarizing phase is also present, during which the action potential is still reduced in amplitude.



Voltage clamp recording.

The voltage clamp technique was used to answer two main questions;

1. How does capsaicin modify the resting conductance of the cell to produce the depolarization ?
2. Is the hyperpolarization induced by capsaicin a consequence of membrane depolarization ?

In voltage clamp recording mode, cells were initially clamped at their resting membrane potential (normally -51 to -55 mV) and membrane conductance assessed by brief (50 to 200 ms) voltage jumps to membrane potentials in the range -110 to -30 mV (Fig.R30). The membrane current at potentials hyperpolarized to rest was devoid of any rectification (over a 200 ms time-scale) , the current-voltage curve being linear within the voltage range -50 to -140 mV. Depolarization did, however, produce a significant increase in membrane conductance and therefore a deviation from simple ohmic behaviour (Fig.R31).

Application of capsaicin resulted initially in an inward current and an increase in the amplitude of the membrane current deflections induced by hyperpolarizing voltage jumps, indicative of an increased membrane conductance. The inward current was often transient and decayed to a net outward current with a further increase in conductance (see Fig.R34). In several cells it was possible however, to record full current / voltage curves both during the early inward current and late outward current phases (Fig.R32) enabling an assessment of the capsaicin-induced current reversal potential (E_{CAP}).

Fig.R30.

The effect of capsaicin on a sensory C-cell recorded using intracellular, single electrode voltage clamp.

I = membrane current.

V = membrane voltage.

The cell has been voltage clamped at its resting potential of -56mV and membrane current responses to a series of voltage steps (shown on a *100 expanded time-base) recorded.

The upper trace shows membrane current responses to the same series of 100 msec voltage steps (shown in the bottom trace), obtained three minutes before the start of the central trace. Downward deflections represent inward current.

Capsaicin induced a monophasic inward current and an increase in the amplitude of the membrane current responses to constant voltage jumps. Further analysis of this record is shown in Fig.R31.

Fig.R30.

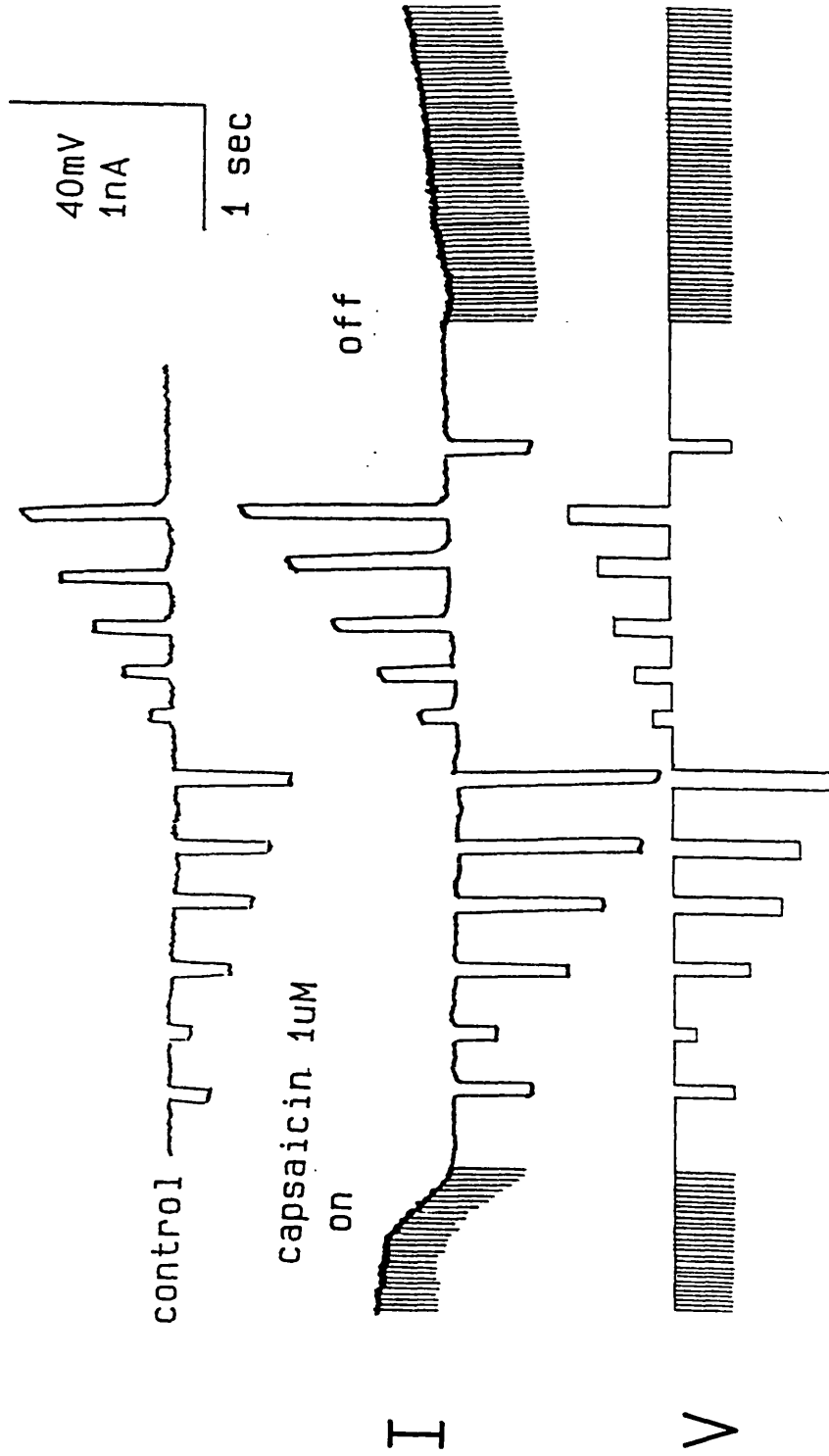


Fig.R31.

Analysis of the monophasic capsaicin response shown in Fig.R30.

The upper graph shows the current / voltage relationship obtained during the control period and in the presence of capsaicin, recorded at the peak of the inward current. Current measurements were made from the instantaneous current deflections induced at the onset of the voltage steps.

The lower graph shows a difference-current / voltage analysis plot. This data was obtained by subtracting the current amplitudes at all membrane potentials obtained in the control curve, from those obtained in the presence of capsaicin at the same potentials.

The data has been fitted by a least-square fit linear regression analysis to measure the slope and intercept when $I = 0$. The data in this example had a correlation coefficient (R) of 0.9985.

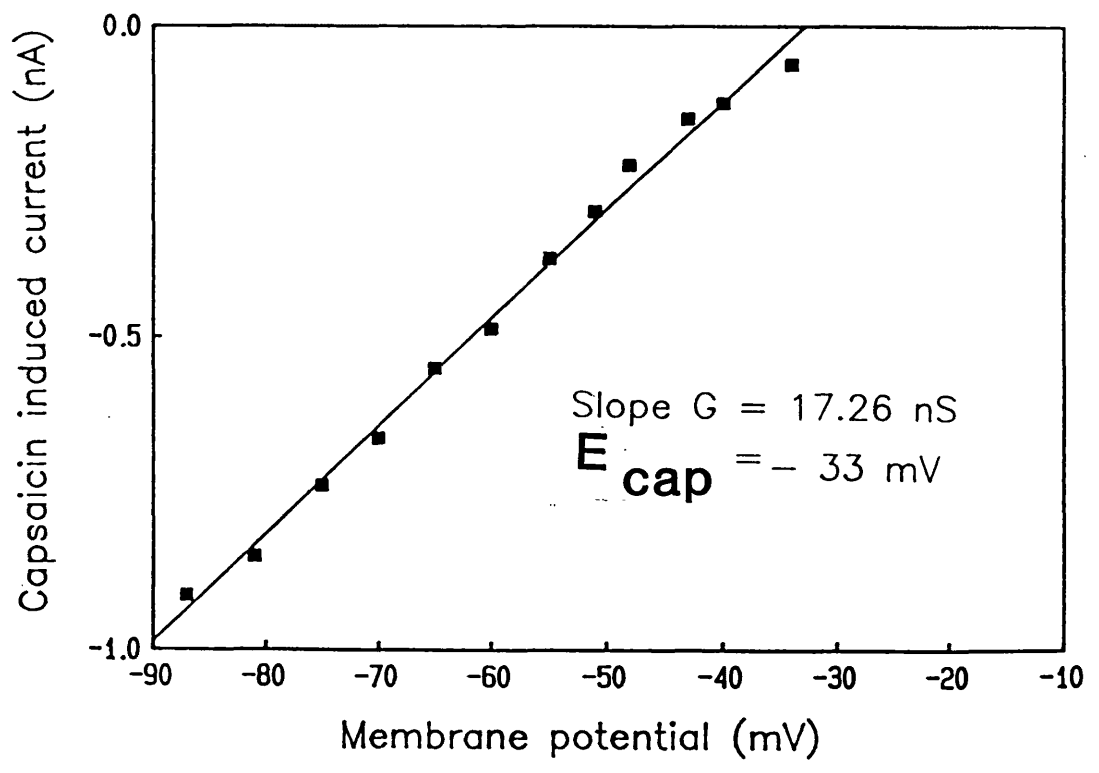
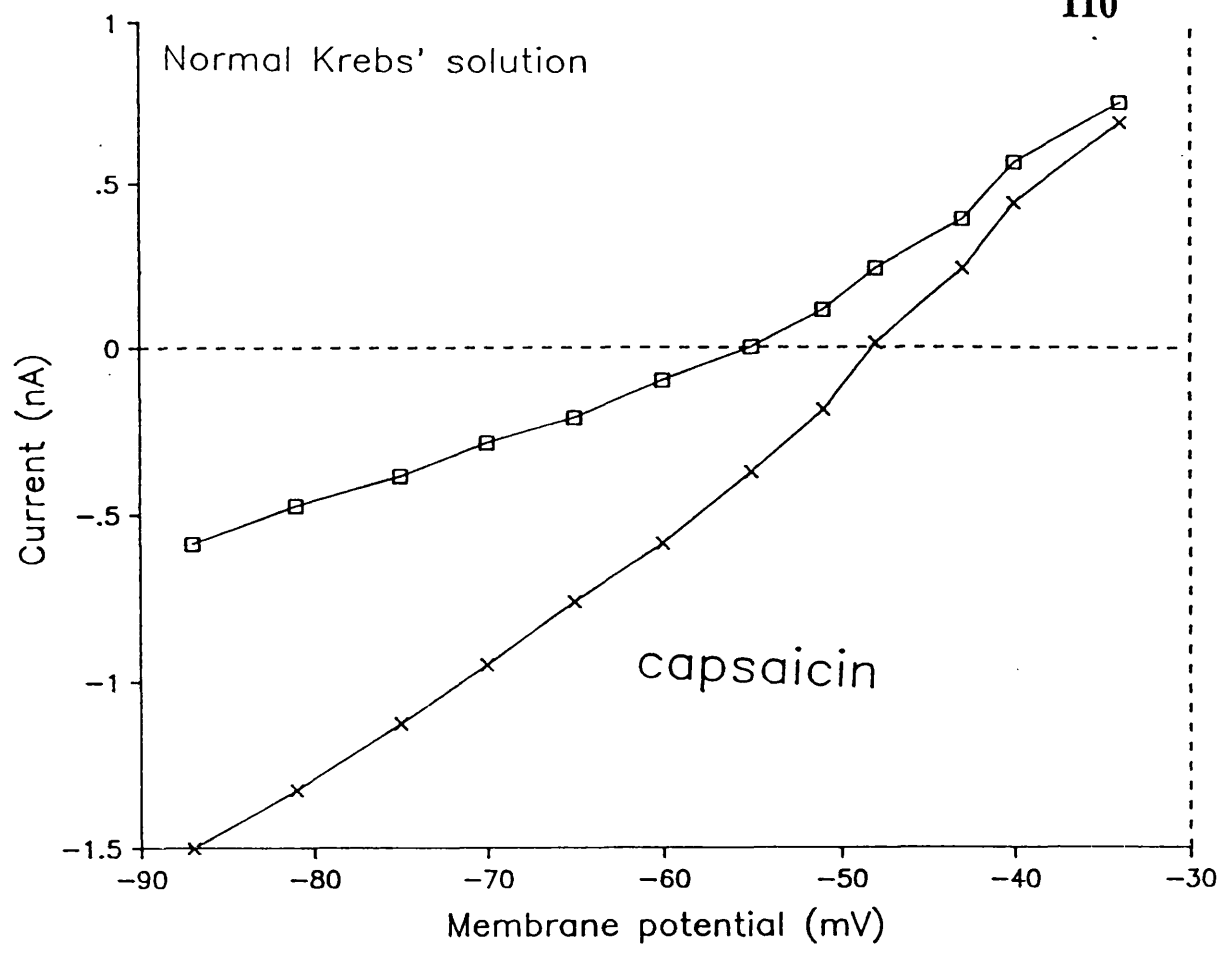


