An electrophysiological investigation of colonic afferent sensitivity in the rat and mouse – *in vitro*

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General Abstract

1. Two novel *in vitro* preparations were developed from which recordings were made from colonic afferents in the rat and the mouse. Fibres with endings in the mucosa are described, along with those in muscle and serosa, and their responses to a range of mechanical and chemical luminal stimuli.
2. Mechanosensitivity was investigated. Stimuli included stretch, mucosal blunt probing and stroking (Von Frey hairs 10-1000mg). 52 fibres were recorded for investigating classification criteria. 12 showed characteristics consistent with the literature of endings in the mucosa, 10 in the muscle and 27 in the serosa. 3 fibres were not mechanosensitive but were chemosensitive.
3. Chemosensitivity was investigated. Stimuli were applied into a ring placed over the mechanoreceptive field of the fibre. 24 serosal, 6 muscular and 20 mucosal fibres were investigated. Mucosal, muscular and serosal fibres were chemosensitive and responded to >1 chemical stimulus. Muscular fibres responded to chemical stimuli independently of muscular activity.
4. Stretch sensitivity was investigated in all classifications of fibres. 0 fibres responded to graded tension, but 1/6 serosal fibres responded to graded length. 2/2 muscular fibres demonstrated a discharge pattern that was most closely correlated to the acceleration phase of an oscillating stretch stimulus.
5. Fibres with multiple receptive fields were investigated. Four fibres showing characteristics of serosal fibres had 2-3 punctate receptive fields otherwise all fibres had single receptive fields.
6. The response to NaCl 308mM in 15 fibres (6 mucosal, 9 serosal) was potentiated in the presence of endogenous prostaglandins (after indomethacin removal). 1/13 fibres responded to mucosal application of PGE_2.
7. A novel *in vitro* preparation of mouse distal colon was developed. 5 recordings were made. Classification criteria appropriate for the mouse are described. Afferents were generally chemosensitive.
8. This is the first characterisation of colonic afferent fibres in an *in vitro* preparation both in the rat and the mouse and first documentation of afferent fibres in colonic mucosa, luminal chemosensitivity of serosal afferents and

potentiation of chemical stimuli by endogenous prostaglandins in the gut. This study contributes significantly to the understanding of normal sensation in the colon.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Penelope Ann Lynn

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Publications Arising From this Thesis

Lynn P.A., Blackshaw L.A., (1999) *In vitro* recordings of afferent fibres with receptive fields in the serosa, muscle and mucosa of rat colon. *Journal of Physiology 518(1):271-282.*

Lynn P.A., Blackshaw L.A. (1998) Colonic afferents sensitive to mechanical and chemical luminal stimuli- *in vitro. Functional Dyspepsia and Irritable Bowel Syndrome- Falk Symposium 99.* Kluwer Academic Publishers. Ch. 8:67-69.

The role of prostaglandins in the hypertonic saline-induced discharge of serosal and mucosal afferents in the rat colon. *(in preparation)*

Lynn P.A., Blackshaw L.A., (1999) A role for prostaglandins in colonic afferent sensitivity-*in vitro. Gastroenterology 116:A959 (abst)*

Lynn P.A., Blackshaw L.A. (1998). Colonic afferents respond to mechanical and chemical stimuli *in vitro. Neurogastroenterology and Motility 10(1):84 (abst).*

Blackshaw L.A., Page A.J., Lynn P.A., (1997). Sensitivity of gastrointestinal spinal afferents determined *in vitro. Gastroenterology 113(4):642 (abst).*

Berthoud H.-R., Lynn P.A., Blackshaw L.A. (2000). Primary afferents with multiple receptive sites in rat colon and stomach. *(accepted Gastroenterology)(abst).*

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Chapter 1. Introduction

1.1 Sensation In the Colon- Anatomy and Basic Physiology

1.1.1 Overview

Sensation in the colon has been subject to investigation for many years. Models of interest have ranged from human perception studies to small animal models of peripheral afferent sensitivity and cell culture work. Yet despite a large body of work, understanding of sensation from the colon has been restricted by the use of too few adequate stimuli. Colonic sensation has been consistently approached using distension of the colon as the predominant or sole adequate stimulus. This is also evident in the literature on primary afferents innervating the colon. Spinal afferents innervating the colon have been characterised previously using the mechanical stimulus of distension (Blumberg *et al.*, 1983; Janig & Koltzenburg, 1991; Sengupta & Gebhart, 1994; Su & Gebhart, 1998). Classifications of afferent fibres have also been based on the responses of spinal afferents to colonic distension. Overall the responses of distension-sensitive fibres to distension and to some chemicals at both physiological and noxious levels have been well documented. In contrast, investigation and classification of afferents in the upper gastrointestinal tract uses a greater number of adequate stimuli including stroking of the mucosa, probing of the tissue and often chemosensitivity. The understanding of the vagal innervation of the upper gastrointestinal tract and circumstantial evidence for other classes of fibres in the colon suggested the necessity for an alternative systematic approach to colonic sensation. This thesis documents the development of a novel *in vitro* technique for the study of colonic sensation in the rat and subsequently in the mouse. In addition, properties of colonic afferents in the rat model relative to previously documented populations of colonic spinal afferents were investigated. In particular, the potential for afferents innervating the colonic mucosa was pursued and the possibility that they could be subject to the same functional classification as upper gastrointestinal vagal afferent fibres. The role of prostaglandins in afferent chemosensitivity was also investigated.

1.1.2 Anatomy of the Colon

Structural and functional differences exist along the length of the colon such that it can be separated into different segments, most easily defined as proximal, mid and distal colon. However, it is not clear in the literature what delineates the distal colon from the mid colon and the rectum in the rat, the area of primary interest in this thesis. Functionally, the regional segments differ, one example being the decrease of sodium and water absorption distally (Binder & Sandle, 1994). This thesis is confined to the study of the distal portion of the colon, but because there is not an adequate standard to define the regions, it has been necessary to create our own definition from the literature. Therefore the decision was made that 4-5cm of colon proximal to the pelvic rim in the rat constituted distal colon.

The luminal surface is made of an epithelium from mainly absorptive cells arranged in crypts (Christensen, 1991). Directly underneath is the lamina propria, under which the muscle cells of the muscularis mucosae lie. The submucosa is the widest portion of the colon, in which the two-layer submucosal plexus can be found. The myenteric plexus lies between the inner circular layer of muscle and the outer longitudinal muscle layer. The serosa, consisting of squamous epithelial cells forms the outer layer of the distal colon.

1.1.3 Innervation of the Colon

Three peripheral neural pathways supply the gastrointestinal tract; the vagal nerves, the spinal nerves (intermesenteric and splanchnic nerves) and the sacral nerves (pelvic and hypogastric nerves). At the level of the distal colon in the rat the combined intermesenteric and splanchnic nerves supply a similar proportion of innervation to that of the pelvic nerve (Luckensmeyer & Keast, 1994). The vagal innervation completes the neural supply to the colon and is proportionally the least significant, the parasympathetic innervation in the distal colon being mostly supplied by the pelvic nerves. Indeed, the vagal innervation progressively decreases down the length of the gut, having less motor influence (Dapoigny *et al.*, 1992) and afferent density (Berthoud *et al.*, 1990).

1.1.4 Anatomy of Afferent Endings

Afferents in the gut largely have unspecialised endings (Cervero, 1994). Indeed, vagal afferents have terminal arborizations ending around the crypts and lamina propria of the villi in the rat duodenal mucosa (Berthoud *et al.*, 1995) and terminal endings that run parallel and in apposition to muscle fibres in the muscle layers of the fundus (Berthoud & Powley, 1992). In addition, afferents in spinal nerves have similar endings in the circular muscle and in the lamina propria and squamous epithelium of the cat oesophagogastric junction (Clerc & Mazzia, 1994). Mechanosensitivity is presumably mediated through deformation of the endings of afferents either within the muscle or the mucosa (and other layers in which afferents have their endings). Chemosensitivity of afferents can be mediated either directly through receptors on the nerve fibre, or through secondary mechanisms such as endocrine or caveolated cells positioned in the epithelium that release mediators in response to luminal stimuli (Mei, 1985).

1.1.5 Anatomical Pathway of Distal Colonic Afferents

The anatomy will be described from the perspective of the afferent pathway (Fig 1-1). The afferent fibre has a receptive field at a site in the gut wall. The afferent fibre then passes through the lumbar colonic nerves that are juxtaposed with the blood vessels supplying the distal colon. The lumbar colonic nerves join together into a bundle about 1cm proximal to the pelvic rim at the level of the abdominal bifurcation, along with the blood vessels. The afferent fibres enter the inferior mesenteric ganglion (IMG). Those that are intestinofugal fibres (with their cell bodies in the myenteric plexus) end in the IMG. Those that are primary afferent fibres pass through the IMG into what is then called the intermesenteric nerve. This nerve contains the lumbar splanchnic nerves, and the efferent contribution from the coeliac ganglion (Harris, 1943). Two small strands of lumbar splanchnic nerve soon diverge from the main bundle, projecting between the abdominal bifurcation of the aorta primarily into L5 (Harris, 1943). This projection contains a negligible number of afferent fibres in the rat (Baron & Janig, 1991). The majority of afferent fibres remaining in the intermesenteric nerve project into L1 and L2 (Baron & Janig, 1991).

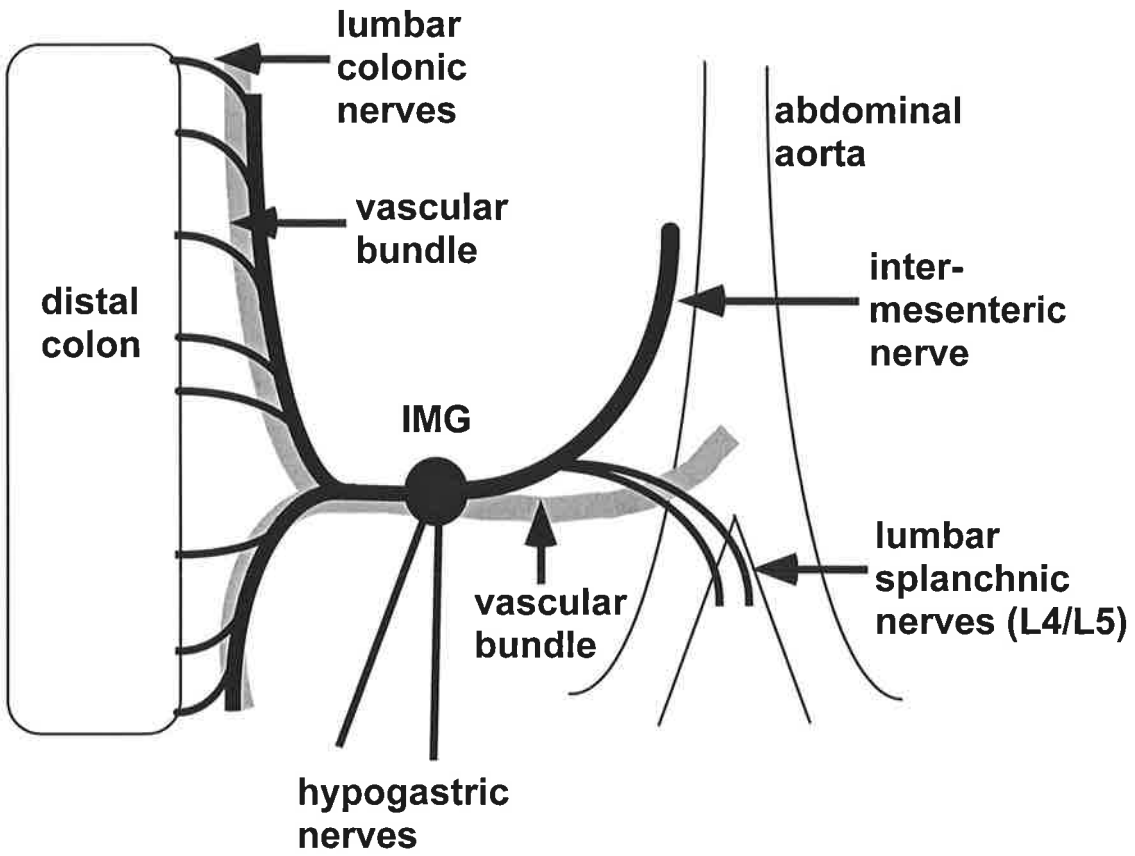

Figure 1-1. Anatomy of the rat distal colon and its neural innervation. 4-5 cm of colon proximal to the pelvic rim is shown. Lumbar colonic nerves join a larger neurovascular bundle which projects to the inferior mesenteric ganglion (IMG). Central to the IMG the intermesenteric nerve bundle contains the lumbar splanchic nerves and nerves projecting up to the superior mesenteric ganglion. Two branches of the lumbar splanchnic nerve separate from the bundle at the level of the abdominal bifurcation of the aorta and project to L4 and L5. The continuing intermesenteric nerve contains lumbar splanchics projecting to L1, L2 and L3.

The vagal innervation of the distal colon (as sparse as it is) also projects through the IMG (Berthoud & Powley, 1993). Thus, in the intermesenteric nerves there is also a very small population of vagal fibres.

The intermesenteric nerve containing the lumbar splanchnic nerves supplying the distal colon contains approximately 1500 afferent and 1250 efferent fibres in the male rat (Baron & Janig, 1991). This abundance of afferent fibres demonstrates the importance of sensory input from the colon, despite the fact that this information rarely reaches conscious perception. In contrast, the pelvic nerve has a predominance of efferent fibres (3195) compared to afferent fibres (1675) in the male rat (Hulsebosch & Coggeshall, 1982). The proportion of vagal afferent fibres compared to efferents supplying the distal colon is unavailable in the literature. There is distinct sexual dimorphism in the spinal innervation of the pelvis in the rat, but this is confined to the efferent innervation; the afferent innervation is comparable between the sexes (Nadelhaft & McKenna, 1987).