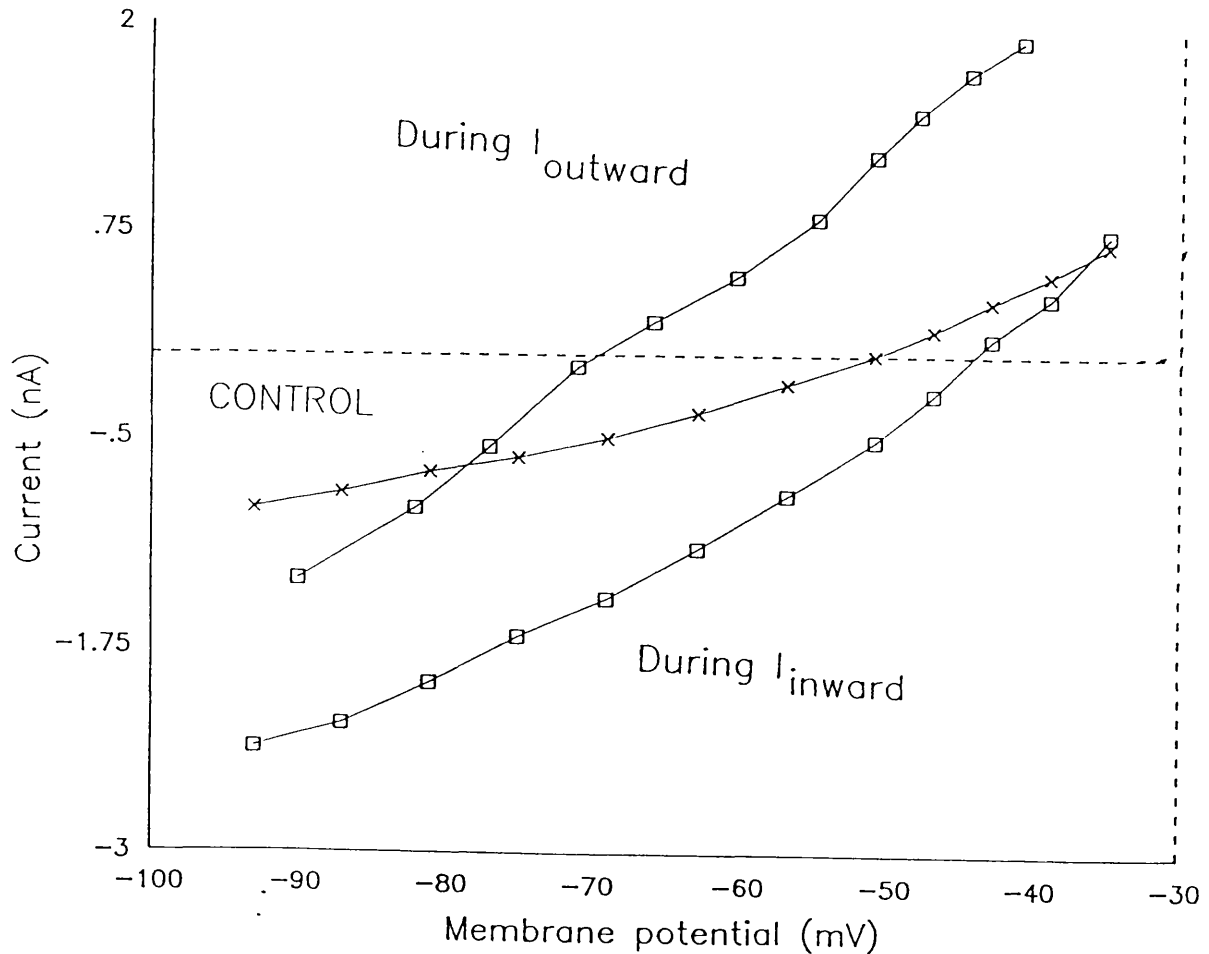
Fig.R32.

Current / voltage analysis of a biphasic capsaicin response.

The majority of C-cells respond to capsaicin in a biphasic manner, with an initial inward current followed by an outward current (Fig.R34).

This graph shows current / voltage curves obtained from a single cell during both the inward and outward current phases. During the inward current phase there is a small increase in conductance and the curves intersect at -35mV .

During the outward phase there is a further increase in membrane conductance and the outward current curve intersects the control curve at -78mV . Note that the curve obtained during the outward current phase does not intersect the current / voltage curve obtained during the inward current phase (see Discussion).



E_{Cap} was estimated from the intercept of the two current / voltage ^{curves} (extrapolated by linear regression analysis) recorded before and in the presence of capsaicin, assuming that the capsaicin-induced conductance is independent of voltage.

The slopes of the two lines and current amplitude at 0 mV (Y intercept) was determined from the linear portion of the current voltage curve (-50 to -100). From these calculations the membrane potential at which $I_{control} = I_{capsaicin}$ can be determined:

$$I_{control} = \text{Slope } G * V_m + I_0$$

$$I_{capsaicin} = \text{Slope } G' * V_m + I_0'$$

Where I_0 = current at 0mV membrane potential

and V_m = membrane potential

$$\text{Slope } G * V_m + I_0 = \text{Slope } G' * V_m + I_0'$$

$$\text{Slope } G * V_m - \text{Slope } G' * V_m = I_0' - I_0$$

$$(\text{Slope } G - \text{Slope } G') * V_m = I_0' - I_0$$

$$V_m = \frac{I_0' - I_0}{\text{Slope } G - \text{Slope } G'} = E_{cap}$$

Alternatively, the capsaicin-induced current reversal potential can be calculated from a difference current determined by simply subtracting the control current at each potential from that obtained in the presence of capsaicin.

A curve of capsaicin-induced current against membrane potential can then be plotted. This calculation involves a single linear regression analysis procedure and also reveals the presence (if any) of a voltage dependence to the capsaicin-induced conductance mechanism (see Fig.R31).

The capsaicin-induced increase in slope conductance and E_{Cap} was determined during both the inward and outward current phases in normal Krebs' solution. Both these parameters proved to be highly variable, particularly in cells that showed a large subsequent outward current.

In 7 cells showing a monophasic inward current, the extrapolated null potential for this current was -4 ± 7 mV (Table 4). In cells showing a biphasic response, the reversal potential during the outward current was -68 ± 6 mV. The determination of E_{Cap} and the capsaicin-induced conductance increase during the outward current phase was easier to determine as in most cells this was often maintained for several minutes. Moreover the conductance increase during this phase was always larger than that measured during the inward current.

Calcium free Krebs' solution.

A series of experiments were undertaken to evaluate the role of calcium ions in the capsaicin-induced primary and secondary conductance mechanisms.

Superfusing impaled neurones with calcium-free Krebs' solution containing elevated magnesium often resulted in a destabilization of the recording and a cell membrane conductance increase. However it was possible in several cells to apply capsaicin and monitor changes in membrane conductance. In all 7 cells in which capsaicin was applied in the absence of external calcium ions only a monophasic inward current was seen. When resubstituting calcium and reapplying capsaicin, a normal biphasic conductance response was seen (Fig.R33). The reversal potential (E_{Cap}), calculated from capsaicin-induced difference current curves, was more positive (+ 32 mV) than that found for a monophasic response in the presence of calcium ions (-4 mV) (Table 4).

The capsaicin-induced conductance increase (4.6 nS) measured in Ca-free Krebs' solution was also significantly lower than that measured for a monophasic inward current (11.32 nS) or during the outward current of a biphasic response (27.43 nS).

Fig.R33.

The effect of calcium-free Krebs' solution on the capsaicin reversal potential and slope conductance.

The upper graph shows the current / voltage curves obtained before and during a monophasic inward current response induced by capsaicin. Note how the curves do not converge within the voltage range studied (compare to Fig.R30).

A difference-current / voltage curve of the data shown in the upper graph. Note the positive reversal potential, the linear difference-current / voltage curve and the reduced slope conductance recorded in a calcium-deficient medium.

Correlation coefficient (R) = 0.9965.

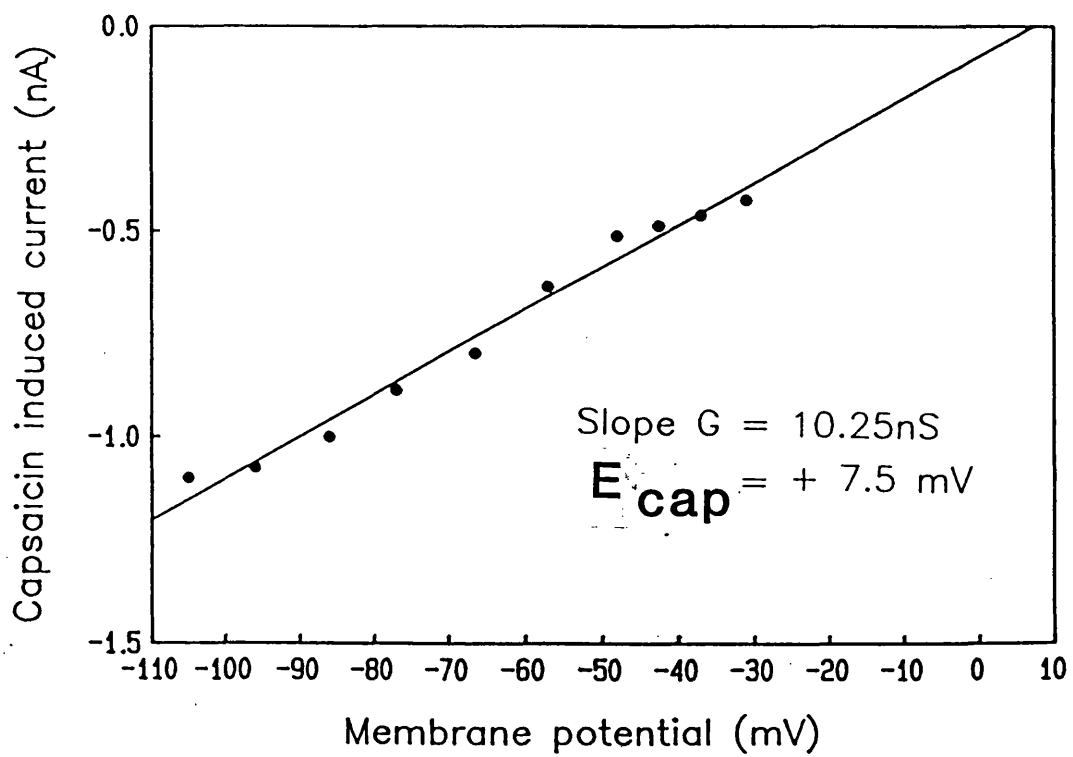
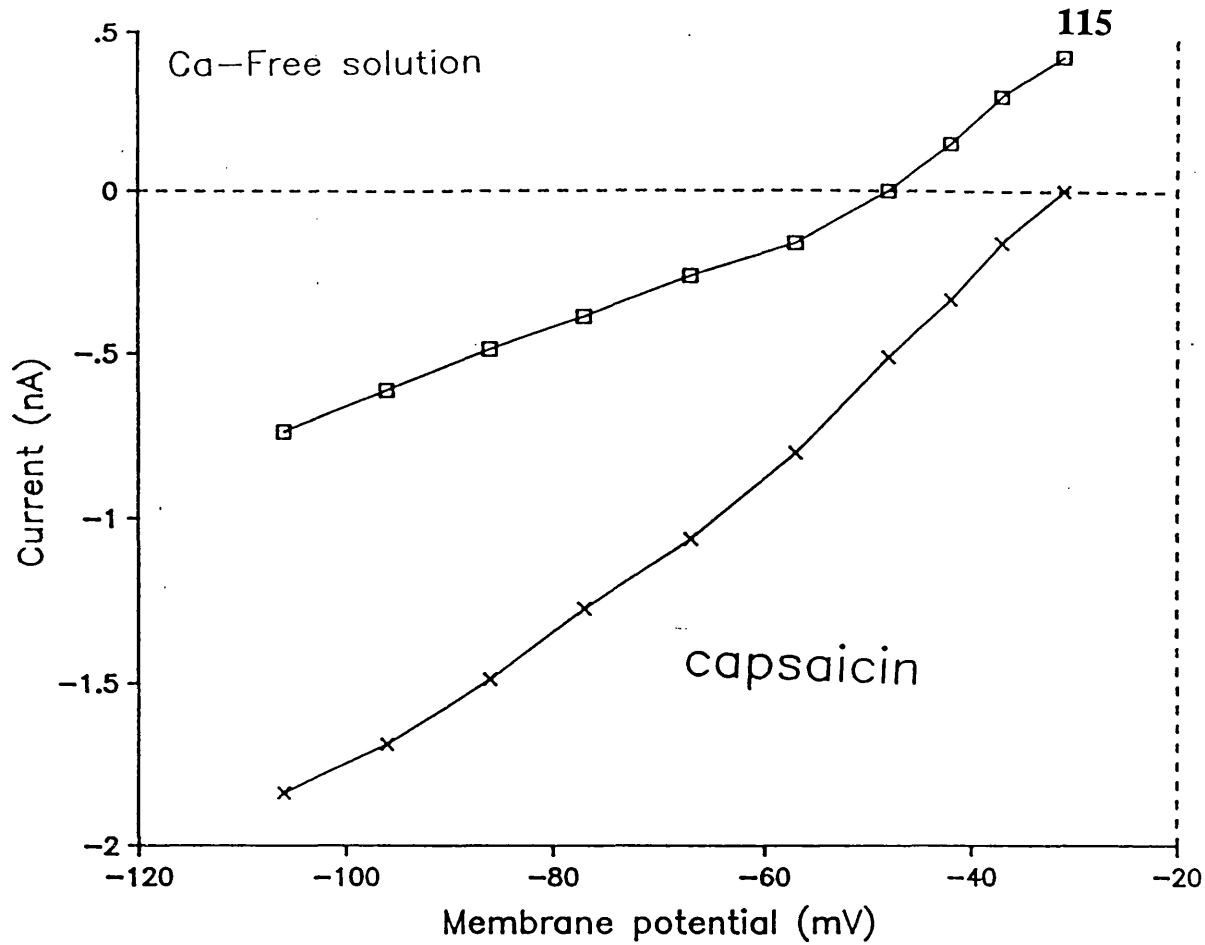
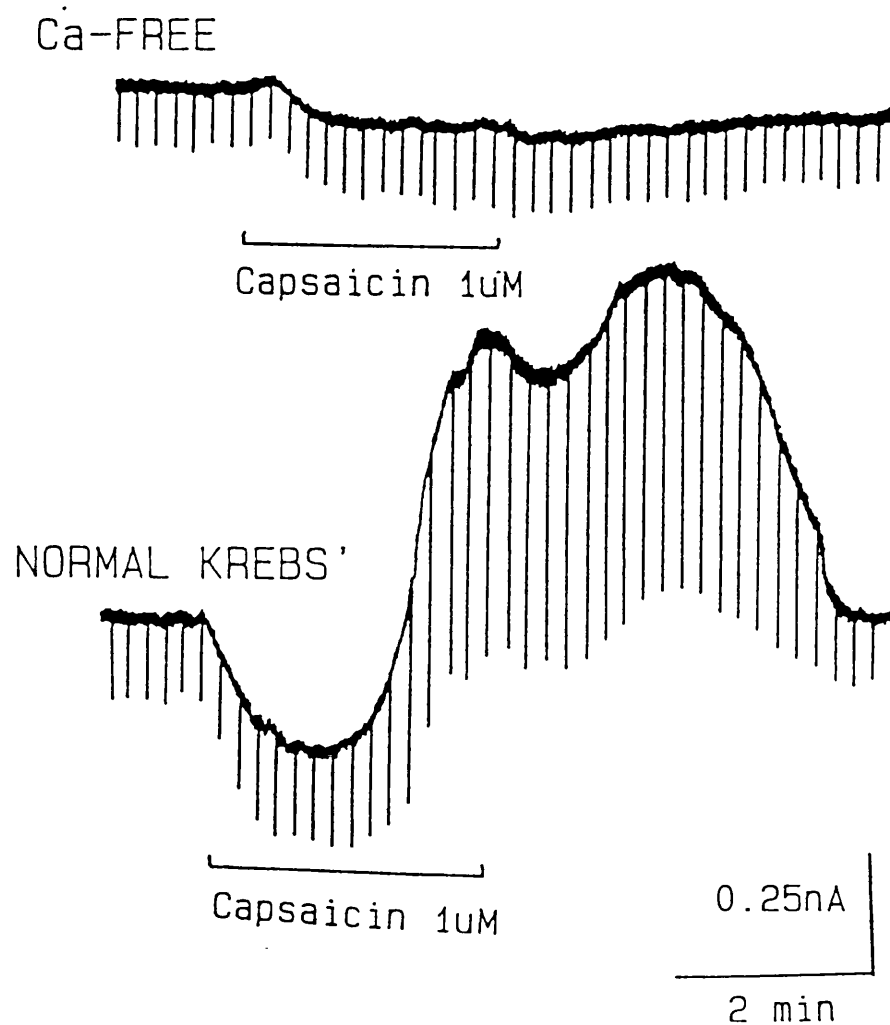


Fig.R34.

The effect of calcium-free Krebs' solution on capsaicin-induced membrane currents. The traces shown are membrane current records obtained from a single cell, voltage clamped at -53mV .

The cell was initially impaled in normal Krebs' solution and then immediately superfused with calcium deficient 10 mM magnesium Krebs' solution for 20 minutes. Capsaicin ($1\mu\text{M}$) induced a small monophasic inward current and an increase in membrane conductance. However when capsaicin was reapplied in calcium containing normal Krebs' solution a typical biphasic ^{response} was seen, which was associated with a much larger increase in membrane conductance.

Downward deflections are membrane current responses to 50 msec , -5mV voltage jumps. $V_{\text{hold}} = -53\text{mV}$.



Caesium loaded cells.

The negative reversal potential for the outward current (-64mV, Table 4) suggests that it might involve an increased potassium conductance. To eliminate this, sensory C-cells were impaled using microelectrodes filled with 4M caesium acetate.

Caesium was injected into cells using short duration (20 - 100 ms) depolarizing (+) current injections and action potential duration monitored. This method, which normally took between 15 - 30 mins, resulted in cell depolarization, a prolongation of the action potential (as voltage-dependent outward potassium currents were reduced) and a large decrease in the resting conductance.

Capsaicin ($1\mu\text{M}$) applied to three Cs^+ -loaded cells produced a monophasic inward current and an increase in membrane conductance. However the capsaicin-induced current / voltage curve no longer appeared to be linear ($R = 0.945$), as rectification was evident at depolarized potentials. Therefore estimates of the reversal potential and slope conductance by linear regression were not valid (Fig. R35). However, the reversal potential for capsaicin, under conditions when outward potassium conductances were substantially reduced, was certainly more positive than +20mV (Table 4) and the average capsaicin-induced slope conductance (7.5nS) was less than that obtained when using potassium acetate filled microelectrodes in normal Krebs' solution (Table 4).

Sodium free Krebs' solution.

Experiments conducted using sodium substitution were extremely difficult to interpret as substitution of sodium ions by either Tris or glucosamine resulted in large junction potentials and spontaneous depolarizations and conductance changes.

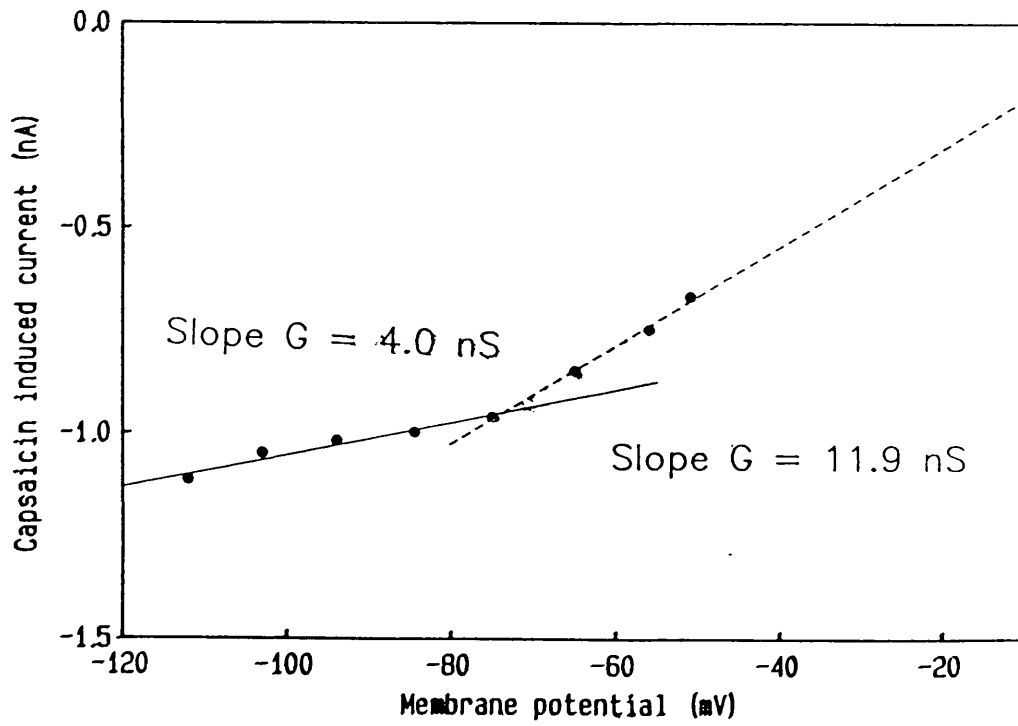
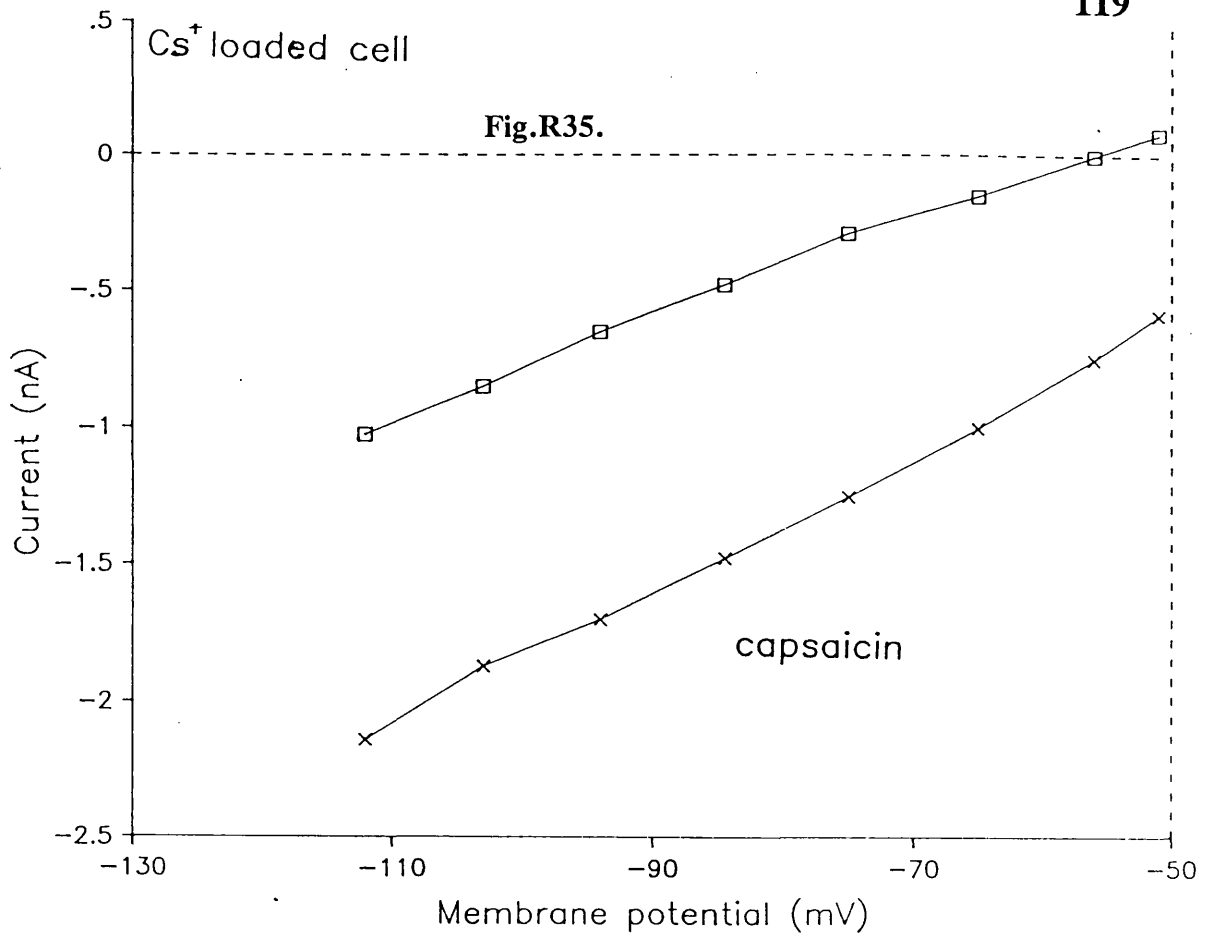
Capsaicin ($1\mu\text{M}$) was successfully applied to three cells, all of which responded in a biphasic manner with an increase in conductance. The amplitude of this conductance increase or the capsaicin reversal potential was not evaluated under these conditions.