Primary afferent fibres supplying the distal colon through the intermesenteric nerve bundle have their cell bodies in the dorsal root ganglia and project to the spinal cord primarily in laminae I and II of the dorsal horn and Lissauers tract (De Groat, 1986). Primary afferents contain many peptides. Dorsal root ganglion cells show immunoreactivity to substance P, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), bombesin, gastrin-releasing peptide and calcitonin gene related peptide (CGRP) (Dockray & Sharkey, 1986).* The majority of studies investigating the contents of afferents involve the study of the dorsal root ganglion of which visceral afferents comprise only a small proportion. Thus, it is less appropriate to extrapolate the results of these studies to subpopulations of visceral afferents than other larger populations, such as cutaneous afferents. However, by a combination or retrograde labelling of the pelvic nerve and immunohistochemistry, dorsal root ganglion cells of visceral afferents in the cat specifically contain VIP, substance P, CCK, leucine and methionine enkephalin but relatively little somatostatin (Kawatani *et al.*, 1986). In addition, they may contain other peptides such as CGRP (De Groat, 1986).

* These transmitters are those presumably involved in transmission from primary afferents in the spinal cord.

1.1.6 Afferent Function

The role of the afferent fibre is to provide a full scope of sensory information to the nervous system where it is then processed and subsequently used to modulate behaviour of the gut itself or of the whole body, whether or not that sensation reaches consciousness. It is necessary to sense many aspects of environment, but particularly those related to the function of the organ in question in order to function efficiently. Therefore, in the gastrointestinal tract it is important to sense the makeup of the luminal contents, the volume of those contents, the mechanical activity and movement of the muscle, the inflammatory state of the tissue and noxious events. It is not always necessary for this environment to be sensed directly. Rather, it can be sensed through secondary mechanisms such as mast cell degranulation (Akoev *et al.*, 1996) and changes in local blood flow (Holzer *et al.*, 1991). In addition, normal sensation can be modulated by mediators such as prostaglandins which can sensitise afferents (Maubach & Grundy, 1999). Having endings in different layers of the gut wall enables the gut to respond to a wide range of chemical and mechanical stimuli simply by virtue of the depth of location of the afferent fibre ending allowing free access to different stimuli. Therefore, with the many different combinations of mechanisms for sensing the state of the environment, it is possible to create a full sensory picture of the events in the gut. This sensory picture enables an appropriate response in the organ or individual that can be initiated either through intrinsic pathways or extrinsic neural pathways respectively.

1.1.7 Reflex Control of Distal Colonic Function

Two sources of control exist for the distal colon, as for the whole gastrointestinal tract. The intrinsic nervous system, comprising the myenteric and submucosal plexuses provide the local control (Kunze & Furness, 1999). The extrinsic nervous system, comprising the vagal and spinal nerves mediate control from the central nervous system. The most local level for neural integration and feedback is in the enteric ganglia. The next level of integration exists in the IMG. Intestinofugal fibres (with their cell bodies in the myenteric plexus) project sensory information to

the IMG (Bywater, 1994) along with collaterals from primary afferent fibres passing through the IMG (Keef & Kreulen, 1990). Thus, reflex loops exist independently of the central nervous system. Two theories of central nervous system interpretation of sensory information from the gut have emerged (Cervero, 1994). The specificity theory, assumes that afferent fibres are heterogeneous, with subpopulations responding to specific stimuli. This includes a role for a specific nociceptor. The pattern and intensity theories rely instead on the pattern of integration in the central nervous system or a threshold of activity in the afferent fibres signifying a change in the sensation that should be experienced. The skin has specific nociceptors (McMahon & Koltzenburg, 1990), but the gut appears not to and many afferent fibres are polymodal. Thus, it is probable that either pattern or intensity summation (or a combination of both) is employed in gastrointestinal sensation rather than the specificity of afferent fibres.

1.2 Sensation in the Gut - Electrophysiological Investigations

1.2.1 Functional Classification of Afferent fibres

An established experimental tool used in the study of visceral sensation is electrophysiology - that is, the study of the activity of a single nerve fibre, a single nerve cell body or the combined activity of a number of fibres together in a strand. These methods of investigation have provided the information to classify groups of nerves on the basis of their pattern of response to various stimuli, be they mechanical or chemical. However, there have been clear differences within the literature in the approach to classification, and the choice of stimuli used. These differences are most obvious between the study of the vagus and the spinal nerves, and because of their relative innervation, the upper gastrointestinal tract and the colon.

The vagal and spinal nerves can be differentiated on the basis of the sensations that they are thought to mediate. In general terms the vagal nerves are involved in non-painful sensations and the spinal nerves, painful sensations (Grundy, 1994). Therefore, much of the literature on vagal afferent fibres contains a broad range of

stimuli used to investigate sensation including noxious and physiological mechanical and chemical stimuli in functional studies. On the other hand much of the investigation into spinal afferent fibres centres on pain thresholds or noxious stimuli.

Nonetheless, between the two approaches it is possible to classify afferent fibres functionally, with reference to the depth of the afferent ending in the gut wall (Grundy, 1988). In addition, functional classification of vagal and spinal afferent fibres innervating the lower oesophageal sphincter of the cat is identical when similar investigative techniques are used (Clerc, 1984). Thus, there are three main classifications that have been put forward; those fibres that have their endings in the mucosa, those with their endings in the muscle layers, and those with their endings in the serosa and mesentery (Fig 1-2). They are traditionally defined by their response to mechanical stimuli. Mucosal fibres respond to stroking or shearing stimuli but not to stretch of the tissue (Blackshaw & Grundy, 1993*a;* Clarke & Davison, 1978; Clarke & Davison, 1974; Davison, 1972; Harding & Leek, 1972*a;* Iggo, 1957; Leek, 1972; Page & Blackshaw, 1998). Muscular fibres do not respond to stroking but rather respond in a linear fashion to the degree of distension or contraction of the gut (Blackshaw & Grundy, 1990; Blackshaw & Grundy, 1993*b;* Clarke & Davison, 1975; Clerc & Mei, 1983; Cottrell & Iggo, 1984*b;* Cottrell, 1984*b;* Harding & Titchen, 1975; Harding & Leek, 1972*a;* Iggo, 1955) and were first described by Paintal in 1953 (Paintal, 1973). Serosal fibres respond to probing of the receptive field and may respond to distension with a rapidly adapting response, particularly at noxious levels of distension (Blumberg *et al.*, 1983). Vagal fibres include mucosal and muscular fibres, but muscular fibres that have a low threshold of response. This is in keeping with the idea that the vagal fibres sense the normal environment: the luminal contents and normal muscular activity. Serosal vagal afferent fibres are rarely encountered and have been documented in only one study (Clerc & Mei, 1983). The spinal afferent fibres include muscular fibres said to have both high and low thresholds (Sengupta & Gebhart, 1994) and serosal and mesenteric fibres. This is in keeping with the idea that spinal visceral afferent fibres (particularly thoracolumbar afferent fibres) may sense noxious events rather than sensing the normal gastrointestinal environment. Mucosal

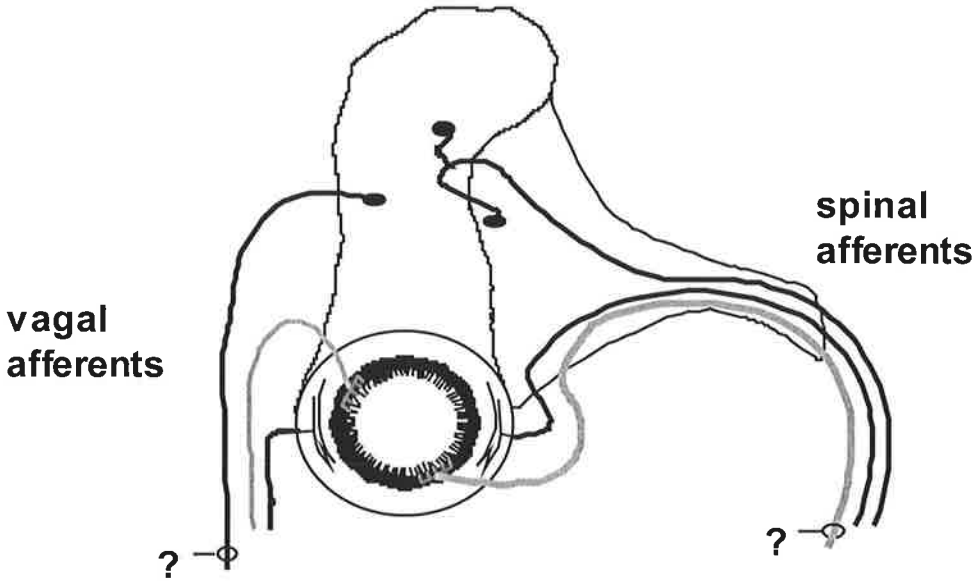

Figure 1-2. Receptive fields of extrinsic afferents in the gastrointestinal tract. Vagal afferents have receptive fields in the mucosa and the muscle of the gut. Serosal fibres in the vagal innervation are less well documented. The spinal afferents have endings in the serosa and mesentery as well as the muscle. Mucosal fibres have not been adequately described in the spinal innervation of the gut. Functional classification of these fibres can be achieved as their mechanosensitivity reflects their depth within the gut wall.

fibres have been documented in spinal afferent fibres in the cloaca of the duck (Koley *et al.*, 1984), the anal canal of the cat (Clifton *et al.*, 1976; Janig & Koltzenburg, 1991) and the rat (Sengupta & Gebhart, 1994). With the exception of the cloacal mucosal fibres, these were spinal pathways through the sacral nerves, not thoracolumbar nerves. The cloacal mucosal fibres had very high mechanical thresholds not consistent with the classification criteria used for mucosal fibres in vagal pathways. Therefore, mucosal fibres fitting the functional criteria used for vagal mucosal fibres have not yet been documented in the thoracolumbar innervation of the gut.

Visceral sensation is relatively under-explored in comparison to cutaneous sensation. Much of the terminology originally used in the study of cutaneous sensation has been subsequently translated or adapted for visceral sensation. In the main part this has been beneficial, but has caused a number of biases to be incorporated into the study of visceral sensation. One such bias arises from the cutaneous nociceptor, receptors designed to transmit exclusively noxious sensations in response to noxious stimuli. There have as yet been few reasons to part from the term nociceptor or the mindset that surrounds the spinal innervation of the gut. Slowly it is being acknowledged that the mechanism for pain in the gastrointestinal system may not mirror those mechanisms for pain in the cutaneous system. This is particularly so with respect to the prevalence of polymodal fibres being identified in the gut in contrast to the highly specific fibres found in the skin (McMahon & Koltzenburg, 1990), suggesting that afferent fibres that signal normal functional events also signal pain in noxious environments (Janig *et al.*, 1993). Thus, with the focus skewed to investigating pain at the expense of non-noxious sensation, there has not been an exhaustive functional classification of the spinal afferent fibres of the gut - particularly of the colon.

1.2.2 Anatomical Support for Functional Classification

There is sound anatomical support for the functional classifications of the vagal innervation. Vagal afferent fibres have been shown to have endings in the muscle (Berthoud & Powley, 1992) and in the mucosa (Berthoud *et al.*, 1995), determined

by retrograde labelling of the vagus by injection of DiI into the nodose ganglion of the rat. As yet there have been no studies combining functional with anatomical techniques demonstrating conclusively that the functional classifications used are correlated directly with the anatomical position of the afferent ending. Nonetheless, these anatomical studies support the functional definition of the site of an afferent ending. Unfortunately, this clarity is not yet available in literature on the spinal innervation. Spinal afferent fibres labelled by injection of choleragenoid horseradish peroxidase into thoracic dorsal root ganglia have been followed to terminations in the mucosa and muscle in the oesophagogastric junction of the cat (Clerc & Mazzia, 1994). There is also indirect evidence from immunocytochemistry studies that spinal afferent fibres have endings in the serosa, muscle and mucosa of the gut (Green & Dockray, 1988; Su *et al.*, 1987). But possibly because of technical difficulties in following spinal afferent fibres to their terminations, the literature does not extend beyond this. Certainly literature is not available for the colon. In addition, functional studies in intestinal spinal afferent fibres have yet to categorically document afferent fibres with characteristics of having mucosal endings despite the fact that histological evidence (Clerc & Mazzia, 1994) does exist.

1.2.3 Electrophysiology of Vagal Afferent Fibres

The majority of the work done in vagal afferent fibres has been carried out *in vivo*, with *in vitro* work beginning to emerge as an alternative (Page & Blackshaw, 1998; Wei *et al.*, 1995). The focus has been on the upper gastrointestinal tract, the primary site of innervation for the vagus. Both mechanosensitivity and chemosensitivity have been explored. The following discussion is separated by virtue of mechanosensitivity. Chemosensitivity is subsequently discussed within each group. A summary of effective chemical stimuli used in a broad range of studies in the gastrointestinal tract can be found in Table 1-1.