Fig.R35.

The effect of caesium-loading on the capsaicin-induced conductance increase.

An example of current / voltage curves obtained during capsaicin-induced monophasic response recorded from a caesium-loaded cell. Capsaicin produced a large inward current at the holding potential (-55mV).

Extrapolation analysis would indicate that the capsaicin-induced current in caesium loaded cells has a very positive reversal potential. However the difference-current / voltage analysis shows that the capsaicin-induced current is no longer linearly related to voltage. This negates the use of linear regression for reversal potential estimates and suggests that, in the absence of outward potassium currents, the capsaicin-induced current does show some evidence of rectification.



Response type	Capsaicin-induced conductance increase	Capsaicin reversal potential (E_{Cap})
Monophasic inward current	$11.32 \pm 2\text{nS}$ (7)	$-4 \pm 7\text{mV}$ (11)
Outward current	$27.43 \pm 6.97\text{nS}$ (8)	$-68.1 \pm 5.6\text{mV}$ (8)
Calcium-free inward current	$4.6 \pm 1.6\text{nS}$ (7)	$+ 32 \pm 8\text{mV}$ (7)
Caesium-loaded inward current	$5.5 \pm 2.34\text{nS}$ (3) [*]	$+ 31 \pm 13.5\text{mV}$ (3) [*]

* These values were determined for comparison only and do not represent accurate determinations (see text)

Table 4.

INTRACELLULAR RESULTS - DISCUSSION.

The experiments in this chapter show that, in normal Krebs' solution, the effect of capsaicin is to produce a membrane depolarization, which is often followed by a hyperpolarization, and that both phases are accompanied by an increased membrane conductance of (on average) 11.3 and 27.4 nS respectively (Table 4). This implies that both phases result from an increased ionic permeability.

The reversal potential for the inward current, driving the initial depolarization, was about -4mV. This suggests that it results from an increased cation conductance, which must include sodium and / or calcium. The reversal potential for the subsequent outward current was about -68mV, suggesting that it results predominantly (although not necessarily exclusively) from an increased potassium conductance. This is substantiated by the observation that the outward current was absent in Cs-loaded cells.

Removal of calcium had three effects. Firstly, it prevented the late phase of hyperpolarization. Secondly, it increased the initial depolarization and shifted the reversal potential for the inward current to + 32mV. Thirdly, it reduced the initial conductance increase from 11.3 to 4.6nS. While the reduced conductance might be interpreted as indicating a large contribution by calcium to the inward current, this is contradicted by the positive shift in the reversal potential: since E_{Ca} is more positive to E_{Na} , removing calcium should produce a negative shift in E_{cap} . Instead, the most plausible explanation of the data is that the primary effect of capsaicin is to open ion channels which are highly permeant to sodium ions, but which are also permeant to calcium ions; and that the influx of calcium ions activates a calcium-dependent potassium current, separate from the primary current.

This latter current has two effects: it partly shunts the primary sodium / calcium current (thus reducing E_{cap} below E_{Na}); and it generates a secondary outward current,

giving the after-hyperpolarization. A calcium-activated potassium current has previously ^{been} reported in sensory neurones (Higashi et al., 1984).

An alternative possibility is that the initial sodium current might generate a calcium-influx by opening voltage-gated calcium channels, but two observations argue against this: the secondary outward current is seen under conditions where the cell is voltage-clamped below the threshold for the calcium-current (about -30mV in these cells: Scott and Dolphin, 1986) and the effects of removing calcium were not imitated by adding divalent cations such as cobalt or cadmium which block the voltage-gated calcium channels. Hence, it seems more likely that calcium enters through capsaicin-activated channels themselves.

In the extracellular recording experiments, it was noted that removing external chloride ions also modified the response to capsaicin. Some sensory neurones exhibit a calcium-activated chloride conductance (Mayer, 1985). This might explain why the reversal potential for the secondary outward current is positive to E_K (ca. -80mV), since E_{Cl} is relatively positive (-35mV in ^{DRG C-cells,} Deschenes et al., 1976).

In unclamped cells, it was noted that the action potential was severely attenuated during the capsaicin-induced depolarization. There are several mechanisms that could induce this effect: firstly, the depolarization could result in inactivation of the sodium channels; secondly, the increase in resting conductance induced by capsaicin could 'shunt' the action potential; thirdly, sodium entry is of such an amplitude that the sodium reversal is shifted more negative and fourthly, the calcium entering can induce inactivation of the sodium channels.

The recovery of the action potential, primarily followed the time course of the repolarization. However, occasionally the action potential failed to recover fully when the cell had repolarized and the conductance increase subsided. This suggests that a long-term non-voltage dependent process can reduce the action potential amplitude, but no attempt was made in the present study to fully evaluate this phenomenon.

CHAPTER 5

HISTOLOGY

Histological results.

Early experiments suggested that the sensory neurotoxic action of capsaicin, induced by subcutaneous injection, was specific to neonatal animals (Jancso et al., (1977).

However more recently the same group have re-evaluated adult rat sensitivity and have shown that capsaicin applied in the same manner can also reduce the numbers of B-type neurones found within spinal dorsal root ganglia and substantially reduce the unmyelinated fibre content of several peripheral nerve bundles (Jancso et al., 1985). A localized application of capsaicin directly onto adult nerve bundles has been also shown to produce a 'long-lasting' reduction in its unmyelinated fibre content (Lynn and Pini, 1985; Jancso et al., 1987).

Sensory C-cells are devoid of dendrites, and synaptic inputs do not directly couple to the sensory cell soma. The primary function of the soma therefore would appear to be the maintenance of cell and axon integrity and the production of cell organelles, transmitter substances and metabolic products. To this end the cell soma contains an abundance of ribosomal material, rough and smooth endoplasmic reticulum (indicative of protein synthesis), Golgi apparatus (glycosylation of proteins and the packaging of transmitter substances) and an abundance of small mitochondria supplying the energy requirements of these processes (Fig.R37).

The peripheral unmyelinated axons of these cells are surrounded over most of their length by Schwann cells and have a simplified internal structure with only a few mitochondria, neurofilaments and microtubules being visualised using electron microscopy (Fig.R41).

A histological study of 62 vagus nerves and nodose ganglia was undertaken to investigate the morphological consequences of the capsaicin induced conductance increase in both nodose C-cells and the unmyelinated fibres of the vagus nerve.

The effect of capsaicin in normal Krebs' solution.

Capsaicin induced gross changes in the complex internal structure of nodose C-cell soma and unmyelinated fibres (Figs.R38 and R41). These effects were clearly visible even under light microscopy where the cytoplasm had an opaque appearance and showed vacuolation (Fig.R36).

Not all of the cells of the nodose ganglion showed these morphological changes; in approximately 25% of the cells no discernible difference could be seen between the cells of the control tissue and that to which capsaicin had been applied.

A critical quantitative assessment of the size of the cells that showed capsaicin sensitivity was not undertaken; however the capsaicin-insensitive neurones showed no obvious size bias (Fig.R36).

The cytotoxic action of capsaicin was readily visualized using the electron microscope where the vacuolation clearly resulted from high amplitude swelling of mitochondria. Mitochondrial diameter often increased four-fold. This resulted in destruction of the internal cristae structure and tiny dense aggregates appear within the mitochondrial matrix. The cytoplasmic damage was not solely confined to mitochondria as both endoplasmic reticulum and Golgi apparatus also showed substantial swelling, distortion and fragmentation (Fig.R38).

Nuclear changes were also evident, with the nuclear membrane convoluted and the nucleus itself becoming granulous.

A high proportion of vagal C-fibres exposed to capsaicin showed pronounced swelling, mitochondrial damage was extensive and a complete disruption of neurofilament organisation was evident (Fig.R41).

Fig.R36.

The histological effects of capsaicin on the sensory neurons of the adult rat nodose ganglion, using light microscopy. Toluidine blue stained light micrographs of nodose ganglion cells taken from three different ganglia. Prior to fixation ganglia had been incubated for 60 min in Krebs' solution under different conditions.

A. A control ganglion incubated in normal Krebs'.

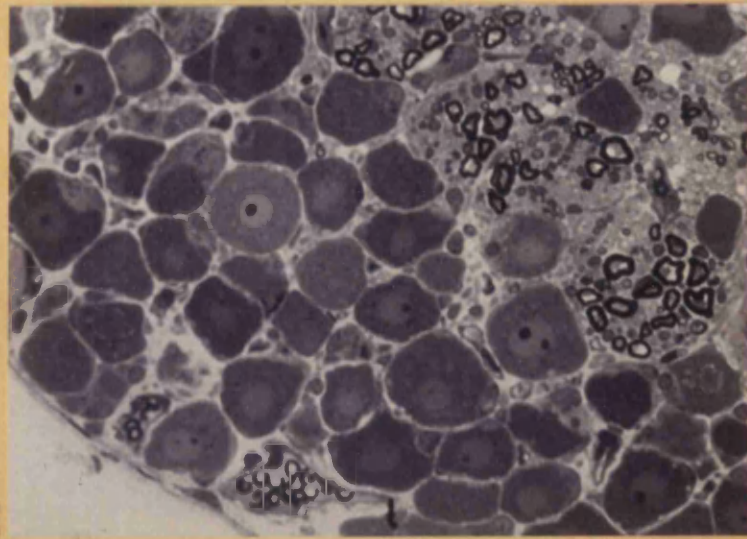
B. A ganglion incubated in normal Krebs' solution to which capsaicin (10uM) was added for the last 30 min.

C. A ganglion incubated in calcium-free solution to which capsaicin (10uM) was added for the last 30 min.

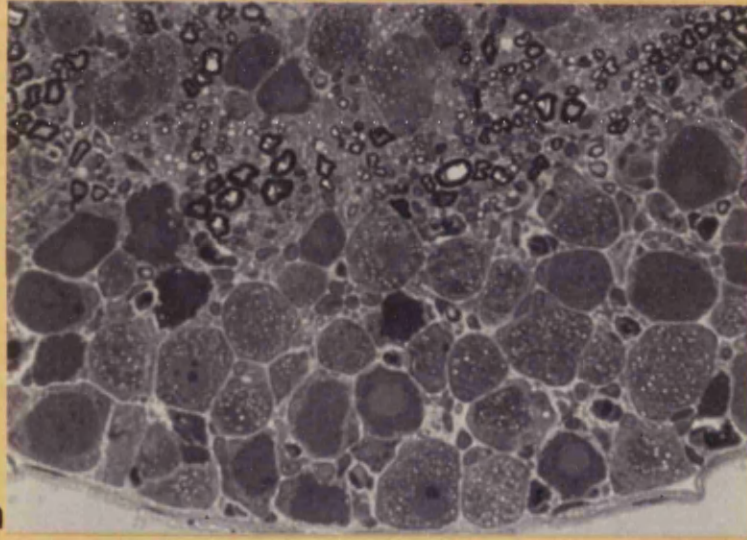
The majority of cells that have received capsaicin treatment have become opaque and vacuolation is evident. There are several cells within this micrograph however that do not take on this appearance indicating some degree of selectivity. There is no obvious selectivity of capsaicin toxicity by cell size.

The ganglion incubated with capsaicin but in the absence external calcium ions (c) does not show the advanced cytotoxic damage evident in B. indicating that, in part, the more advanced stages of cell vacuolation might be due to a calcium-dependent process.

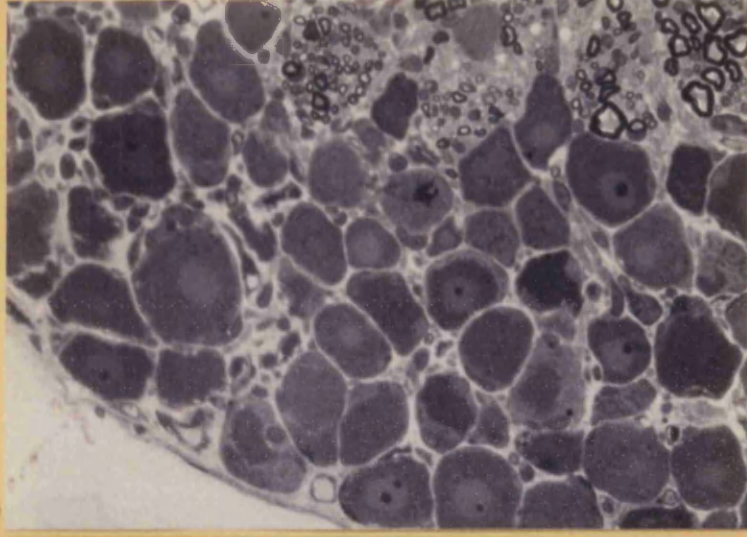
A



B



C



20 μ

Control

CAPSAICIN (10 μM)

CAPSAICIN (10 μM)
CALCIUM FREE
(10 mM Mg⁺⁺)

Fig.R36

Experiments in which the period of capsaicin application was varied showed that substantial cytotoxic damage could occur during five mins incubation. However the cells most affected were those at the periphery of the ganglion. Longer duration incubations (> 10 mins) resulted ⁱ a uniform dispersion of capsaicin-sensitive neurones. This probably reflects the slow diffusion and action of capsaicin.

The cytotoxic effects of capsaicin could be induced within the same concentration range (0.3 - 10 μ M) and over the same time course, at which conductance changes could be measured electrophysiologically (see Chapter 4).

The effects of capsaicin in calcium-free Krebs'.

Large increases in intracellular free calcium ions are believed to be a prerequisite for cell death (Trump et al, 1980, 1984). To examine the effects of transmembrane calcium flux on the capsaicin-induced morphological changes, a series of experiments were conducted in which calcium ions in the incubation medium were replaced with magnesium. In all of the experiments, cytotoxicity was far less advanced in the absence of external calcium ions (Fig.R39). Mitochondrial swelling was not evident and, even during prolonged periods of application (> 30 mins) with high concentrations of capsaicin (10 μ M), the internal structure of the mitochondria was maintained (Fig.R39).

Some swelling of the endoplasmic reticulum and Golgi apparatus did occur; however this was far less than that found in the control nodose ganglia incubated with capsaicin (10 μ M) in the presence of external calcium.

Fig.R37.

An electron micrograph taken from a control nodose ganglion incubated in normal Krebs' solution for one hour at 27⁰ C, before fixation, showing a typical C-type neuron.

Nodose neurons are metabolically highly active and have a complex internal structure.

N = nucleus; M = mitochondria, ER = endoplasmic reticulum, G = Golgi apparatus, L = liposomal bodies, NM = Nuclear membrane, C = Unmyelinated fibres.

Scale bar = 2 μ m.

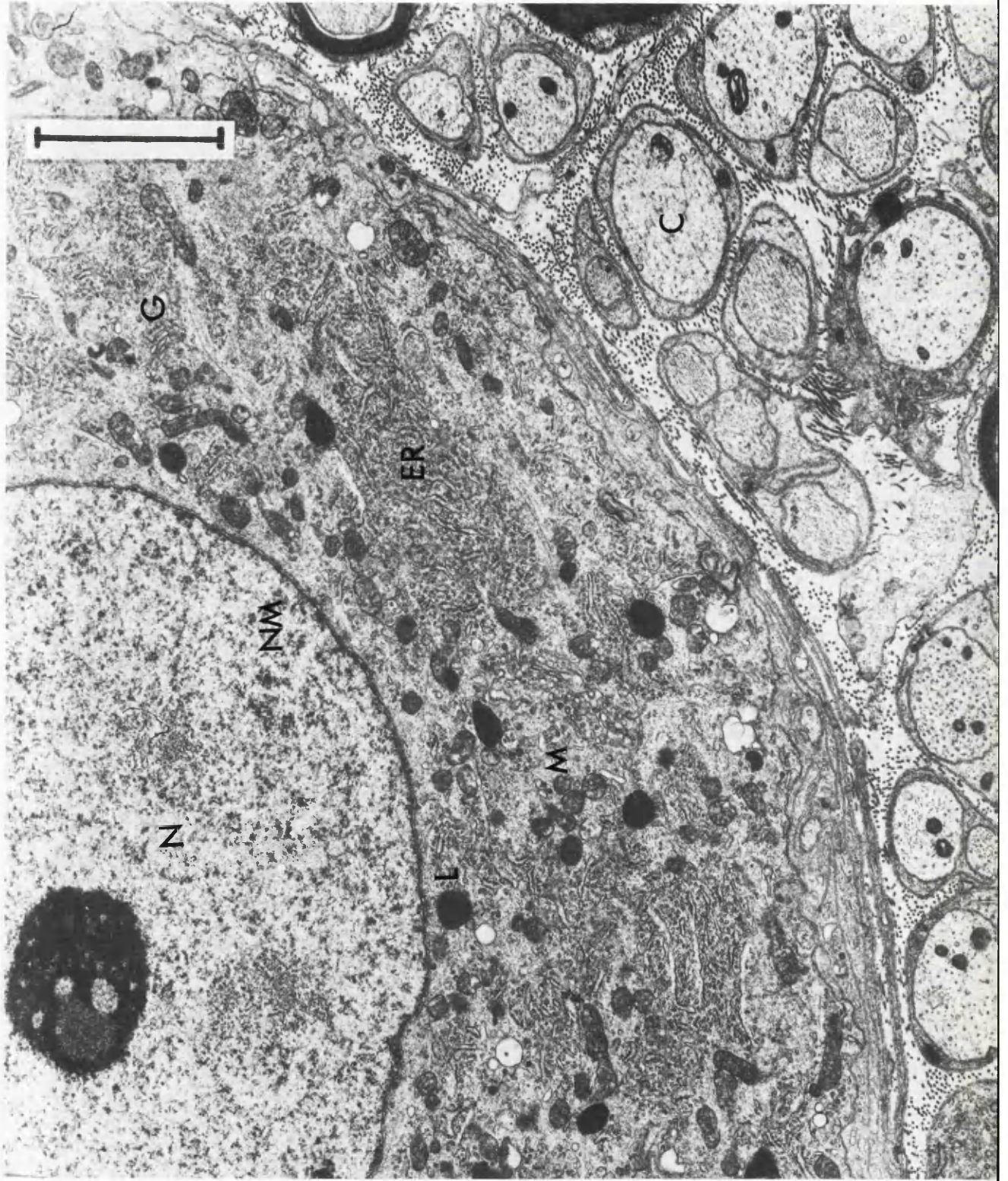


Fig. R3

Fig.R38.

Electron micrograph of a capsaicin-sensitive C-cell.

This shows the results of a 30 min incubation in normal Krebs' solution in the presence of capsaicin ($10\mu\text{M}$).

It is clear that the vacuolation noted under light microscopy results from extensive, high amplitude mitochondrial swelling (M). Both endoplasmic reticulum and Golgi apparatus are also subject to substantial swelling, distortion and fragmentation. The nucleus has become granular and the nuclear membrane is distorted.

Magnification = 22,500 *

Fig. R3

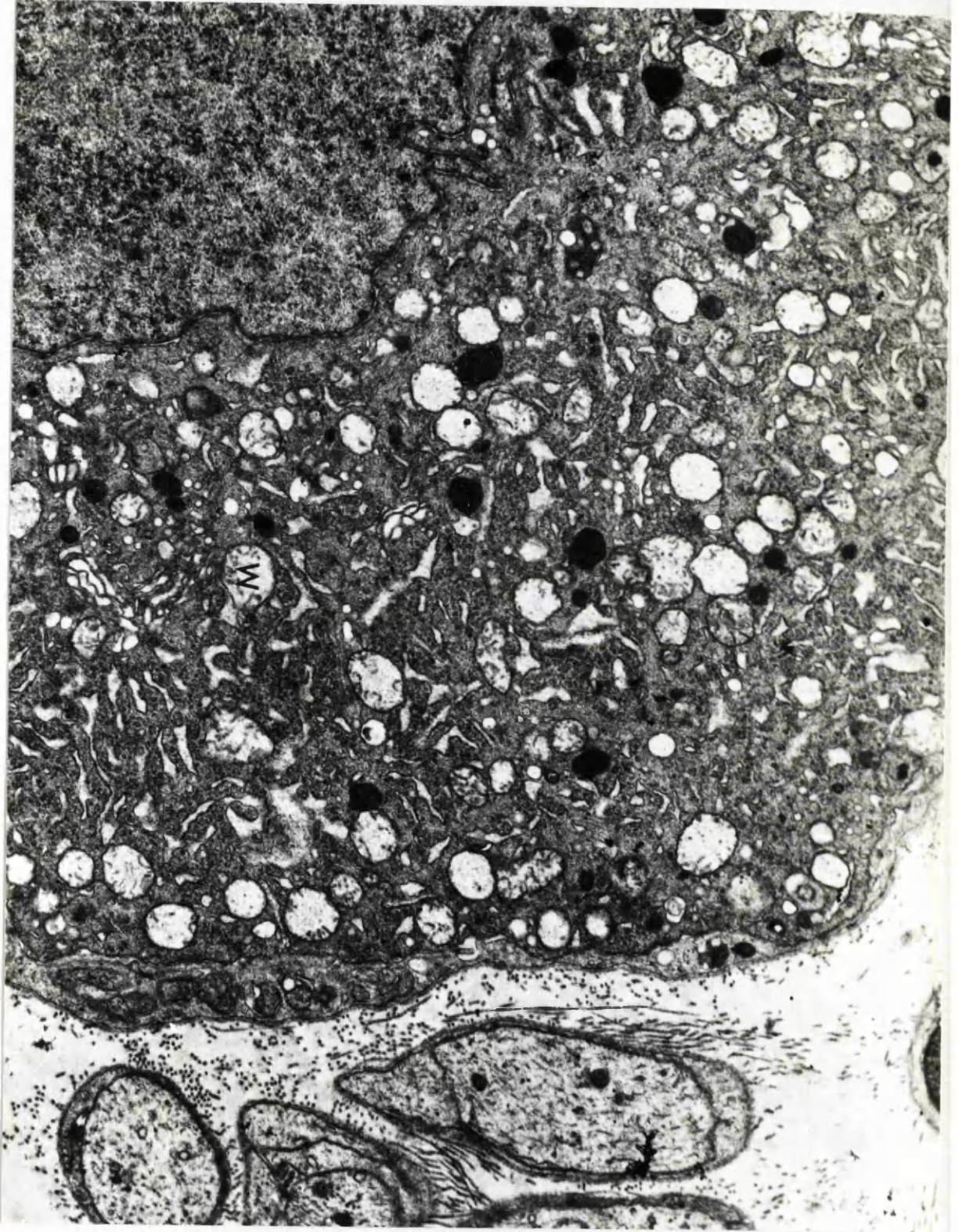


Fig.R39.