1.2.4 Vagal Tension Afferent Fibres

As was the case with spinal nerves, the first documented afferent fibres from the gut were distension-sensitive afferents (Iggo, 1955). They were initially classified

Location	Luminal stimuli		i.a./i.v. external pledget stimuli		Species
Vagal mucosal afferent fibres					
Oesophagus/ stomach	Water (+) H^+ (+) NaOH (+) Casein (+)	Hypertonic saline (+) Glucose (0) Capsaicin (+) PGE_2 (+)	CCK (+) 5-HT (+) adrenaline (+) bradykinin (+)		Rat Ferret Cat
Small intestine	Lipids (+) H^+ (+) Hypertonic saline (+) Glucose (0) Water (0) PGE_2 (0) PGF_2 (+) KCl (+) Magnesium sulphate (0) Sodium bicarbonate (0)	Acetylcholine (+) Noradrenaline (+) Phenylbiguanide (+) Papaverine (+) 5-HT (0) bradykinin (0) acetic acid (+) butyric acid (+) propanoic acid (+)	CCK (+) 5-HT (+) adrenaline (+) bradykinin (+)		Cat Ferret Sheep
Vagal muscular afferent fibres					
Oesophagus/ stomach	H^+ (0) water (0) 5-HT (+)	bradykinin (+) PGE_2 (+) Capsaicin (+)	CCK (+) 5-HT (+) bradykinin (+)		Rat Ferret Rabbit Opossum
Small bowel	HCl (+) Fatty acids (+) Alkali (+) Amino acids (+)		CCK (+) 5-HT (+) bradykinin (+) insulin (+)	prostaglandins (+) acetylcholine (+) ferret (+)	Sheep Ferret
Spinal muscular and serosal afferent fibres					
Small bowel	Casein (+) Proprionate (+) Butyrate (+) Acetate (0) Caprylate (0)	Glucose (+) Mannitol (0) Hexose (0) Mannose (0)	HCl (0) Hypoxia (+) Histamine (+) Bradykinin (+)	Adenosine (+) LTB_4 (+) Ischaemia (+) Capsaicin (+)	Cat Rat
colon	inflammatory soup (+) bile salts (+)	H^+ Krebs (0) ^-OH Krebs (0)	Bradykinin (+) KCl (+)	Hypertonic saline (+) Ischaemia (+)	Rat Cat
Rectum/ anus/cloaca	Chemical irritants (+)		HCl (0) NaOH (0) PGE_2 (0)	$PGF_{2\alpha}$ (0) Phenyldiguanide (+)	Duck Cat

Table 1-1. Summary of chemical stimuli used in the study of gastrointestinal afferent fibres. (+) indicates an effective stimulus, (0) indicates an ineffective stimulus. A stimulus has been identified as effective if one study cites its efficacy. Thus when studies disagree, only those that show stimuli to be effective are represented accurately. A list of publications from which this table was developed follows; Blackshaw & Grundy, 1993*a;* Blackshaw & Grundy, 1990; Blackshaw & Grundy, 1993*b;* Brunsden & Grundy, 1999; Clarke & Davison, 1975; Clarke & Davison, 1974; Clifton *et al.*, 1976; Cottrell & Iggo, 1984*b;* Cottrell, 1984*a;* Cottrell, 1984*b;* Davison, 1972; Grundy *et al.*, 1994; Hardcastle *et al.*, 1978; Haupt *et al.*, 1983; Iggo, 1957; Koley *et al.*, 1984; Koley *et al.*, 1982; Longhurst *et al.*, 1984; Melone, 1986; Morrison, 1973; Page & Blackshaw, 1998; Pan *et al.*, 1994; Pan *et al.*, 1995; Richards *et al.*, 1996; Richards *et al.*, 1991; Sengupta & Gebhart, 1994; Sengupta *et al.*, 1992; Stahl *et al.*, 1993; Su & Gebhart, 1998).

using criteria used in the study of somatic afferent fibres innervating skeletal muscle. Consequently, the nomenclature now includes 'in-series' tension receptors defined by their ability to respond in a linear fashion to distension, and phasic afferent fibres thought to have endings not aligned with the direction of muscle fibres. Tension receptors within the muscle respond not only to distension of the gut but also contraction (Grundy & Scratcherd, 1989). They have also been documented as being chemosensitive although there is discord in the literature as to whether muscular fibres respond to chemical stimuli or as a secondary response to muscular activity initiated by the chemical stimulus. In the rabbit duodenum responses in muscular fibres to stimuli such as glucose and hypertonic saline coincided with induced muscular activity (Cottrell, 1984*a*). A study in ferret upper gastrointestinal tract aimed at investigating the relationship between the response of muscular fibres to 5-hydroxytryptamine and the muscular activity elicited by the stimulus concluded that 5-hydroxytryptamine did not act directly on the fibre, but indirectly through muscular activity (Blackshaw & Grundy, 1993*b*). This was also demonstrated in the same species in gastroduodenal afferent fibres in response to cholecystokinin (Blackshaw & Grundy, 1990). This is in contrast to an earlier study in the rat that concluded that the response to cholecystokinin in gastric mechanoreceptors was independent of muscular activity and therefore direct (Davison & Clarke, 1988).

Subsequently, another classification of afferent fibres emerged. These were termed mucosal afferent fibres because they responded to stimuli applied to the luminal surface, notably stroking of the luminal surface. They were also tension insensitive, suggesting that their endings were not within the muscle wall (Iggo, 1957). Many mucosal fibres are polymodal, also being chemosensitive. More recently, with the development of an *in vitro* preparation of the ferret oesophagus, another class of vagal afferent fibres has been defined. These have properties of both tension-sensitive and mucosal afferent fibres, the role for which is not yet clear (Page & Blackshaw, 1998). This study by Page and Blackshaw however, clearly highlights the advantages of having an *in vitro* preparation complementing *in vivo* work, allowing controlled stimuli to be applied and therefore apply tighter

classification criteria, in this case identifying a hitherto unrecognized class of afferent fibre.

1.2.5 Vagal Mucosal Afferent Fibres

Mucosal afferent fibres are insensitive to distension of a hollow organ and are also generally silent. First described in 1957 (Iggo) in the gastric mucosa of the cat, they are characterised by rapidly adapting responses to light stroking of the mucosal surface. They have been functionally described using single unit recordings in the oesophagus (Bitar *et al.*, 1975; Clerc, 1984) stomach (El Ouazzani & Mei, 1982; Iggo, 1957) and duodenum (Clarke & Davison, 1978; Davison, 1972) of the cat, antrum and small intestine of the rat (Clarke & Davison, 1978; Harding & Leek, 1972*a*), duodenum (Cottrell & Iggo, 1984*a*) and stomach (Harding & Leek, 1972*b;* Leek, 1972) of the sheep and the oesophagus (Page & Blackshaw, 1998), stomach and duodenum (Blackshaw & Grundy, 1990; Blackshaw & Grundy, 1993*a*) of the ferret.

Mucosal fibres form the first line of sensation in the gut, responding to many luminal stimuli and mucosal humoral stimuli. Extensive study of the chemosensitivity of mucosal afferent fibres in the upper gastrointestinal tract has taken place over the years. Many mucosal afferent fibres are shown to be polymodal, responding to more than one chemical stimuli if indeed the right combination of chemicals are applied in addition to mechanical stimulation of the mucosa (Grundy & Scratcherd, 1989; Mei, 1985). Not surprisingly, there appears to be regional specificity of the stimuli effective in activating mucosal afferent fibres and these are related to the function of that particular part of the gut. For instance, gastric mucosal afferent fibres show sensitivity to pH (Blackshaw & Grundy, 1990; Blackshaw & Grundy, 1993*a;* Davison, 1972) but not to bile (Davison, 1972) or glucose (Blackshaw & Grundy, 1990). Afferent fibres from the small intestine have been shown to be sensitive to nutrients such as glucose (Mei, 1978), lipids (Melone, 1986) and amino acids (Jeanningros, 1982); stimuli more appropriate to the physiology of the small intestine. Nomenclature in this body of work provides some confusion. Early studies allowed the idea of specific chemoreceptors such

as glucoreceptors or acid receptors to emerge – possibly because the range of stimuli used were often not extensive enough to demonstrate polymodality. Increasingly, studies demonstrate that chemosensitivity extends beyond one type of mediator but has not yet surplanted the original nomenclature. Eventually a knowledge of transduction mechanisms – particularly for luminal stimuli – will help to clarify this issue. Nonetheless, this broad approach to the study of vagal afferent fibres compared to spinal afferent fibres has given greater insight into their physiological role. It would be beneficial to apply this principle to spinal afferent fibres of the colon in order to gain a broader understanding of the functional role of spinal afferent fibres.

1.2.6 Visceral Spinal Afferent Fibres

Work on visceral afferent fibres is not confined to the study of the gastrointestinal tract. Of particular interest are those studies that have investigated spinal visceral innervation throughout the abdomen and pelvis, comparing and contrasting the afferent fibres arising from different organs but travelling within the same nerve. The classification procedure for afferent fibres differs from that described so far for the gastrointestinal tract in that responses to probing of the serosal surface (Morrison, 1974; Morrison, 1973), along with conduction velocity of the fibre (Longhurst *et al.*, 1991; Pan *et al.*, 1995) are used rather than distension of the hollow organ. This follows the classification used for cutaneous afferents rather than gastrointestinal afferents. Two foci have emerged from these studies. The first is the study of the number, site, and size of receptive fields for these fibres. It is evident that unlike vagal fibres that have single punctate receptive fields (Grundy & Scratcherd, 1989), serosal afferent fibres can have between one and seven punctate receptive fields that are associated with blood vessels (Morrison, 1973). The second focus of these studies has been the chemosensitivity of afferent fibres. Up to 50% of splanchnic visceral afferent fibres (which correlate to the serosal or mesenteric afferent fibres discussed earler) are sensitive to ischaemia (Longhurst & Dittman, 1987). Ischemically sensitive visceral afferent fibres are also sensitive to bradykinin (Pan *et al.*, 1994) and a lesser proportion to prostaglandins (Longhurst & Dittman, 1987) although prostaglandins play a pivotal role in

mediating the response to ischemia itself (Longhurst *et al.*, 1991). Of particular interest to the overall discussion here is the observation that ischemically sensitive serosal afferent fibres in the stomach and small intestine have a high (in the noxious range) threshold to distension of the gut (Pan & Longhurst, 1996). This observation has contributed significantly to the idea that a proportion of spinal afferent fibres are primarily nociceptive.

1.2.7 Electrophysiological Investigations in the Distal Colon

Functional investigation of the afferent innervation of the distal colon has included work on the splanchnic and pelvic nerves; loosely categorised into sympathetic and parasympathetic innervation respectively. *In vivo* preparations have consistently been used. Most of the work has been carried out in the cat (Bahns *et al.*, 1987; Blumberg *et al.*, 1983; Floyd *et al.*, 1976; Floyd & Lawrenson, 1979; Haupt *et al.*, 1983; Janig & Koltzenburg, 1991; Morrison, 1973) and to a lesser extent the rat (Sengupta & Gebhart, 1994; Su & Gebhart, 1998).

1.2.8 Colonic Distension-Sensitive Afferent Fibres

Distension of the colon has been the primary adequate stimulus of choice in studies on the splanchnic as well as the pelvic innervation and is used to classify afferent fibres along with nerve stimulation. Consequently, a number of classifications have been formulated on conduction velocity and type of response to distension.

Distension-sensitive fibres in the pelvic nerves of the cat have been shown to have relatively low thresholds to intraluminal pressure, certainly within the normal physiological range (Janig & Koltzenburg, 1990; Janig & Koltzenburg, 1991). The responses were either rapidly adapting phasic responses or tonic responses. The fibres with tonic responses respond in a linear fashion to increasing intraluminal pressure, even at noxious levels. Similar findings in the rat support this data (Sengupta & Gebhart, 1994) although this group supports the addition of a high threshold group of distension sensitive fibres. It is possible however, that these high threshold fibres are fibres homogeneous with the low threshold fibres, but with

receptive fields beyond the end of the intraluminal balloon stimulated indirectly by secondary mechanical distortion. It was certainly shown that noxious levels of distension can trigger a response in normally low threshold fibres in another area of the distal colon (Janig & Koltzenburg, 1991).

Distension-sensitive afferent fibres supplying the distal colon have also been documented in the lumbar splanchnic nerves (Blumberg *et al.*, 1983). There was no identifiable difference between the responses of the lumbar splanchnic nerves (Blumberg *et al.*, 1983) and the responses of the sacral pelvic nerve (Janig & Koltzenburg, 1991) – both studies carried out by the same group. The distension-sensitive afferent fibres in the lumbar splanchnic nerves were generally spontanously active whereas those in the sacral pelvic nerve were silent. Generally the response threshold is lower in lumbar splanchnic afferent fibres than in the sacral pelvic afferent fibres. In addition the afferent fibres in the lumbar splanchnic nerve could be split into four categories ranging between tonic and phasic including those which responded with a steady state discharge, a transient response which adapted to a steady state discharge, an adapting response and a rapidly adapting response. In contrast, the sacral pelvic afferent fibres could be characterised as clearly being only phasic or tonic.

Whether distension sensitive afferent fibres are primarily sensitive to tension within the wall of the organ rather than the length of the tissue is still open to some debate. It is generally accepted that tension is the primary adequate stimulus, but recently work on intestinofugal fibres projecting to the IMG from the colon of the guinea-pig has described a sub-population of neural cell bodies that respond to changes in volume not tension. That is, that when the volume of an *in vitro* colonic segment was held constant, the discharge of the cell also became constant despite changing intracolonic pressure (Anthony & Kreulen, 1990), particularly at low physiological levels. This suggests that it may be possible for extrinsic afferents also to be sensitive to changes in volume (length of the tissue) rather than exclusively pressure (wall tension).

1.2.9 Afferent Chemosensitivity Pertinent to this Thesis

The majority of all investigations into afferent fibre chemosensitivity in the gut have involved vagal fibres from the upper gastrointestinal tract. Therefore, the discussion of the literature largely involves vagal afferent fibres, particularly mucosal afferent fibres. An overview of chemical stimuli used in the study of gastrointestinal afferent chemosensitivity can be found in Table 1-1. The following section contains a discussion of afferent sensitivity to chemical stimuli that have been used in the studies documented in this thesis.

Vagal mucosal fibres are chemosensitive to both luminally applied stimuli and to perfused or blood-borne stimuli. Effective stimuli include alcohol, pepper, thiourea and mustard powder in the antrum and small intestine of the rat (Clarke & Davison, 1978); glucose and various carbohydrates in the small intestine of the cat (Mei, 1978); amino acids in the small intestine (Jeanningros, 1982); intra-arterial cholecystokinin (Blackshaw & Grundy, 1990) and adrenaline (Blackshaw & Grundy, 1993*a*) in the gastroduodenum of the ferret. A more detailed account of chemical stimuli pertinent to the current project follows.

Hyperosmotic stimuli have been used with mixed responses in the past. They are one of the few stimuli that have been preferentially used as luminal stimuli. Duodenal mucosal receptors are not sensitive to the osmolarity of a solution (Blackshaw & Grundy, 1993*b;* Blackshaw & Grundy, 1993*a;* Cottrell & Iggo, 1984*a*) nor are ileal mesenteric afferent fibres (Hardcastle *et al.*, 1978) although others (Clarke & Davison, 1978; Davison, 1972; Harding & Leek, 1972*b*) found that some gastroduodenal mucosal receptors were sensitive to hypotonic stimuli. In contrast, one study that attempted to define possible specific osmoreceptors (Garnier & Mei, 1982), found that mucosal duodenal-jejunal afferent fibres were sensitive to hypoosmotic and hyperosmotic stimuli, but not to osmolarities that were within the normal range for duodenal chyme. This is supported by the observation that mucosal receptors in the gastric antrum and small intestine may respond to both water and hyperosmotic NaCl (Clarke & Davison, 1978). Garnier and Mei (1982) found that responses varied according to the type of chemical used to make up the osmolar solution, two of which were

mannitol and NaCl. The issue of whether or not specific osmoreceptors exist - an issue that has been thus far confined to the small intestine - has been argued for a number of years. It is unlikely however, that osmoreceptors would be truly specific and have no polymodal functions, because it is clear that some fibres that respond to osmotic stimuli are also mechanosensitive (by which they were classified as mucosal fibres) and chemosensitive to other chemical stimuli (Clarke & Davison, 1978; Harding & Leek, 1972*a*). It has been argued that some fibres are specific because they respond to only one of the chemical stimuli that were applied (Mei, 1985). This may well be true resulting in two groups of fibres, those that are specific and those that are polymodal. Alternatively it may well be that there is not a broad enough range of chemical stimuli applied to fully elucidate the polymodal nature of what are otherwise described as specific fibres as discussed earlier.