Electron micrograph of cytotoxic action of capsaicin in the absence of external calcium ions.

Nodose ganglia incubated in calcium-free (10mM magnesium) Krebs' solution (27°C) for 1 hour. Capsaicin (10 μ M) was added for the last 30 mins before fixation.

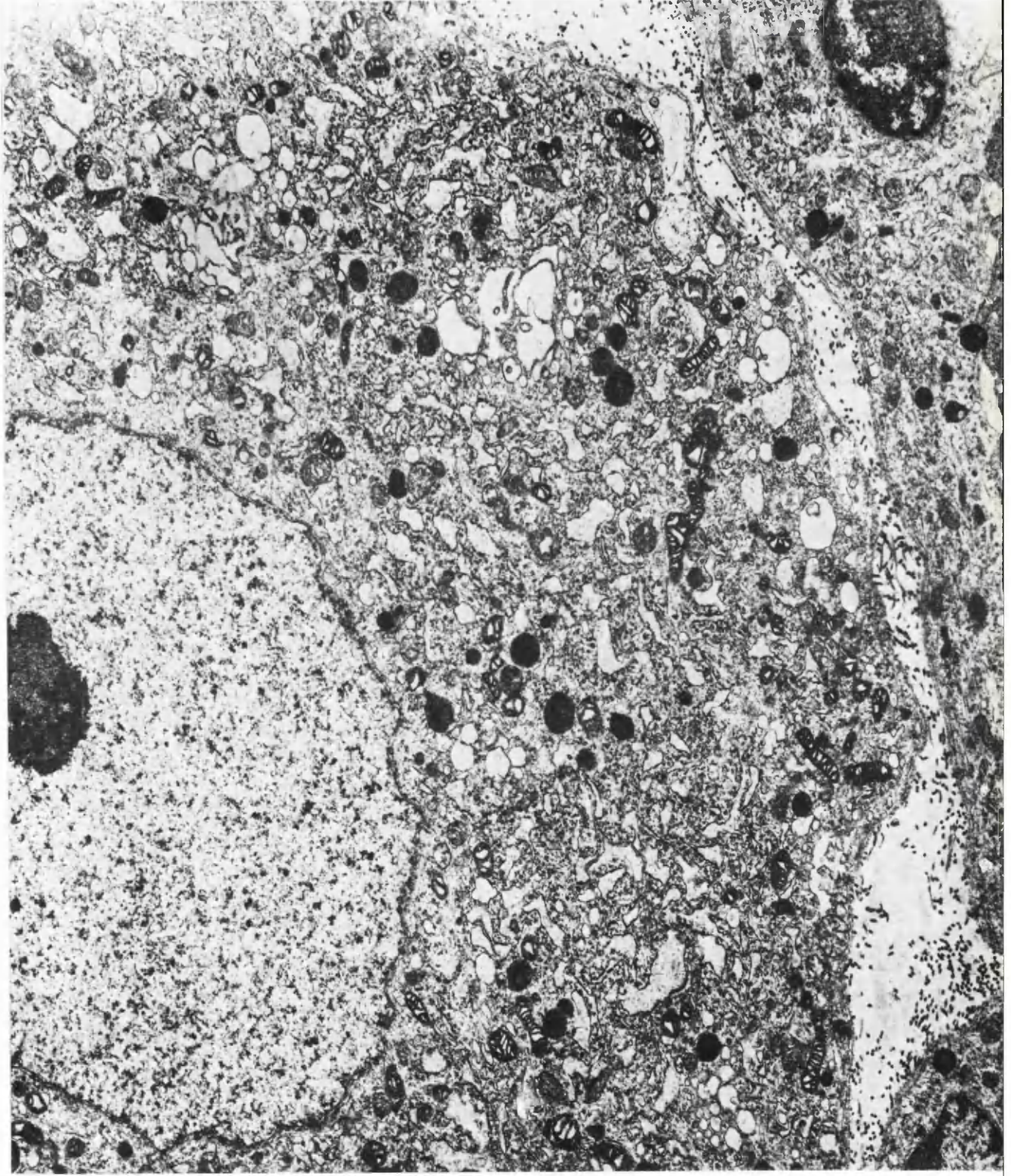
The cytoplasmic disruption induced by capsaicin is far less advanced, mitochondrial swelling is not apparent and effects on endoplasmic reticulum and other subcellular organelles are not so prominent.

Magnification = 22,500 *

Scale: 2 μ m = 4.5 cm



Fig. R3



The effects of A23187.

The calcium ionophore A23187 is a lipophilic compound capable of forming a reversible complex with calcium allowing calcium to be transported across cell membranes, a process driven by the transmembrane calcium concentration gradient.

A23187 has been used successfully to examine the pathological consequences of facilitated calcium influx in peripheral nerves (Schlaepfer 1977) where it was concluded that 'calcium influxes are determinants in the degeneration of peripheral nerve'.

The effects of A23187 ($10\mu\text{M}$) was investigated in three nodose ganglia and vagus nerves. A23187 induced morphological changes to the cytoplasmic structure identical to those produced by capsaicin (Fig.R40). However, unlike capsaicin A23187 did not appear to differentiate between subpopulations of neurones or axons, as some degree of cytotoxic damage was evident in all of the cells and axons scrutinised.

The effect of raised potassium solution.

Rat sensory neurones contain several voltage-dependent calcium currents (Peterson et al., 1989) that could be activated by the large depolarization induced by capsaicin in unclamped neurones.

Morphological consequences of persistent depolarization ^{were} investigated by elevating the potassium concentration in the incubating medium from 5.9 mM to 30 mM. No appreciable changes in mitochondria or endoplasmic reticulum in C-cells or oedematous swelling of the unmyelinated fibres of the vagus nerve was evident (Fig.R42).

Consistent with this result, the voltage-activated calcium channel blockers, cadmium ($200\mu\text{M}$) and gallopamil ($100\mu\text{M}$) failed to prevent or reduce the capsaicin-induced neurotoxic effects. The inference from these experiments is that calcium entry through voltage activated channels plays but a small part in the sensory neurotoxic process of capsaicin.

Fig.R40.

The effect of A23187 on sensory cell morphology.

A 30 min incubation in A23187 ($10\mu\text{M}$) resulted in a profound cytotoxic effect, which was essentially the same as that produced by capsaicin, with high amplitude swelling of mitochondria, endoplasmic reticulum and Golgi apparatus.

Magnification = 22,500 *

Scale: $2\mu\text{m} = 4.5\text{cm}$

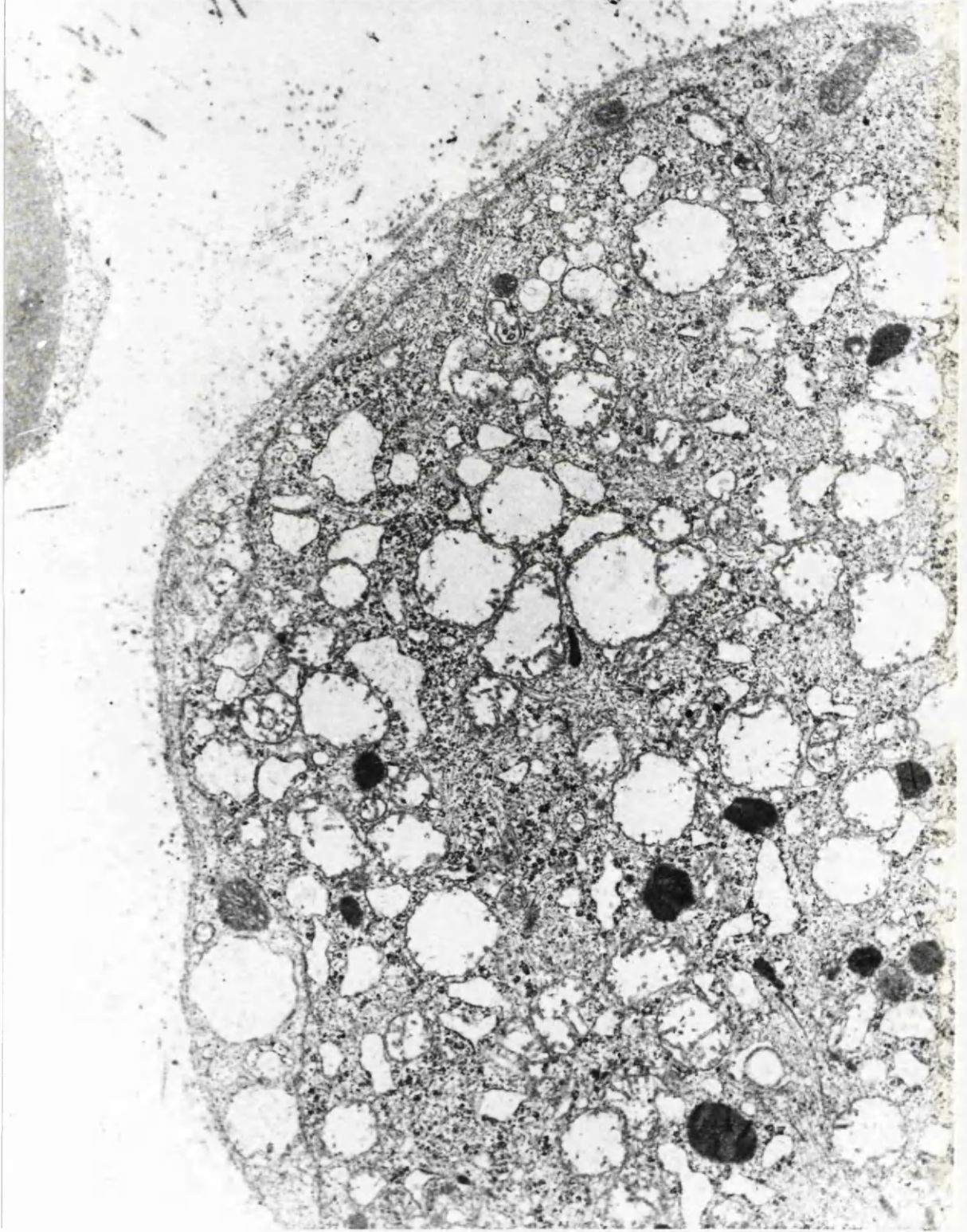


Fig. R40

Fig.R41.

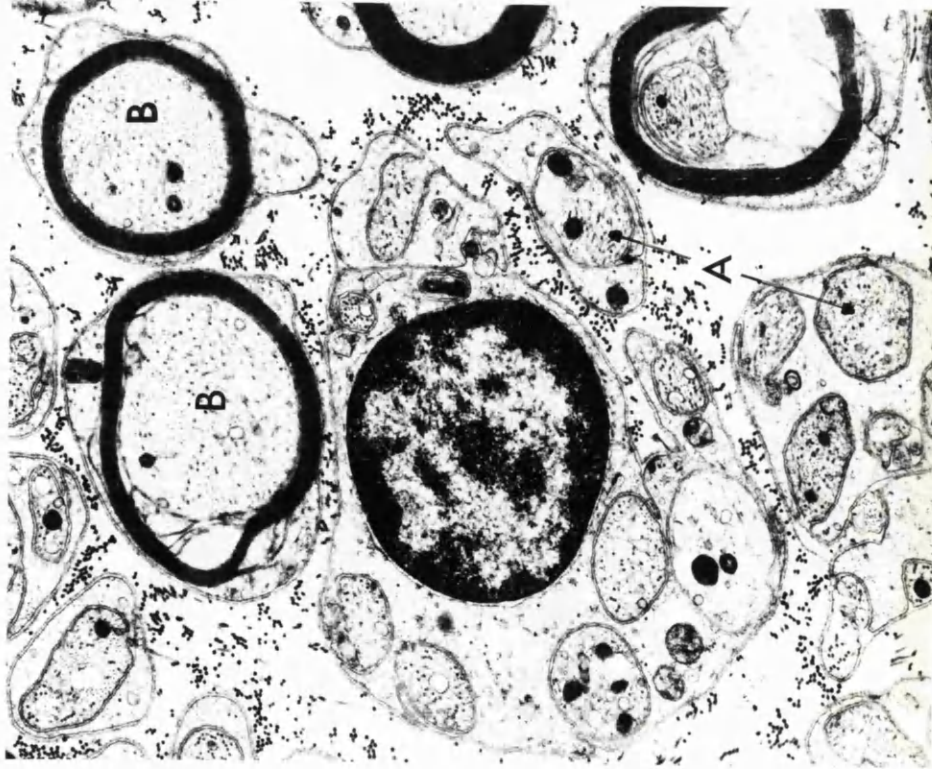
Electron micrographs of the neurotoxic effects of capsaicin on the unmyelinated fibres of the vagus nerve.