The investigation of the role of protons in activation of gastrointestinal afferent fibres started - not surprisingly - in the stomach (Iggo, 1957). In this instance, mucosal afferent fibres responded to either acidic or alkaline solutions, but never to both. The concentrations of these substances had to be high in order to elicit a response. Further work investigated protons as luminal stimuli both in the small intestine (Blackshaw & Grundy, 1990; Clarke & Davison, 1978; Cottrell & Iggo, 1984*a;* Davison, 1972) and in the oesophagus (Page & Blackshaw, 1998). With the emerging role for protons in pain generation for hyperalgesia and inflammation (Steen *et al.*, 1992), it is necessary to see acid not only as a luminal content, but the level of acidity in the tissue as a potential indicator of noxious events. Tissue acidosis accompanies ischaemia, inflammation and tissue damage (Chen *et al.*, 1998; Kress & Zeilhofer, 1999). A decrease in extracellular pH has been shown to have two opposing effects on neuronal excitability. In small diameter dorsal root ganglion neurons – considered to be nociceptors – excitability is increased with decreasing pH. In other neurons a decrease in pH reduces excitability (Kress & Zeilhofer, 1999). Thus, there are two perspectives on the study of proton sensitivity in gastrointestinal afferent fibres. The first is an investigation of acidic conditions generated in the lumen by luminal contents which may have direct or indirect effects on afferent fibres for the generation of normal sensation. The second is an investigation of acidic conditions generated in the mucosa and

muscle by damage and inflammation which may have direct effects on afferent fibres for the generation of noxious sensation.

Capsaicin sensitivity in the gut - investigated electrophysiologically - has received little interest, despite the large amount of literature to be found on skin afferent sensitivity and its role in nociception. Capsaicin is believed to specifically activate nociceptors in skin via the vanilloid receptor (Caterina *et al.*, 1997). This same receptor is believed to involve a heat activated ion channel (Caterina *et al.*, 1997). There have been suggestions that capsaicin sensitive fibres are also proton sensitive, but this has been challenged as not being universal in the skin (Steen *et al.*, 1992) and also in the gut (Blackshaw *et al.*, 2000). This introduces one of two studies investigating gastrointestinal afferent fibres and their sensitivity to capsaicin. In this case, mucosal fibres were sensitive to capsaicin at a rate of around 30%, and this was similar in mucosal and muscular fibres. Therefore there was no difference in the response to capsaicin with respect to their functional classification, or to their classification by conduction velocities. This finding was robust across *in vivo* and *in vitro* preparations and also different methods of administration of the drug. In contrast, spinal afferent fibres supplying all visceral organs demonstrated a rate of capsaicin sensitivity of 38% in A-fibres, but 100% in C-fibres (Longhurst *et al.*, 1984).

5-hydroxytryptamine (5-HT) is involved in many aspects of gastrointestinal function, of which one is an effect on afferent nerve endings (Blackshaw & Grundy, 1993*b*). Mucosal afferent fibres in the gastroduodenal region of the ferret are sensitive to 5-HT (Blackshaw & Grundy, 1993*a*). 5-HT is found mainly in the enterochromaffin cells of the mucosa, but is found to be released by a number of different luminal stimuli such as hypertonic, acidic and possibly mechanical stimuli (Larsson, 1981). Therefore, 5-HT is a good candidate for a second messenger in sensing luminal stimuli. However, whilst intrinsic primary afferents have been shown to be dependent on 5-HT for signal transduction from luminal stimuli, extrinsic primary afferents are not (Grundy *et al.*, 1994). 5-HT also elicits responses in muscular fibres in the gastroduodenal region of the ferret (Blackshaw & Grundy, 1993*b*). However, the authors suggest that the responses seen to 5-HT

in muscular fibres are secondary to muscular activity induced by 5-HT and is not a direct effect on the afferent fibre. 5-HT is believed to activate extrinsic primary afferents through 5-HT_3 receptors (Grundy *et al.*, 1994).

Muscular fibres elsewhere have been shown to respond to chemical stimuli. Colonic pelvic afferent fibres in the rat respond to bile salts and an inflammatory soup (Su & Gebhart, 1998). This was the first documentation of the effects of bile salts on colonic afferent sensitivity, despite the fact that bile salts present in the colon are known to cause pain in man (Edwards *et al.*, 1989). Intracolonic instillation of bile salts activated stretch-sensitive afferents but did not sensitise the afferent to colorectal distension. The inflammatory soup (included bradykinin, PGE_2, 5-HT, histamine, KCl and low pH) both activated the afferent fibres and sensitised them to subsequent colorectal distensions. However, in this study as in many others, it is not known whether the response to chemical mediators is direct or secondary to muscular activity caused by the chemical mediators. In contrast, bradykinin elicits identical responses in distension-sensitive splanchnic afferent fibres supplying the colon during a bradykinin-induced contraction and when the muscle is paralysed – suggesting a direct effect (Haupt *et al.*, 1983; Sengupta & Gebhart, 1994).

1.2.10 Silent Nociceptors

The literature on spinal visceral afferent fibres contains many references to a large group of afferent fibres called 'silent nociceptors'. Silent nociceptors carry similar characteristics to mucosal afferent fibres in the vagal innervation of the viscera. First described in the bladder (Habler *et al.*, 1990) then in the colon (Janig & Koltzenburg, 1991), they are silent and are insensitive to distension - even noxious distension. These afferent fibres form up to 50% of all afferent fibres arising from the colon in cat splanchnic nerves (Blumberg *et al.*, 1983). Being silent, it seems unlikely that these fibres would have a role in the normal sensing of the environment, as they are unable to send a tonic message of surrounding events. In the bladder, 10% of these fibres develop background activity and some of them mechanosensitivity after inflammation of the mucosa has been induced

(Koltzenburg & McMahon, 1995). Consequently, it is believed that these fibres are involved in nociception. However, given the limitations of the techniques and perhaps the focus on pain, it is not known whether they respond to non-noxious or physiological stimuli such as normal luminal stimuli and stroking.

1.3 Advances In Methodology, Models and Techniques

1.3.1 In Vivo and In Vitro Preparations

In vitro techniques in the study of visceral sensation have been largely unexplored. *In vitro* preparations of bronchi-nerve (Fox *et al.*, 1993), uterus-pelvic nerve (including the colon) (Berkley *et al.*, 1990), testicular-superior spermatic nerve (Sato *et al.*, 1989), ureter-ureteric nerve (Cervero & Sann, 1989), ileum-mesenteric nerve (Cervero & Sharkey, 1988) and two preparations of oesophagus-vagus nerve (Page & Blackshaw, 1998; Wei *et al.*, 1995) have allowed single unit recordings from extrinsic nerves in a whole organ preparation. Many of the organ baths used for these studies show similarities to the first successful *in vitro* skin-saphenous nerve preparations (Reeh, 1986). Most of the knowledge of visceral sensation has been gained from *in vivo* preparations as only three productive whole organ gastrointestinal preparations (Cervero & Sharkey, 1988; Page & Blackshaw, 1998; Wei *et al.*, 1995) have been published. The wealth of information gained from *in vivo* preparations cannot be underestimated. However, there are advantages to working *in vitro*, the obvious being the control one has over the environment of the tissue without complications arising from the whole body. In addition, not all surfaces of the tissue are easily accessible in an *in vivo* preparation, leading to data being distorted or incomplete – particularly responses to mechanical stimuli. With greater control over the tissue, it should be possible to create tighter classification criteria of the function of afferent fibres. The main reason for the delay in effective gastrointestinal *in vitro* preparations being established for the study of single afferent fibres has been the difficulty in keeping the tissue alive and viable *in vitro*, particularly the mucosa. After removal of the tissue from the animal the mucosa of the gut begins to deteriorate (see Kunze & Furness, 1999), secreting many different metabolites and mediators into the surrounding area. In stripped preparations of the mucosa, this can be washed

away more effectively than it can in a whole organ preparation. In muscle strips, by virtue of the absence of the mucosa, secretions from the mucosa do not cause deterioration. Consequently, if the integrity of the mucosa is a priority in a whole organ preparation, alternative approaches to the standard organ bath methods must be employed to prevent mucosal deterioration. One such method has been the use of perfusion systems.

1.3.2 Mucosal Viability In Vitro

One study, using the whole organ of the colon, took considerable care in maintaining the viability of the mucosa (Roediger & Moore, 1981). It was found that the tissue remained viable *in vitro*, so long as ischaemia to the tissue was less than 40 minutes, before having the vascular bed perfused. In addition, superfusion of the lumen with short chain fatty acids improved normal function of the mucosa *in vitro*. One of the results of ischaemia is an increase of prostaglandin levels – namely PGE_2 - in the small intestine (Rendig *et al.*, 1994). The role of prostaglandins in mucosal viability and afferent sensitivity is discussed in detail later.

In vitro preparations have become more popular with the study of intrinsic nerves of the gut. These preparations come in many and varied designs, providing a wealth of ideas that can be utilised in the study of extrinsic afferent fibres. Of particular interest, but little use, are studies that use the whole organ - mucosa intact. When the gut is maintained as a tube (Tonini & Costa, 1990) it is difficult to care for the mucosa. Preparations that have the gut opened and lying flat (Grider, 1994; Kunze *et al.*, 1997) have the advantage of allowing some nutrients to the mucosal surface. However, even studies that rely on mucosal stimulation do not take specific measures to preserve the mucosa (Grider, 1994). In the study of intrinsic primary afferent fibres supplying the mucosa, intracellular recordings were made in a whole organ setup of fibres with endings in the mucosa (Kunze *et al.*, 1997). No alterations were made to accommodate the need for mucosal viability but successful recordings were made. Nonetheless, in this study there was no

mention of length of time the preparation was viable or how long it was possible to continue making recordings.

1.3.3 Colonic Mucosal Viability

The colonic mucosa has specific nutrient needs. It relies heavily on nutrients being provided from the lumen rather than the vascular system (Scheppach, 1994). It preferentially uses a variety of short chain fatty acids produced from anaerobic fermentation. Seventy percent of energy is taken from butyrate even though butyrate does not make up a large proportion of luminal short chain fatty acids. The remaining 30% of energy is taken mainly from acetate - the predominant luminal short chain fatty acid - and proprionate (Scheppach, 1994). Glucose is used only when access to the short chain fatty acid is denied and causes a detrimental buildup of lactate in the colonocytes (Krishnan *et al.*, 1998), thereby causing damage to the tissue. Consequently, the commonly used solutions used in *in vitro* work, namely Ringers and Krebs solutions, both of which use glucose as the main nutrient source, are unable to provide the correct nutrient balance to the colonic mucosa. Therefore, an ideal situation would allow the mucosa access to a mixture of short chain fatty acid's and allow muscle and nerves access to glucose. This could readily be achieved *in vitro* but has not yet been pursued.

When the tissue is separated out into the mucosal and muscular layers and investigated independently, preparations are run at 37° Celsius (Goerg *et al.*, 1991; Keenan & Rangachari, 1989; Levi *et al.*, 1996; Wardle *et al.*, 1993). But on occasion whole organ gastrointestinal preparations are run at a lower temperature such as 34°C (Maubach & Grundy, 1999; Page & Blackshaw, 1998) or even 31°C (Wei *et al.*, 1995), presumably to preserve the mucosa. Consequently, mucosal integrity is often compromised for viability of the muscle and preparations are run at 37°C (Benard *et al.*, 1997). Considering that intrinsic primary afferent fibres with endings in the mucosa influence normal motor activity of the tissue (Grider *et al.*, 1996), it is unfortunate that experimental conditions cannot suit both concurrently and so optimise the investigative environment. Whilst the temperature cannot be manipulated to suit both tissues optimally other determinants of tissue viability can.

Prostaglandins have multiple roles within the gut. Not only are they potent inflammatory mediators, but they are also involved in the modulation of sensory information both directly and through secondary mechanisms. *In vivo*, PGE_2 is shown to be protective to the mucosa, decreasing cell clearance and increasing cell proliferation in rat antral, duodenal and jejunal mucosa (Uribe, 1992). In addition, an increase of endogenous production of PGE_2 accelerates healing of ulceration in rats with colitis (Fedorak *et al.*, 1992), further supporting its role in maintenance of the healthy mucosa *in vivo*. However, in the rat distal colon *in vitro*, this positive effect of endogenous prostaglandins on the proliferative activity of the mucosa appears to be lost - in direct contrast to the *in vivo* data from the same study. This implies that *in vitro* prostaglandins are not beneficial to the mucosa *in vitro* as they are *in vivo*. This pivotal evidence supports the procedure of adding indomethacin to the bath superfusate (despite its ulcerative effect *in vivo*) to prolong the life of the colonic mucosa *in vitro*.

1.3.4 Novel Mice Preparations

It is of interest to note that thus far, there is no literature cited for the mouse. The cat, rat and ferret have figured highly in the study of visceral sensation, but to date, the mouse has not been the animal of choice for investigation of gastrointestinal sensation. In the study of cutaneous sensation the mouse is beginning to gain favour as a suitable model and used for single fibre recordings of cutaneous afferent fibres (Koltzenburg *et al.*, 1997; Stucky, 1997) and other studies of sensation (Basbaum, 1999; Khasar *et al.*, 1999). Mice have advantages over other animal models because their size results in less bulky preparations. This is particularly useful when viability of *in vitro* preparations is a concern because a thinner tissue and smaller preparation means that penetration of nutrients and chemical stimuli through the layers of the tissue is more efficient. Although mouse models for gastrointestinal function and disease have not been developed, they are being used increasingly for the study of the sensory function of dorsal root ganglion neurons (Cummins *et al.*, 1999; Khasar *et al.*, 1999). With the tools of gene

manipulation currently being available preferentially for the mouse, there is scope for novel investigation into the function of afferents in the gastrointestinal system.