The unmyelinated axons (A) of the vagus nerve have a very small diameter ($< 1\mu\text{m}$) and several of these axons are often enclosed together, along their length, within a single Schwann cell sheath. Capsaicin (1 μM) induced large increases in the diameters of a population of unmyelinated C-fibres (C), with profound effects on the neurotubule and neurofilament system.

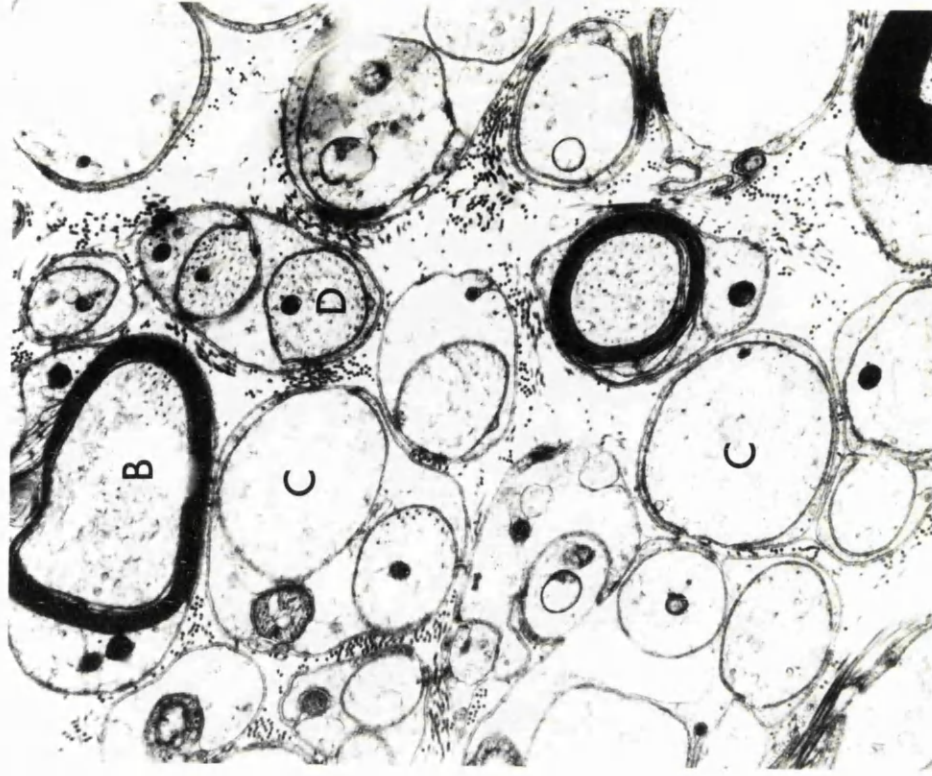
Other unmyelinated fibres (D) and myelinated fibres (B) within the same nerve bundle appeared to be unaffected.

Magnification = 16,000 *

A



B



capsaicin

Fig.R41

Fig.R42.

The effect of elevated potassium ions on the morphology of vagal unmyelinated fibres.

Capsaicin induces a large depolarization in non voltage clamped sensory C-type neurons and axons. In order to evaluate the morphological consequences of prolonged axonal depolarization, vagus nerves were incubated for 30 mins at 28°C in an elevated potassium solution, containing 30 mM KCl, isotonicly balanced by reducing NaCl.

Although slight swelling of the axons did occur, no cytotoxic changes in mitochondria and neurotubule system was evident.

Magnification = 16,000 *

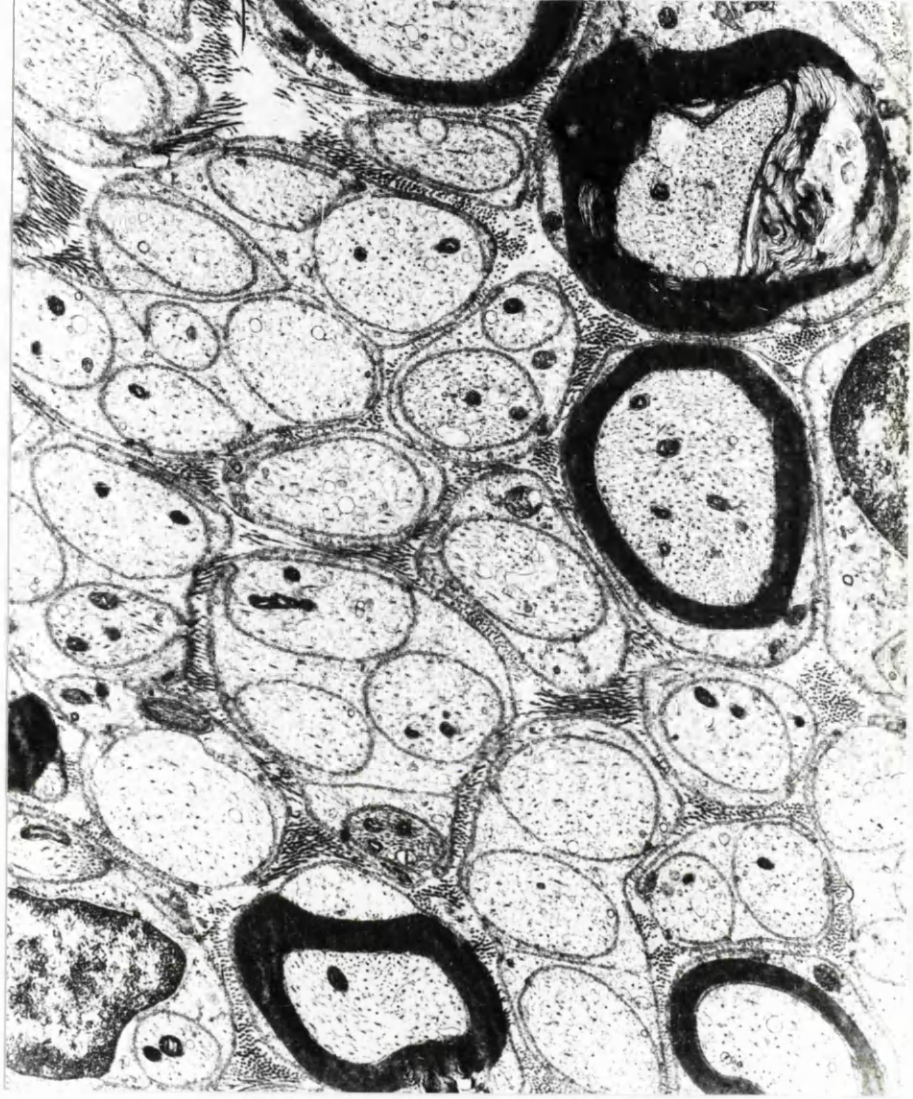


Fig. R42

Histological results discussion.

The results of the histological experiments conducted have shown that capsaicin can, in the same concentration range and over the same time-period required to induce a membrane cationic conductance increase, also induce substantial cytoplasmic damage to a subpopulation of sensory neurones. Further, the entry of calcium ions plays a major role in the cytotoxic process.

This appears to involve entry through capsaicin-activated channels rather than through voltage-gated channels since the histological changes were reduced by omitting external calcium but not by calcium channel blockers. Although an additional effect of capsaicin, not involving an increased membrane conductance, cannot be discounted, it seems likely that the cytotoxic process is contingent on the activation of cationic channels by capsaicin.

The question arises of whether the cytotoxic actions of capsaicin seen within this study would ultimately progress to cell death.

Trump et al (1981), using an ischaemic model, have defined morphological and biochemical stages that can lead to cell death. Following the cytotoxic insult, the initial stages are thought to be reversible and are summarized by a low amplitude swelling of endoplasmic reticulum and mitochondria. The calcium-dependent low-amplitude swelling of mitochondria results in the inhibition of mitochondrial phosphorylation and a reduction in ATP levels. Under these conditions, anaerobic metabolism of cell glycogen occurs, which results in the accumulation of both lactate and inorganic phosphate (from ATP hydrolysis) which decrease intracellular pH. The change in the intracellular pH is reflected by clumping of nuclear chromatin which is known to be associated with a decrease in RNA synthesis. If the pH of the cell continues to fall anaerobic glycolysis is also impaired as the enzyme phosphofructokinase is inhibited and the production of ATP decreases.

In the absence of sufficient ATP, the various energy-requiring processes such as protein synthesis and electrogenic ion pumps are inhibited. This leads to the intracellular accumulation of ions and then water (which osmotically redistributes across

the cell membrane) and is reflected in cell oedema.

As mitochondrial swelling increases, a stage where 'the point of no return' is achieved. High amplitude swelling of mitochondria appears at this stage; the mitochondria also contain tiny dense aggregates and the inner membrane is distended. This increases mitochondrial membrane permeability which releases the matrix enzymes and stored cofactors such as calcium. The high concentrations of free intracellular calcium ions can then activate many enzymes such as proteases, phospholipases and nucleases which can induce increases in cell membrane permeability and initiate karyolysis.

The role of calcium in capsaicin-induced cell death has long been a contentious issue, as it was not known if the accumulation of intracellular calcium ions was the cause or result of cell death (Jancso et al., 1978). The partial protective effect of removing calcium, together with the replicating effect of A23187 suggests that the induction of a transmembrane calcium ion influx by capsaicin is a prerequisite for the advanced stages of cytotoxicity.

Using the above criteria, it is evident that the cytotoxicity induced by capsaicin would eventually lead to cell death.

CHAPTER 6
GENERAL DISCUSSION AND SUMMARY

General discussion and summary

The experiments described in this thesis suggest the following sequence of events consequent upon application of capsaicin to visceral sensory neurones and unmyelinated fibres:

1. The primary effect is to increase Na and Ca conductance presumably by opening a set of cation-selective channels.
2. The influx of Na leads to a membrane depolarization, with a reversal potential of about +32 mV.
3. The influx of calcium has 2 consequences:
 - (1). It activates a Ca-dependent K-conductance and (probably) a Ca-dependent chloride-conductance.

This has 2 effects:

- (a). The initial depolarization is partly shunted, reducing the apparent reversal potential from +32 mV to -4 mV.
 - (b). It induces a variable-amplitude after-hyperpolarization with a reversal potential of about -68 mV.
- (2). The influx of Ca also induces a neurotoxic effect, leading to cytotoxic damage and cell death.

These effects are restricted to a subpopulation of visceral sensory C-type neurones and their axons. This suggests the presence of a site-specific capsaicin "receptor".

It is presumed that the initial depolarization may be responsible for the excitatory (irritant) effect of capsaicin and that calcium influx during this depolarization can initiate transmitter release both in the periphery and the spinal cord. Thereafter, action potentials are attenuated or blocked by the sodium-inactivation and increased

conductance, which may, in part, account for subsequent analgesia.

Since these experiments were completed, several studies by others have, in a substantial part, confirmed and extended the above inferences.

A capsaicin receptor ?

Both electrophysiological and binding data have shown that a reversible receptor site for capsaicin and its analogues exists (Szallasi and Blumberg, 1989). However it would seem unlikely that such a site would be constructed for 'chilli pepper' but for an endogenous ligand. The nature of this ligand is unknown. Selective antagonists at the capsaicin receptor site have yet to be described but it is with these compounds that the role of the capsaicin-receptor-ion channel complex in nociceptive pain perception needs to be pursued.

Ruthenium red is an organic dye that has been shown to reduce transmembrane calcium flux in neuronal tissue (Swanson et al., 1974) and also reduces a variety of capsaicin-induced responses, such as calcium uptake into dorsal root ganglion cells (Wood et al., 1988), CGRP release in the guinea pig ileum (Jin et al., 1990) and substance P release from the guinea pig urinary bladder (Maggi and Meli, 1988). No clear evidence is available, at present, if ruthenium red is acting as a capsaicin-receptor antagonist or simply blocking the capsaicin channel. However it would appear to act, functionally, as a capsaicin antagonist, so the use of this compound in experiments that monitor nociceptive pain induction might prove interesting.

A proton-activated inward current in cultured dorsal root ganglion neurones has recently been described (Bevan and Yeats, 1989). This is not only inhibited by ruthenium red and is absent in capsaicin-insensitive neurones but has identical single channel characteristics to that of the capsaicin channel (S. Bevan, personal communication). Within the histological discussion it was suggested that calcium-dependent mitochondrial impairment could lead to a decrease in intracellular pH; if this

proves to be so, then the initial cytotoxic effects of capsaicin could be compounded by additional capsaicin channel activation.

This result is also interesting, in that it shows that the capsaicin channel might be opened by means other than a direct agonist/receptor interaction and raises the question of whether the capsaicin channel might be activated during the transduction of other nociceptive stimuli such as intense heat or mechanical stimuli. Dray et al., 1990, using the intact spinal cord with the functionally connected tail preparation, have shown that ruthenium red can selectively attenuate the ventral root depolarizing responses induced by peripheral capsaicin receptor activation, sparing those induced by noxious heat, bradykinin or 5HT. This would appear to discount a common transduction mechanism for capsaicin and noxious heat, but it is evident from previous experiments that capsaicin fails to reduce the response to subsequent noxious heat stimuli (Bettany et al., 1988) which might be expected if they were mediated by a common pathway.

Maggi and Meli (1988), suggest that capsaicin-sensitive neurones may operate as a low threshold component of a two-part sensory system and that unless noxious stimuli were optimized, one could invariably obtain discrepancies when evaluating if the noxious stimuli are being transduced via capsaicin-sensitive neurones.

An analogous form of Ca-dependent neurotoxicity is produced by activation of central NMDA receptors. In vitro experiments on hippocampal and cerebellar slices (Meldrum and Garthwaite, 1990) have shown that NMDA-induced cell death not only has an absolute requirement for extracellular calcium ions but also is dependent on the concentration of excitant. The actions of NMDA can be reversed by the application of sub-lethal doses or the use of selective antagonists. It would appear, therefore, that a reversible sub-pernicious cytotoxic state exists.

It is suggested that excitatory amino acid-induced accumulation of intracellular calcium ions needs to reach a critical level (the point of no return ?) before cell death develops.

Measurements of excitatory amino acid induced-increases in intracellular free

calcium are limited, but both calcium-sensitive fluorescence indicators such as arsenazo III (MacDermott et al, 1986) and ion sensitive electrodes (Buhrle and Sonnhof, 1983) suggest that intracellular free calcium could rise to $10\mu\text{M}$ within two minutes, for moderate doses of NMDA or glutamate.

Although no measurements of capsaicin-induced increases in intracellular free calcium concentration were attempted in the present study, it is possible to estimate a rate of calcium ion flux from the voltage clamp data.

Assuming, under voltage-clamp, that the calcium conductance contributes 10% of the total current (0.5nA) measured at -50mV . The charge carried ($0.05 * 10^{-9} \text{ C} \cdot \text{sec}^{-1}$), which when converted to moles sec^{-1} [by simply dividing by the ionic valency (2) * Faradays constant (96495)] results in a transmembrane flux of $2.59 * 10^{-16} \text{ moles} \cdot \text{sec}^{-1}$. A $20\mu\text{m}$ diameter spherical cell has a cell volume ($4/3 \pi r^3$) of $4.2 * 10^{-9} \text{ cm}^3$ and therefore one would predict that calcium is accumulating within the cell at a rate of $60 \mu\text{M} \cdot \text{sec}^{-1}$. This calculation is obviously a gross over-simplification as it does not take into account the contribution of the cell's own buffering capacity or the fact that a histological assessment is not normally undertaken on voltage clamped neurones, but it could explain the large capsaicin-induced accumulation of ^{45}Ca ($12 \text{ mmole} / \text{L}$) in capsaicin-sensitive dorsal root ganglion neurones (Wood et al, 1988) and the increases in free calcium ($> 1\mu\text{M}$) measured by the fluorescent calcium indicator, Fura-2 (Dray et al., 1990). These increases in free intracellular calcium are equivalent to, or greater than, those associated with the excitotoxic death of hippocampal and spinal cord neurones.

The precise role of calcium in capsaicin-induced cell death of sensory neurones is obviously not known, but extrapolating from the excitotoxic example of the NMDA-induced cytotoxicity data, it is apparent that the levels of intracellular calcium achievable by capsaicin (albeit theoretical) would activate a multitude of calcium dependent enzymes such as proteases, protein kinase C (PKC), calmodulin (CaM), calmodulin-dependent protein kinases II (CaM KII) and phospholipase A_2 .

A role for each of these enzymes has been proposed in calcium-dependent cell

necrosis (Orrenius et al, 1989) but as of yet no evidence is available as to which, if any, of these enzymes are involved in capsaicin-induced cell death.

Capsaicin-induced increases in intracellular cGMP levels have been described (Winter et al., 1989).

Experiments by Robertson et al (1989) have also shown that the inward current induced by capsaicin in dorsal root ganglion neurones can reduce the amplitude of subsequently recorded whole-cell calcium currents. This process may involve a phosphatase enzyme or a channel inactivation mechanism that is activated by raised levels ^{of} intracellular calcium ions (Eckert and Chad, 1984). h

It is suggested that an electrophysiological study of cultured sensory neurones pretreated with sub-lethal doses of capsaicin might give an insight into the non-degenerative desensitization process suggested by Bevan and Szolcsanyi, 1990. By studying the enzymatic process induced by a potentially lethal capsaicin application, it might be possible, using specific enzyme inhibitors, to prevent cell death but maintain the reversible non-degenerative desensitised state of the nociceptive transduction mechanism, which is preferable if one is to extend the therapeutic uses of capsaicin treatment (Lynn, 1990).

Thus the present studies of the electrophysiological and histological effects of capsaicin on vagal nerve fibres and nodose sensory neurones, has allowed the proposition of a hypothetical unifying excitotoxic mechanism, which is compatible with subsequent studies.

Epilogue

The first competitive antagonist of capsaicin-induced responses has been recently described by Bevan et al. (1991). Capsazepine (2-[2-(4-chlorophenyl)ethylamino-thiocarbonyl]-7,8-dihydroxy-2,3,4,5-Tetrahydro-1H-2-benzapine) was found to competitively inhibit (Schild Plot, Slope = 1) both capsaicin-induced $^{86}\text{Rb}^+$ efflux from dorsal root ganglion neurones ($K_d = 100\text{nM}$) and ^{14}C -guanidinium efflux from vagus nerve ($K_d = 690\text{nM}$). Capsaicin-induced membrane current and $^{45}\text{Ca}^{2+}$ uptake and accumulation into dorsal root ganglion neurones was also antagonised.

The ability of capsazepine to inhibit capsaicin-induced responses in a wide range of *in vitro* and *in vivo* preparations was undertaken by Dray et al (1991) and Dickenson et al. (1991). The results confirmed the effectiveness of this compound seen in the electrophysiological and ion-flux studies and supports the idea that capsaicin interacts with a specific membrane receptor.

Bleakman et al, (1991) have used a fluorescent Ca^{2+} indicator (fura-2) to monitor capsaicin-induced changes in intracellular free calcium concentration $[\text{Ca}^{2+}]_i$. Capsaicin (1 μM) applied to voltage-clamped cultured dorsal root ganglion neurones, increased membrane conductance with a concomitant increase in $[\text{Ca}^{2+}]_i$ from a basal level of 125nM to a peak of 600nM, within 1 minute. They also found that this increase in $[\text{Ca}^{2+}]_i$ was capable of decreasing the amplitude of a simultaneously recorded whole cell voltage-activated calcium current (I_{Ca}). The inhibition of the calcium current was not, however, wholly dependent on an increased $[\text{Ca}^{2+}]_i$ as some inhibition persisted when barium ions were used as the charge carrier or when the cell was loaded with a high affinity calcium buffer, BAPTA.

Docherty et al, (1991) found that substituting barium or magnesium for calcium ions prevented the long-term inhibition of the whole cell calcium current and concluded that the inhibition of voltage-activated calcium current by capsaicin was secondary to an increased $[\text{Ca}^{2+}]_i$ mediated by activation of a cation selective membrane conductance by capsaicin and that this long-lasting inhibition of I_{Ca} might contribute to the analgesic actions of capsaicin through inhibition of neurotransmitter release.

Appendix 1.

Equation (2D) describes the relationship of the theoretically derived ED₅₀ values for depolarization to the agonist-induced conductance ratio and the dissociation constant Ka.

Eqn. (2D) .

$$ED_{50} = K_a * \frac{G}{G + g_{MAX}}$$

G = Resting conductance
g_{Max} = Maximal agonist induced conductance.
K_a = dissociation constant

The ED₅₀ value is the agonist concentration (x) that produces a 50% maximal depolarization and can be defined by combining Equations (2B) and (2D) and solving for (x).

ie $\frac{V}{V_{max}} = 0.5$ ED₅₀ = Agonist concentration (x) producing V amplitude depolarization.

Eqn. (2B) $V = (e-E) \frac{g}{G + g}$

Eqn. (2D) $V_{max} = (e-E) \frac{g_{Max}}{G + g_{Max}}$

So:

Eqn. (3A) . $\frac{V}{V_{max}} = 0.5 = \frac{(e-E) \frac{g}{G + g}}{(e-E) \frac{g_{Max}}{G + g_{Max}}}$

Therefore:

$$\text{Eqn. (3B).} \quad \frac{g}{g + G} * \frac{g_{\text{Max}} + G}{g_{\text{Max}}} = 0.5$$

Dividing by g .

$$\text{Eqn. (3C).} \quad \frac{1}{1 + \frac{G}{g}} * \frac{g_{\text{Max}} + G}{g_{\text{Max}}} = 0.5$$

From equation (2C) define g .

$$\text{Eqn. (2C).} \quad \frac{g}{g_{\text{Max}}} = \frac{x}{x + K_a}$$

Dividing by x and rearranging.

So:

$$\text{Eqn. (3D).} \quad g = \frac{g_{\text{Max}}}{1 + \frac{K_a}{x}}$$

Substitute for g in Eqn.(3C) and rearrange.

$$\text{Eqn. (3E).} \quad \frac{1}{1 + \frac{G * (1 + k_a / x)}{g_{\text{Max}}}} * \frac{g_{\text{Max}} + G}{g_{\text{Max}}}$$

Redefine 1 as $\frac{g_{\text{Max}}}{g_{\text{Max}}}$, invert divisor and multiply.

$$\text{Eqn. (3F).} \quad \frac{g_{\text{Max}}}{g_{\text{Max}} + G * (1 + K_a / x)} * \frac{G + g_{\text{Max}}}{g_{\text{Max}}} = 0.5$$

Therefore:

$$\text{Eqn. (3G). } \frac{G + g_{\text{Max}}}{g_{\text{Max}} + G * (1 + K_a/x)} = 0.5$$

And:

$$\text{Eqn. (3H). } 2G + 2 g_{\text{Max}} = g_{\text{Max}} + G + G * k_a / x$$

Solve for value of x.

$$\text{Eqn. (3I). } 2G + 2g_{\text{Max}} - g_{\text{Max}} - G = G * K_a / x$$

$$\text{Eqn. (3J). } \frac{G + g_{\text{Max}}}{G} = \frac{K_a}{x}$$

$$\text{Eqn. (3K). } x = K_a * \frac{G}{G + g_{\text{Max}}}$$

Where $x = \text{ED}_{50}$

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