1.4 Prostaglandins and Sensation in the Colon

1.4.1 Prostaglandins in the Colon

The predominant prostaglandin being produced by the rat distal colon *in vitro* is PGE_2, accounting for 55% of all eicosanoids. PGD_2 and $PGF_{2\alpha}$ account for the bulk of the remaining mediators being produced from arachidonic acid in an *in vitro* preparation (Craven *et al.*, 1983). The role of prostaglandins and other eicosanoids is complex, with opposing and interacting effects between them all. Indeed, PGE_2 and PGD_2 have contrasting direct effects on the proximal colonic mucosa (Keenan & Rangachari, 1991). The interactions between prostaglandins and other inflammatory mediators can be so complex that indomethacin, a cyclooxygenase inhibitor (decreasing the amount of PGE_2 being produced in the tissue), can potentiate the overall response of PGE_2 on the mucosa - the opposite of the predicted effect. This is presumably by the interaction of either a disturbed balance of inhibitory prostaglandins or the potentiating effect of leukotrienes (Keenan & Rangachari, 1989). Despite these complicated interactions, in an inflammatory state, the levels of all of these mediators increase. The inflamed human colon has higher secretion levels of prostaglandins and leukotrienes but again interactions between these mediators are observed (Wardle *et al.*, 1993). Prostaglandins have a complex role in normal and inflammatory states in the gastrointestinal system.

1.4.2 Prostaglandins and Afferent Nerves

Prostaglandins also have a role in afferent sensitivity. PGE_2 and $PGF_{2\alpha}$ (more potent) stimulate duodenal distension-sensitive and mucosal receptors (Cottrell & Iggo, 1984*a;* Cottrell & Iggo, 1984*b*) and mucosal PGE_2 stimulates oesophageal mucosal receptors (Page & Blackshaw, 1998). Prostaglandins also have a modulatory role in the afferent fibre response to other mediators, as is the case with bradykin-induced visceral-cardiac reflexes (Stebbins *et al.*, 1985) and in the

saphenous nerve where prostaglandins are necessary for the action of bradykinin on cutaneous afferent fibres (Chahl & Iggo, 1977).

Of great interest recently has been the role of sensitisation of afferent fibres to the application of chemical and mechanical stimuli by prostaglandins. This has been primarily investigated in the skin sensory system, and has only recently been described in the gastrointestinal system (Maubach & Grundy, 1999). In this case, an interaction between bradykinin and PGE_2 was described, where the response to bradykinin in serosal afferent fibres was dependent on the presence of PGE_2. PGE_2 and PGI_2 cause hyperalgesia to mechanical force in the absence of all other known indirect hyperalgesic mechanisms when injected intradermally in rats (Taiwo & Levine, 1989). PGE_2 along with 5-HT sensitises muscle nociceptors to bradykinin, thereby having a role in pain sensation (Mense, 1981). An understanding of the role of prostaglandins in sensation has yet to be investigated.

1.5 Summary

The gastrointestinal tract is innervated by vagal and spinal neural pathways. Afferents in both pathways can be functionally classified by their response to mechanical stimuli applied to the gut. Vagal afferents have endings in the mucosa, muscle and only very few in the serosa. Spinal afferents have their endings in the muscle and in the serosa and mesentery but have not been documented as having endings in the mucosa using the criteria employed in classifying vagal afferent fibres. The study of mechanosensitivity in the spinal innervation has not been as comprehensive as in the investigation of vagal innervation of the gut. This has had most impact on the understanding of colonic afferent mechanosensitivity as the vagal innervation in this region of the gut is sparse. Vagal mucosal afferents are often polymodal, their chemosensitivity investigated by luminal application of stimuli in addtion to other means of stimulus application such as intra-arterially. Using identical methods, vagal muscular fibres also show chemosensitivity but often as a secondary response to muscular activity. In the small bowel, chemosensitivity of the spinal innervation of the gut has been investigated in similar manner to the vagal innervation, but the large bowel has been largely

ignored, with few studies investigating chemosensitivity of muscular and serosal afferents.

Recording extracellularly from gastrointestinal afferents has been primarily carried out in *in vivo* preparations. Novel *in vitro* nerve-gut preparations have recently enabled increasingly controlled study of afferent fibre mechano- and chemosensitivity in the gut. The deterioration of the mucosa *in vitro* is problematic in gastrointestinal preparations, particularly in the colon as normal buffer solutions do not provide adequate nutrients specifically required by colonic mucosa. Whole organ *in vitro* preparations have not yet been used to investigate afferent mechano- and chemosensitivity in the colon in any species.

Prostaglandins have a complex role in the normal physiological function of the colon. They have a role in afferent sensitivity in the cutaneous and gastrointestinal system and have been shown to be involved in modulating both mechano- and chemosensitivity of cutaneous afferents and more recently, the chemosensitivity of mesenteric gastrointestinal afferents. Prostaglandins have an integral role in inflammation and disease, implicating them also in pain generation and disordered sensation.

1.6 Aims

The aims of the project were as follows.

1. Develop a novel *in vitro* preparation that enabled electrophysiological investigation of the functional properties of colonic afferent fibres in the rat.
2. Explore the properties of colonic afferent fibres in this model relative to previously documented populations of lumbar colonic afferent fibres, and determine whether these colonic afferent fibres could be subject to the same functional classification as upper gastrointestinal vagal afferent fibres, particularly if mucosal fibres were encountered.
3. Explore the chemosensitivity of colonic afferent fibres.
4. Investigate the properties of muscular fibres and their adequate stimulus.
5. Explore the role of endogenous prostaglandins in chemosensitivity of colonic

afferent fibres.

6. Develop a novel *in vitro* preparation that enabled electrophysiological investigation of the functional properties of colonic afferent fibres in the mouse.

Chapter 2. Methods and Development of Rat Preparation

2.1 Abstract

1. A novel *in vitro* preparation of rat distal colon allowing recordings from extrinsic afferent fibres was developed.
2. An organ bath was designed that allowed discrete, controlled mechanical and chemical stimuli to be applied to the mucosal surface of the colon.
3. Krebs solutions were modified to prevent mucosal deterioration. This was achieved by replacing glucose with short chain fatty acids in the superfusate of the mucosal surface whilst the serosal surface was superfused with glucose supplemented Krebs solutions. Indomethacin was added to both Krebs solutions to prevent buildup of endogenous prostaglandins.
4. Other protocol procedures were employed to prevent tissue deterioration. These included variable temperatures, and fast superfusion rates.

2.2 Introduction

The first part of this project was spent designing the technique that was subsequently used successfully for the data generated from this project.

Traditionally, the sensory innervation of the colon has been investigated using *in vivo* methods. To date there has been no documentation in the literature of *in vitro* preparations in the colon utilising extracellular recordings from extrinsic nerves. Yet there are many advantages to being able to utilise both *in vivo* and *in vitro* techniques to look at the same physiological system. This is particularly true when there are aspects of *in vivo* data that have not been resolved successfully using current techniques of investigation. For example, muscular fibres have been described for the vagal innervation of the oesophagus (Sengupta *et al.*, 1989; Sengupta *et al.*, 1992), but it has only been as a result of an *in vitro* preparation that the presence of mucosal fibres was functionally confirmed and a third group of afferents - termed tension-mucosal afferents - have been documented (Page & Blackshaw, 1998). Thus, the aim of developing a new technique was to design a system whereby some of the unresolved issues in the understanding of colonic sensation could be investigated with the use of very discrete and controlled stimuli.

2.3 Methods

2.3.1 Animal Preparation

Female Sprague Dawley rats (180-220g) were sedated with ether and anaesthetized with Nembutal (60mgkg^{-1} ip) (or anaesthetised with Nembutal (60mgkg^{-1} ip) only). After a midline laparotomy, 4-5 cm of distal colon lying oral to the rim of the pelvis was removed, along with the lumbar colonic nerves and the neurovascular bundle containing the inferior mesenteric ganglion (IMG), the intermesenteric nerve and the lumbar splanchnic nerves (according to the classification of Baron *et al.*, 1988). The pelvic and hypogastric nerves were not included in the preparation. The tissue was transferred into ice cold, carbogenated modified Krebs' bicarbonate buffer for dissection. Animals were then killed by severance of the abdominal aorta. Composition (mM) of the Krebs' solution used during dissection was as follows; NaCl 117.9, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.3, $MgSO_4(H_2O)_7$ 1.2, $CaCl_2$ 2.5, sodium butyrate 1, sodium acetate 10, glucose 5.55, and Indomethacin (3μM).

The distal colon was opened longitudinally off-centre to the antimesenteric border in order to orientate lumbar colonic nerve insertions to lie along the edge of the opened preparation. The faecal pellets were removed. After this dissection approximately 1cm of colon lay below the insertion point most closely related to the IMG and 4 cm lay above this point. Connective tissue was dissected away from the neurovascular bundle. Blood vessels were not dissected away from the nerve, but were left adjacent to the nerve to add support to the preparation. The neurovascular bundle was cut and tied at the level of insertion of the artery into the abdominal aorta. All studies were performed within the guidelines of the animal ethics committees of the University of Adelaide and the Institute for Medical and Veterinary Science.

2.3.2 Bath Layout

The organ bath consisted of two compartments (Fig 2-1). One compartment superfused with Krebs' solutions, accommodated the colon. The other, filled with paraffin oil, contained the neurovascular bundle.

The colonic compartment was further divided into two chambers, one above the other and separated by a mesh. The colon was mounted mucosa side up, so that the serosal surface - supported by the mesh - lay directly over the basement chamber. In this position the insertion points of the lumbar colonic nerves lay along the edge of the tissue abutting the wall separating the two compartments of the bath. After the tissue had been positioned, strips of PVC plastic were pinned over the remaining exposed areas of mesh to prevent movement of solutions between the two chambers.

The smaller basement chamber, over which the colon was pinned, provided superfusion of Krebs' solution to the serosal surface of the colon, whilst the chamber above provided superfusion to the mucosal surface of the tissue.

The colon was pinned down along the side closest to the nerves. The opposite edge of the colon was attached at 1cm intervals to silk threads, which were passed through a pulley system. During baseline conditions the tissue was maintained at approximately *in situ* longitudinal and circumferential length. Each thread could be attached to isometric or isotonic force transducers to measure local motility changes and to apply mechanical stimuli.

Short chain fatty acids replaced glucose in the mucosal superfusate (2mM butyrate, 20mM acetate). Glucose (11.1mM) was the nutrient in the superfusate of the serosal surface. Both Krebs' solutions had the following composition (mM) in common; NaCl 117.9, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.3, $MgSO_4(H_2O)_7$ 1.2, $CaCl_2$ 2.5 and Indomethacin (3μM) and were bubbled with carbogen (O_2 95%,CO_2 5%).

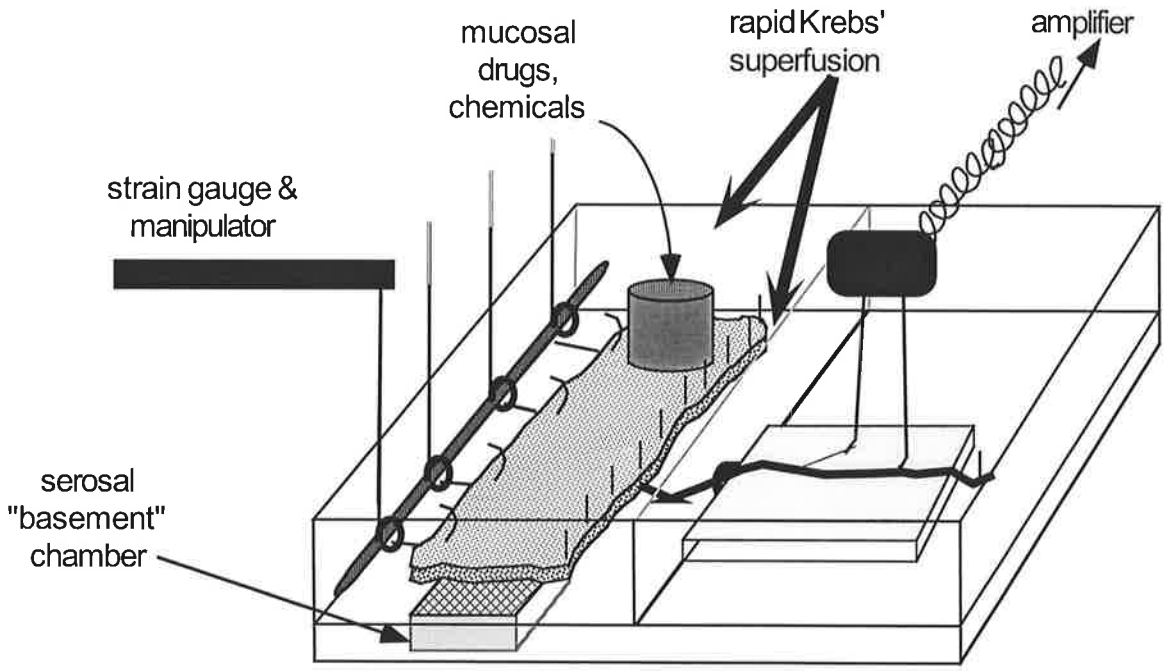

Figure 2-1. Diagram of the organ bath used to record from colonic afferent fibress. The bath had two compartments. The compartment on the left with two chambers held the colon, which was opened out and placed mucosa side uppermost over the basement chamber. The distal colon was pinned down on the right (mesenteric) side. The left side was attached to a pulley system via silk thread. Modified Krebs' solution was superfused over both surfaces of the colon. Drugs were applied locally to the site of the receptive field which was isolated with a metal ring. Single fibre recordings were taken from the intermesenteric/lumbar splanchnic nerve bundle which had been passed through into the right hand oil-filled compartment.

2.3.3 Nerve Recording

The neurovascular bundle was pulled through a hole into the paraffin filled compartment. The bundle lay over a mirror in a small blister of Krebs' solution that was continuous with the solution in the colonic compartment. Under a dissecting microscope, afferent strands were teased away from the neurovascular bundle (which comprised lumbar splanchnic nerves and intermesenteric nerve – Fig 2-2) and placed on a platinum wire recording electrode (0.25mm diameter). A reference electrode was positioned in the blister of solution in which the neurovascular bundle lay. Neural activity was differentially amplified (JRAK, Melbourne), filtered (JRAK, F-1) and displayed on an oscilloscope (Yokogawa DL1300A, Japan). Strands of nerve were discarded or separated into smaller bundles if spontaneous activity of more than three different action potential profiles was observed. Data was not included if the shape and character of two units merged with another such that they could not be discriminated confidently by visual inspection of rapid oscilloscope sweeps. Amplified neural recordings were recorded onto hard disk, along with amplified length and/or tension recordings from muscle via a µ1401 interface (CED, Cambridge, UK). Single fibre activity was discriminated off-line for analysis using Spike2 for Macintosh software (CED).*

2.3.4 Experimental Protocol

After a receptive field had been identified, the temperature of the bath was increased from room temperature (18 – 21°C) to 32°C and routine tests were begun. Mechanical sensitivity of fibres was similar at cold and warm temperatures, and no units showed direct thermosensitivity. In earlier experiments focal electrical stimulation of the colon was used as a search stimulus for afferent fibre endings. This was discontinued because considerable pressure had to be exerted on the tissue by the electrode before an orthodromic action potential could be elicited which could damage the fragile colonic mucosa. Data on conduction velocity of fibres is therefore unavailable.

* Fibres were disconnected from their cell bodies. The effects of this are unknown but assumed to be minimal.

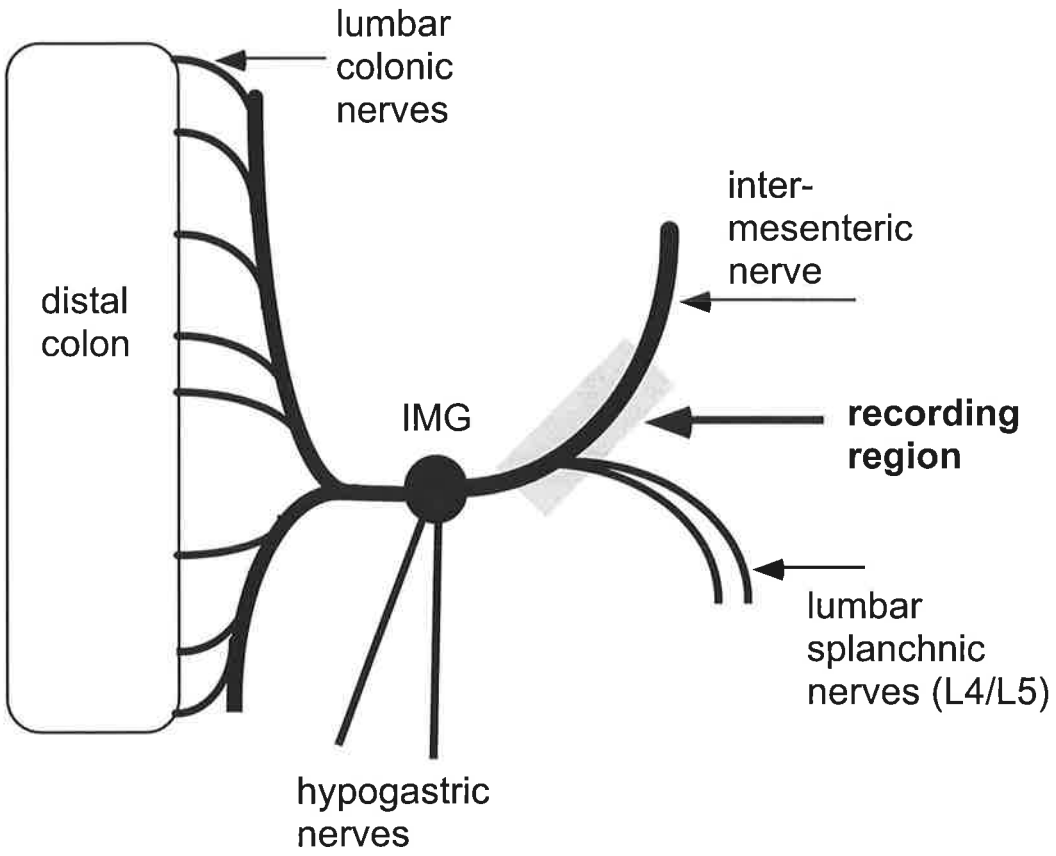

Figure 2-2. Site of afferent fibre recordings. The grey box indicates the region from which afferent fibres were teased. Central to the IMG, the recordings should not include intestinofugal fibres. Due to dissection techniques, afferents from L4 or L5 would have been rarely encountered.

2.3.5 Protocol

A piece of silk thread was attached to the edge of the tissue either with a small fish hook or by sewing a loop into the tissue at the level down the length of the colon closest to the receptive field of the fibre under investigation. The thread was then passed through the pulley system and connected to either the isotonic or isometric transducer.

Mechanical Stimuli

Mechanical stimuli applied manually to the identified receptive field included:

1. Ungraded, manual circumferential stretch, such that the tissue was stretched to twice the approximate *in situ* length.

2. Change of circumferential length (0-10mm from tissue resting length). This was achievied manually using a micromanipulator attached to an isometric transducer to incrementally increase (2mm at a time) the length of the tissue (Fig 2-3).

3. Change of circumferential tension (0, 1, 2, 5 and 8g) applied in a stepwise manner with weights attached to an isotonic transducer (Fig 2-4).

4. Stroking the mucosal surface of the receptive field of a fibre with calibrated Von Frey hairs (10, 50, 200, 1000mg) (Fig 2-5). Von Frey hairs were designed to deliver a constant force when stroking across the surface of the receptive field when held at 45° at bending force (Page & Blackshaw, 1999).

5. On a number of occassions the colonic tissue was turned upon itself exposing the serosal surface. This enabled the application of blunt probing and Von Frey hairs to the receptive field of serosal fibres to identify more accurately the sensitivity and site of a receptive field.

6. A later protocol of circumferential length and tension included the incorporation of a mechanical ventilator providing a sinusoidal wave stimulus whilst still

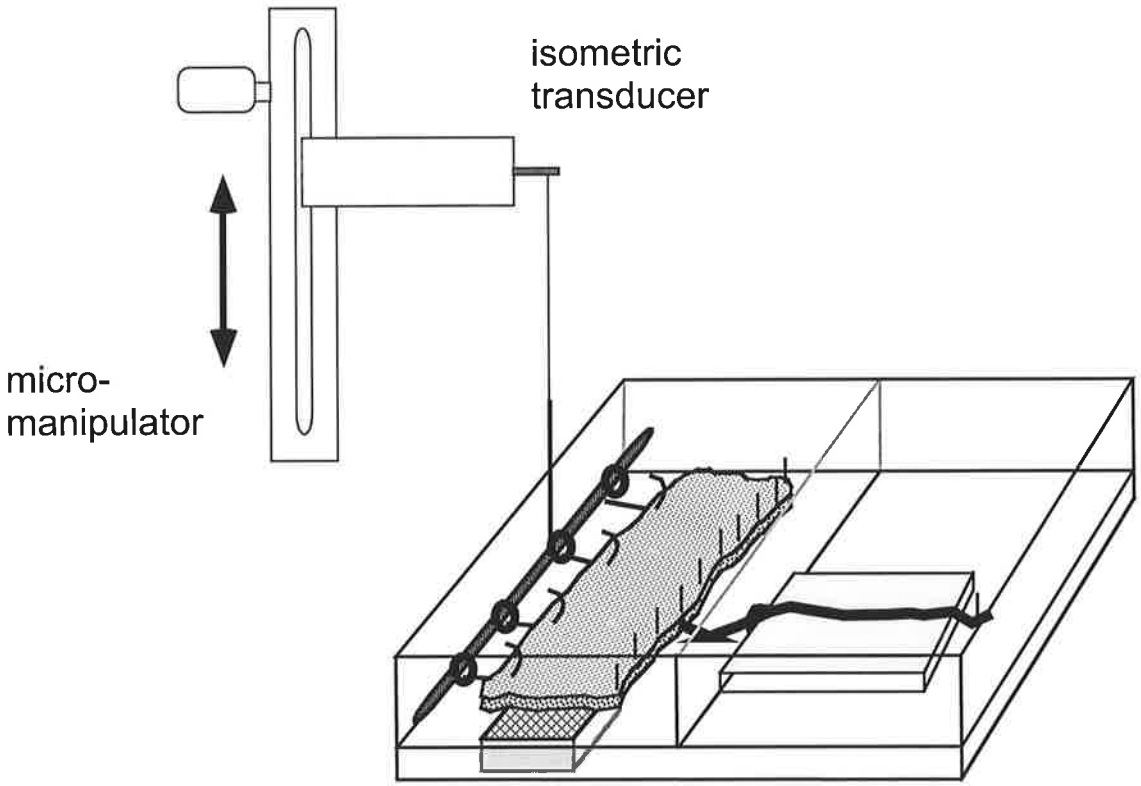

Figure 2-3. Isometric transducer with organ bath. A thread was attached to the tissue adjacent to the receptive field of the fibre under investigation. The thread was attached to an isometric transducer and micromanipulator. The tissue was stretched circumferentially 2mm at a time providing a graded static length stimulus whilst concurrently recording spontaneous muscular tension.

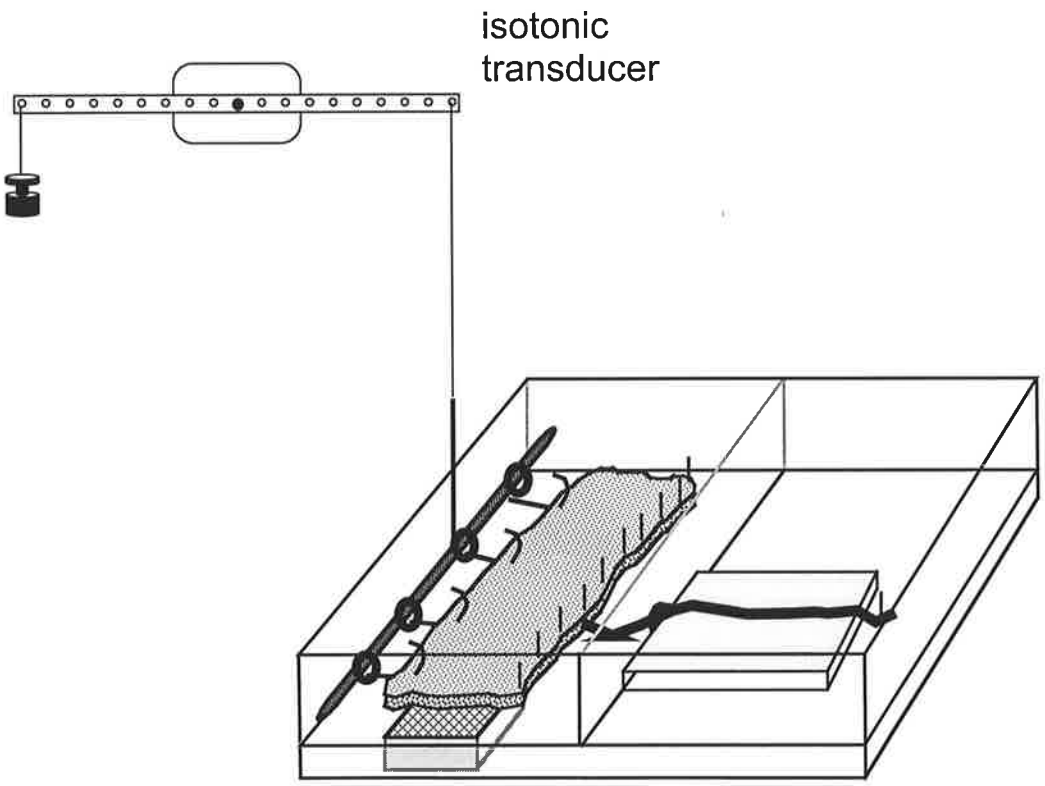

Figure 2-4. Isotonic transducer with organ bath. A thread was attached to the tissue adjacent to the receptive field of the fibre under investigation. The thread was attached to an isotonic transducer. The tissue was stretched circumferentially by adding weights to the other side of the fulcrum, thus providing a graded static tension stimulus whilst concurrently recording spontaneous muscular length.

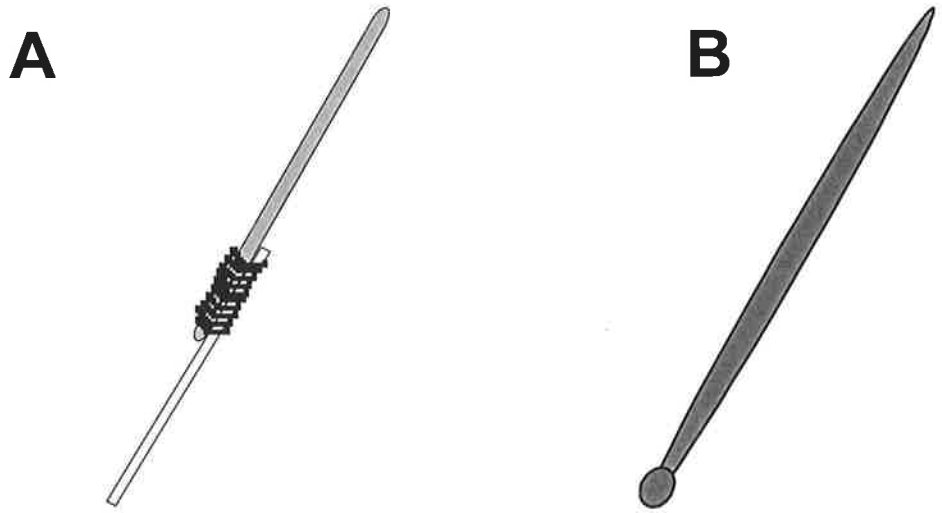

Figure 2-5. Probing implements. A. Von Frey hairs were constructed with a PVC tube tip attached to a wooden handle. The PVC tube was of differing size and length depending on the force intended. They were calibrated to the correct force by probing the surface of a balance and adjusting the length of the tube accordingly. The probe was held at 45 degree angle and pressed to bending point. **B.** The blunt probe was a glass thermometer with ball point.

allowing concurrent recording of length and tension changes using both isometric and isotonic transducers (Fig 2-6).

Chemical Stimuli

Chemical stimuli applied to the site of the receptive field included:

distilled water, isotonic NaCl (154mM), hypertonic NaCl (308mM),
D-mannitol (isotonic 308mM, hypertonic 462mM and 616mM),
ferret bile (removed from the gallbladder of ferrets used for other studies in our laboratory) full strength or diluted by 50% with distilled water,
deoxycholic acid (DCA) 3mM,
HCl 50mM,
capsaicin (caps) 100μM,
5-hydroxytryptamine (5-HT) 10μM, 100μM,
bradykinin (BK) 1μM,
noradrenaline (NA) 1μM,
prostaglandin E_2 (PGE_2) 1μM, 10μM, 100μM,
prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 1μM, 10μM, 100μM, 1mM,
prostaglandin D_2 (PGD_2) 1μM, 10μM, 100μM, 1mM.

Bradykinin was purchased from Auspep, Parkville; PGE_2, $PGF_{2\alpha}$, PGD_2 from Sapphire Bioscience, Alexandria; all other chemical agents were purchased from Sigma, Sydney. All drugs were prepared in normal saline, diluted to the required concentration with normal saline and frozen in 1ml aliquots at –20°C until required. The exception was capsaicin, which was prepared in 1:1:8 of ethanol, tween and normal saline to a stock solution of 1M before being diluted to required concentrations and frozen. Any drug that was to be applied to the tissue by either superfusion or spritzing of the serosal surface was added to the Krebs' solution at a concentration allowing a dilution ratio of 1:1000 to reach the final desired concentration.

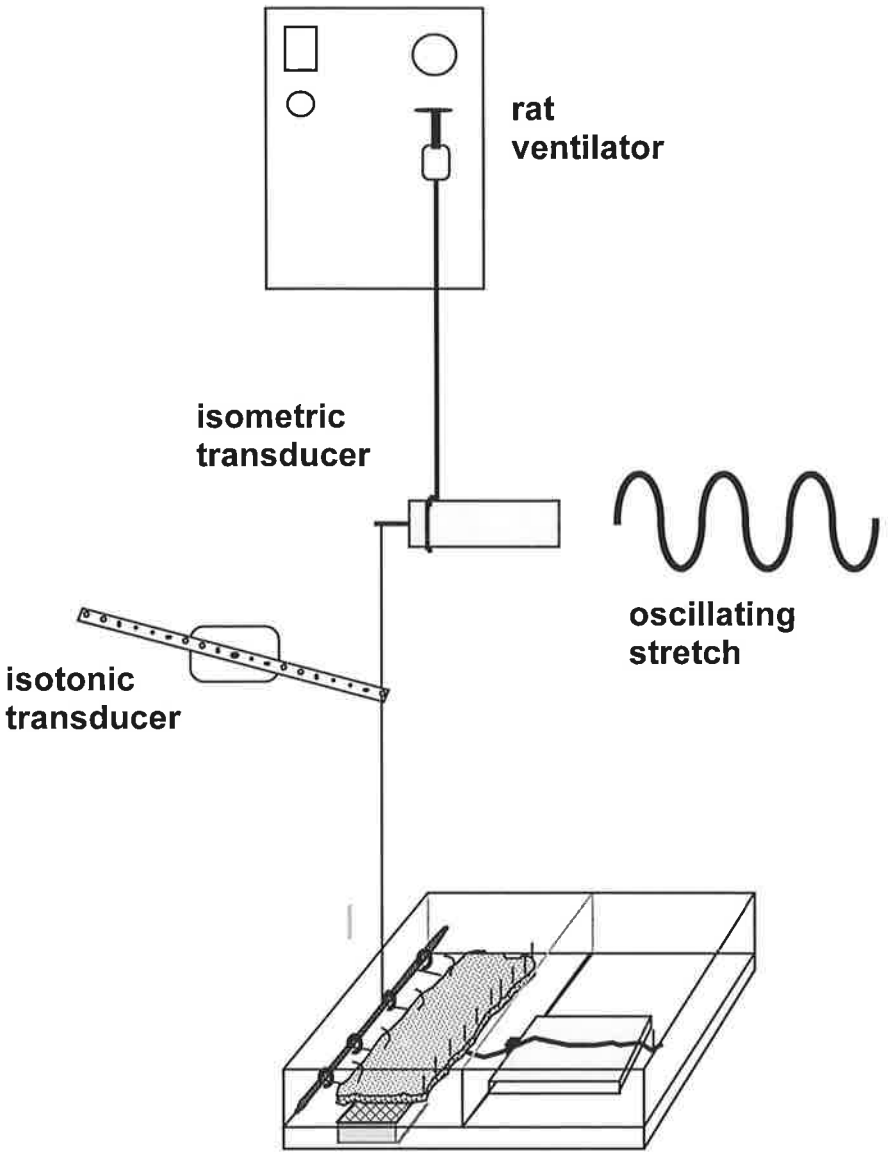

Figure 2-6. Oscillating stretch stimulus. A thread was attached to the tissue adjacent to the receptive field and attached to first an isotonic transducer and then an isometric transducer that was held by a rat ventilator. The rat ventilator allowed a sinusoidal oscillating stimulus to be applied to the tissue.

Chemical stimuli were applied to the site of the receptive field on the mucosal surface into a 1cm-diameter ring that was placed directly around the receptive field. Chemical stimuli were applied usually for 2 minutes before being aspirated from the ring. The ring was then lifted and the area of tissue superfused with Krebs' solution for at least three minutes before re-application of the ring for administration of another stimulus. Chemicals were administered in the following order; distilled water, isotonic NaCl (154mM), 308mM NaCl, undiluted or 50% diluted ferret bile, 50mM HCl and 100μM capsaicin. This order was generally followed so that noxious stimuli were given later in the order to avoid damage or desensitisation earlier in the protocol. Exceptions to this were 12 fibres where bile was given before 308mM NaCl. This did not affect the pattern of results when compared to the other protocol. When the same chemical stimuli were administered a second time to the majority of responsive afferent fibres, no desensitisation of responses was evident, with the exception of bile (see later). Chemosensitivity was routinely investigated in mucosal and serosal colonic afferent fibres. In order to exclude the possibility that chemically induced responses were secondary to induced muscular activity, sensitive strain gauge recordings of muscular activity were routinely made concurrently with neural recordings. Chemosensitivity in muscular afferent fibres was not as routinely investigated, as the focus in this respect was on mucosal and serosal fibres.

On occasion, serosal application of chemicals was achieved by adding the chemical stimuli into the reservoir of the serosal chamber, the tubing was rerouted and the chemical solution superfused across the surface of the serosa (through the basement chamber) at 32°C. Alternatively, the tissue was turned upon itself, thus exposing the receptive field and the chemicals were added to the serosal surface into a ring placed onto the tissue in identical manner to that used for mucosal application.

Chemical Treatments

Indomethacin (3μM) was used as a pharmacological treatment. Indomethacin was present in the Krebs' solution at the beginning of the study and removed to

essentially allow endogenous prostaglandins to be the pharmacological treatment. Indomethacin was then reintroduced into the Krebs' solution during the recovery period. With the use of dye, it was established that 30mls (2 min) of solution was required to displace the original solution in the bath. Thus, treatment and recovery periods were 12 min in duration allowing 10 min of superfusion at the correct concentration of indomethacin.

2.3.6 Definitions of Fibre Classifications

The criteria used for classification of afferent fibres were based on those used previously for vagal afferent fibres, particularly those with their endings in the mucosa, and observations already documented for spinal afferent fibres from the colon. All criteria are based on responses to mechanical stimulation.

Mucosal afferent fibres were defined by their ability to respond to stroking with a 10mg Von Frey hair (Page & Blackshaw, 1998), and a lack of response to circumferential stretch or spontaneous activity in the muscle (Blackshaw & Grundy, 1993*a*).

Muscular afferent fibres were defined by their ability to respond readily to circumferential stretch of the tissue with a response that adapted slowly over the duration of the stretch. Traditionally, the activity of muscular afferent fibres has been correlated with the spontaneous activity in the muscle (Cottrell, 1984*b*). As the colonic tissue in this study demonstrated very little spontaneous contractile activity, I was unable use this criterion in the classification of these afferent fibres.

Serosal afferent fibres were defined by their ability to respond to firm blunt probing on the mucosal surface, but not light mechanical stimuli, and a rapidly adapting (if any) response to circumferential stretch. Another characteristic of these afferent fibres was that they responded at lower mechanical thresholds to stimulation of the reflected serosal surface than stimulation of the mucosal surface. This procedure was, however, not feasible on a routine basis.

Other fibres were grouped according to similarities in their patterns of responses to particular stimuli after all data were gathered. Two groups were identified. One group contained fibres with multiple receptive fields. In the other group, fibres were recruited in response to locally applied chemicals, but no mechanoreceptive field was identifiable at the beginning of the experimental protocol.

2.3.7 Data Analysis

Data was analysed off-line using Spike2 for Macintosh (CED, Cambridge). On the occasion that there were multiple unitary action potentials recorded from the same strand of nerve tissue, the software was set up to discriminate between them and generate an independent histogram of the frequency of activity in each unit. The software was also set up to track the changes in the action potential profile of a unit over the course of a study. Spontaneous discharge was measured as the mean over one minute as soon as possible after identification of units and equilibration of the bath. Responses to chemicals were counted when a maintained >50% increase in discharge above basal levels occurred. To be counted, smaller responses than this (>25%) had to meet the additional criteria that they were superimposed on a particularly steady level of resting activity and were highly reproducible on repetition of the stimulus. Latency was determined as that point where there was a 50% increase in the mean discharge rate from basal discharge rate and is expressed in seconds (s). Data are expressed as mean±SEM.

2.4 Discussion

There were some theoretical issues of particular interest that motivated the design of the bath finally used for this project. These issues were not necessarily those that were subsequently investigated, but are relevent to the documentation of the design process. The first issue related to the lack of reports of mucosal fibres in nerves innervating the colon. There had been reports of mucosal fibres in spinal nerves innervating the cloaca of the duck (Koley *et al.*, 1984), and the rectal and anal canal (Clifton *et al.*, 1976; Janig & Koltzenburg, 1991; Sengupta & Gebhart, 1994) but apart from that the documentation of mucosal fibres were isolated to the vagus. One notable absence in the literature on colonic sensation is the use of

adequate mucosal mechanical stimuli - the primary stimulus most often used being distension of the gut. Thus, this *in vitro* preparation needed to allow distension-like stimuli along with discrete stimuli to the mucosal surface, a technique that had proved particularly useful in investigating accurately the chemo and mechano sensitivity of individual receptive fields of vagal afferent fibres arising from the oesophagus of the ferret (Page & Blackshaw, 1998). By opening the tissue out flat on a firm surface (the mesh overlaying the basement chamber) and exposing the mucosal surface, it was possible to apply a shearing or stroking stimulus to the mucosal surface of the colon and accurately quantify the sensitivity of fibres to mucosal stimuli with Von Frey hairs. This had not been possible with previous methods.

2.4.1 Colonic Mucosal Viability

Related to the search for mucosal fibres was the necessity of maintaining the integrity of the mucosa, an often ignored but vital issue when applying mucosal stimuli. *In vitro* preparations of the colon are often either stripped of the muscle for mucosal studies or stripped of the mucosa. It very quickly became clear that using a whole organ system needed additional attention to the nutrient requirements to prevent the mucosa sloughing off. The colonic mucosa uses short chain fatty acids as its primary nutrients rather than glucose (Scheppach, 1994) which causes a detrimental buildup of lactate in colonocytes (Krishnan *et al.*, 1998). This was taken into account firstly with the physical design of the bath. The two-chamber design allowed superfusion of the tissue on both the mucosal and serosal surface. The thicker the tissue, the more difficult is passive penetration of nutrients to the centre of the tissue. Therefore, superfusion of nutrients over both surfaces of the tissue was beneficial. Secondly, two superfusion chambers allowed different nutrient solutions to be used for each surface of the tissue. For this reason a mixture of short chain fatty acids replaced glucose in the mucosal superfusate (see Scheppach, 1994). Thus, the mucosa received nutrients appropriate to its requirement as did the serosa and muscular layers. Preliminary studies with dye demonstrated that there was remarkably little mixing of solutions between the two chambers even though flow in both chambers was not obviously laminar.

Nonetheless, the exposed pieces of mesh not covered with colon were routinely covered with a strip of PVC plastic to prevent mixing of the two solutions.

Indomethacin (3μM) was added to regular and modified Krebs' solutions to reduce prostaglandin synthesis. Although prostaglandins are thought to reduce colonic epithelial proliferation and thereby to have therapeutic effects *in vivo* (Craven *et al.*, 1983), effects of local accumulation of prostaglandins as may occur *in vitro* are more incompletely understood. When colonic mucosa is incubated *in vitro*, there is a very high basal production of PGE_2 in the first few hours which reaches a much lower and stable level after 5 hours (Nygård & Berglindh, 1989). The high initial basal production is attributed to stress on the tissue during removal and dissection, a situation reminiscent of the current preparation. Indeed, trauma caused by pressure and manipulation significantly increase the levels of PGE_2 in gastric mucosa of humans *in vitro* (Bennett *et al.*, 1977). I therefore reduced prostaglandin synthesis with indomethacin as a precautionary measure to avoid possible pro-inflammatory influences often associated with prostaglandins (e.g. Su & Gebhart, 1998). Indomethacin at a high concentration of 0.25mM abolishes production of all prostaglandins in rat distal colon *in vitro* (Craven *et al.*, 1983). A concentration of 66nM decreased the initial surge of PGE_2 production in rabbit colonic mucosal explants by 50% and by 100% at 10μM (Nygård & Berglindh, 1989). Consequently, an intermediate dose of 3μM was chosen to add to the superfusate of the bath. At the beginning of the development process, when the colon was pinned out flat in a normal organ bath, with only glucose Krebs being superfused across the mucosal surface visible sloughing of the mucosa would begin within an hour of harvesting the tissue. After making the changes described here so far, visible sloughing of the mucosa would not occur until 4-6 hours after harvesting of the tissue. No doubt there were other pharmacological measures that could have been taken to help prevent mucosal deterioration. Indeed, 5-HT is probably released in response to mechanical manipulation (Larsson, 1981) as are other pro-inflammatory mediators. However, the improvements at this stage were substantial and it was felt that any gain from further pharmacological interference would not outweigh the disadvantages of manipulating the system further.

2.4.2 Other Specific Novel Features of the Preparation

Both surfaces of the tissue were superfused with Krebs' solution at the rate of 15ml/min. A high rate of superfusion has been used previously in *in vitro* preparations, notably in the guinea pig airways (Fox *et al.*, 1993) and the ferret oesophagus (Page & Blackshaw, 1998). Bath temperature was maintained at 21°C prior to the commencement of the experimental protocol and raised to 32°C only following identification of a viable fibre; this procedure was followed to reduce the rate of deterioration of the mucosa. The only disadvantage for pursuing this protocol was that the spontaneous activity in the muscle was considerably decreased than if the preparation was run at 37°C as was the case in preliminary studies not described here. Those fibres identified at room temperature did not obviously change their mechanosensitivity when the temperature of the bath was raised. There were, however, a number of fibres that were silent at room temperature but became spontaneously active when the temperature of the tissue was raised to a more physiological level. This phenomenom was not investigated in this study, but has been subject to investigation elsewhere. Pelvic afferent fibres (Su & Gebhart, 1998) and gastroduodenal splanchnic afferents (Gupta *et al.*, 1979; Riedel, 1976) have been described as being specifically cold or heat sensitive. In addition, intracolonic heat and cold was shown to potentiate and inhibit, respectively, mechanosensitivity of distension-sensitive afferents (Su & Gebhart, 1998). The estimated threshold for cold fibres was 28°C - a temperature that was crossed between the identification of receptive fields and the subsequent onset of the experimental protocol. Some of the fibres that developed spontaneous activity as the temperature increased have been included in the studies presented here. It is not known whether the temperature change in the current study biased the recruitment of afferents by affecting mechanosensitivity. However, all mechanical stimuli used to characterise fibres were repeated at the higher temperature, thus submitting these fibres to the full characterisation protocol.

Other *in vitro* gastrointestinal techniques have utilised some similar aspects of the technique developed here. With the aim of refining the application of stretch to a piece of tissue, the ileum has been opened out and placed flat in the organ bath (Brookes *et al.*, 1999). In their study, the tissue was pinned down along one edge

and stretch was applied to the opposite side. This was achieved with a rake like arrangement that allowed a long portion of the tissue to be stretched at a time. My technique employed only one thread attached to the site nearest the receptive field. This ensured that the stretch stimulus was applied locally to the receptive field only, but because of that there was some distortion longitudinally as the edge was distorted. The Brookes method compromises on the discrete nature of the stretch, but because it includes a larger area of tissue included in the stretch, the stretch that a receptive field would experience would include more circumferential influence and less longitudinal influence.

In the light of some recent evidence suggesting that a population of nerves arising from the colon of the guinea-pig are volume-sensitive rather than tension-sensitive (Anthony & Kreulen, 1990), it was considered necessary to be able to mimic distension of the gut, with the ability to control and record both tension and length of the tissue. Three different options were pursued and refined for investigating this issue. Chapter 5 is devoted to this part of the project and a full discussion of this can be found there.

The cat (Blumberg *et al.*, 1983; Floyd *et al.*, 1976; Floyd & Lawrenson, 1979; Haupt *et al.*, 1983; Morrison, 1973) and the rat (Sengupta & Gebhart, 1994; Su & Gebhart, 1998) are the favoured models for investigation of colonic sensation. The rat was chosen for this project for a number of reasons. It has been used before for investigation of colonic afferent fibres and perhaps more importantly for future studies beyond this thesis, the rat has been firmly established as a model for colonic inflammation (Buell & Berin, 1994; Yamada *et al.*, 1992). The rat had a size advantage over the cat or species used for colonic inflammation such as the rabbit (Eysselein *et al.*, 1991). As mucosal viability was a priority, thinner tissue (from the smaller animal) was preferred to maximise absorption of nutrients in the tissue. Young adult rats (180-220g) were used for this reason. These rats were also lean, thus aiding the ease of dissection. The anatomy of the rat distal colon was also ideally suited to this preparation. Unlike the guinea pig and ferret (personal observations from preliminary studies), the rat mesentery in the distal colon is not arranged in an arcade. Instead, the lumbar colonic nerves and

associated blood vessels exit the colon and join a single main bundle that lies in relatively close apposition to the gut itself. Therefore, when laid out in the organ bath in this study, the main neurovascular bundle lay in a straight line next to the edge of the basement chamber underneath the colon. This meant that the neurovascular bundle was readily exposed to the glucose supplemented Krebs' and interfered with only a small portion of the colon which an arcade would not. Only female rats were used. No control was made for the oestrus cycle.

The methods and the development of the technique discussed in this chapter formed a significant part of the overall project in terms of time and thought. However, it is not the only part of the overall project that was devoted to technique development. A similar mouse preparation was also developed in the final stages of this project. The mouse preparation presented different challenges to the ones described here, despite the many eventual similarities between the two techniques. Therefore, Chapter 8 has been devoted to the development and utilisation of the mouse preparation and a full discussion of the comparison between the rat and the mouse preparation can be found in that chapter.

Chapter 3. Mechanosensitivity and Classification of Colonic Afferent fibres

3.1 Abstract

1. The mechanosensitivity of 53 afferent fibres with single punctate receptive fields in the distal colon was investigated.
2. Twelve fibres showed characteristics of having endings in the mucosa. Mucosal afferent fibres were sensitive to stroking with a 10mg Von Frey hair and mucosal probing, but were not sensitive to circumferential stretch.
3. Ten fibres showed characteristics of having receptive fields in the muscular layer. These fibres responded readily to circumferential stretch, as well as to blunt probing of the mucosa. They were not sensitive to a 10mg Von Frey hair.
4. Twenty-seven fibres showed characteristics of having endings in the serosal layer. 26 fibres did not respond and 1 fibre adapted rapidly to circumferential stretch. All responded to blunt probing of the mucosa and were generally sensitive to light stroking on the serosal surface.
5. Three fibres were unresponsive to all mechanical stimuli but were recruited by chemical stimuli.
6. One fibre responded to all mechanical stimuli applied; a 10mg Von Frey hair, blunt probing of the mucosa and circumferential stretch.
7. This is the first characterisation of colonic afferent fibres using an *in vitro* method and the first documentation of afferent fibres with their endings in the mucosa of the colon.

3.2 Introduction

All afferent fibres documented in this thesis were characterised by their mechanosensitivity. This was the first time that this range of mechanical stimuli has been used for characterisation of spinal afferent fibres innervating the distal colon of the rat. For this reason and because this was a novel preparation it was necessary to discuss the mechanosensitivity of these afferent fibres as an investigative measure as well as using these criteria to characterise the fibres.

3.3 Methods

A full description of the methods used in this chapter can be found in Chapter 2.

3.4 Results

3.4.1 General Topographical Features of Colonic Afferent Fibres

Fifty-three afferent fibres were used for this study of mechanosensitivity. Fifty were identifiable as single units that had a single mechanoreceptive field (Table 3-1). Mechanoreceptive fields were 1-4mm^2 in size, according to mapping with a blunt probe, and were clustered along the mesenteric border of the colon (Fig 3-1). They were found above and below the point of exit of the main neurovascular bundle and therefore ascended or descended in the lumbar colonic nerves before projecting centrally.

3.4.2 Mucosal Afferent fibres

Twelve of the 50 fibres described above fitted the classification criteria for mucosal afferent fibres. Each afferent fibre had only one receptive field. The size of the receptive fields for mucosal afferent fibres was generally smaller than other classes, not exceeding 2mm^2, although precise quantification was not possible using blunt probing. Seven afferent fibres initially showed no resting activity. Three of these did not develop any resting activity over the course of the study, and four showed sporadic resting activity by the end of the study. The other 5 mucosal afferent fibres were all spontaneously active, but none with a rate exceeding 1 spike s^{-1} (mean 0.30±0.09 spike s^{-1}; see Fig 3-1 for data on all mucosal afferent fibres).

All mucosal afferent fibres responded to stroking and were sensitive to the full range of Von Frey hairs when moved across their receptive field (10, 50, 200 and 1000mg; e.g. Fig 3-2). Responses were of short latency and short duration, not exceeding 4 seconds. Eleven out of twelve fibres tested responded to blunt probing, but none responded to circumferential stretch of the tissue. Mechanical

Unit	Probe	Stretch	Stroke 1000mg	Stroke 10mg	Chemo-sensitive	Spontaneous Activity
Serosal						
72-1	+	0	+	0		+
82-1	+	0	+	0		+
96-3	+	0	+	0		+
100-2	+	0	0	0		+
50-1	+	0	0	0		+
59-1	+	0	0	0		+
135-1	+	0	0	0		+
116-3	+	0	0	0		+
130-1	+	0	0	0	0	+
130-3	+	0	0	0	0	+
123-1	+	+	0	0	+	+
138-1	+	0	0	0	+	+
128-1	+	0	0	0	+	+
125-1	+	0	0	0	+	+
114-1	+	0	0	0	+	+
71-4	+	0	0	0	+	+
74-1	+	0	0	0	+	0
69-2	+	0	0	0	0	+
83-1	+	0	+	0	+	+
87-1	+	0	0	0	+	+
48-1	+	0	0	0	+	+
55-2	+	0	+	0	+	+
61-1	+	0	0	0	0	+
61-2	+	0	0	0	+	0
64-1	+	0	0	0	+	+
64-2	+	0	0	0	+	+
68-1	+	0	+	0	+	0
Muscular						
15-1	+	+				+
1-1	+	+				0
50-3	+	+				+
102-2	+	+				+
59-2	+	+	0	0		+
106-4	+	+	+	0		+
71-3	+	+			+	+
57-1	+	+	0	0	0	0
116-1	+	+	0	0		0
130-2	+	+	0	0	+	+
Mucosal						
91-1	+	0	+	+		0
121-2	+	0	+	+	0	0
137-1	+	0	+	+	+	0
106-2	+	0	+	+	+	+
106-3	+	0	+	+	+	+
47-2	+		+	+	+	+
69-1	0	0	+	+	+	+
55-1	+	0	+	+	+	0
58-1	+	0	+	+	+	0
58-2	+	0	+	+	+	0
76-1	+	0	+	+	0	0
84-1	+	0	+	+	+	+
114-2	0	0	0	0	+	+
125-6	0	0	0	0	+	+
121-1	0	0	0	0	+	0

Table 3-1. Summary of mechanosensitivity of individual afferent fibres. A response to a stimulus or presence of spontaneous activity is indicated with **+**, no response or activity with **0**. A blank is left when a particular stimulus was not tested in a study. Afferents are grouped in classifications of serosal, muscular, mucosal and other chemosensitive afferents (see methods for classification criteria). Mechanical sensitivity is shown as that present at the beginning of studies.

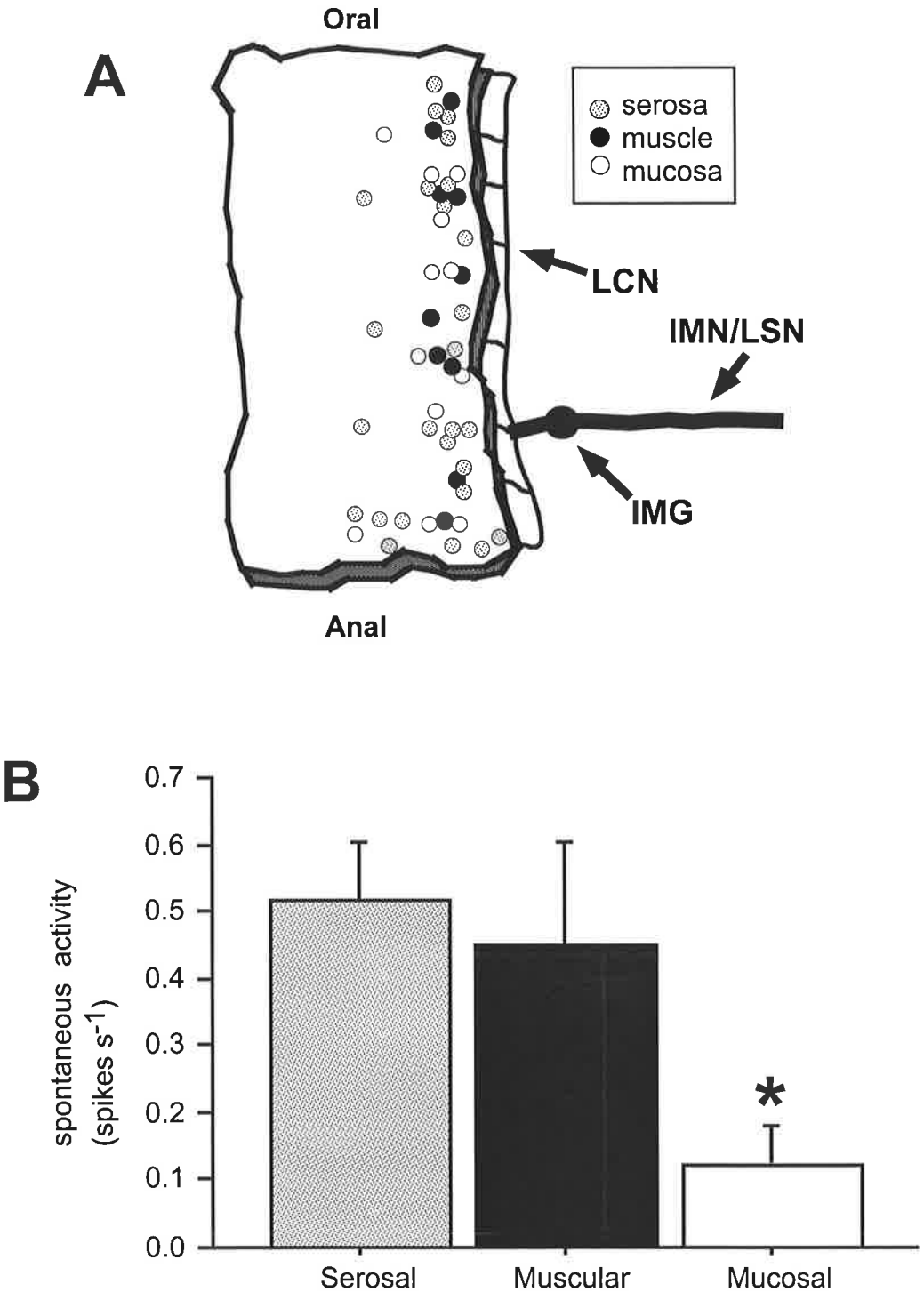

Fig 3-1. A schematic diagram of the location of receptive fields of all fibres in the serosa, mucosa and muscle in the distal colon and the spontaneous activity of these fibres determined at the beginning of the study. A. Each dot represents the site, not the size of the receptive field. **B.** There is no statistically significant difference between the spontaneous activity of the serosal and muscular fibres. Mucosal fibres, however, have a lower spontaneous activity than serosal fibres ($p<0.01$) and muscular fibres ($p<0.05$).

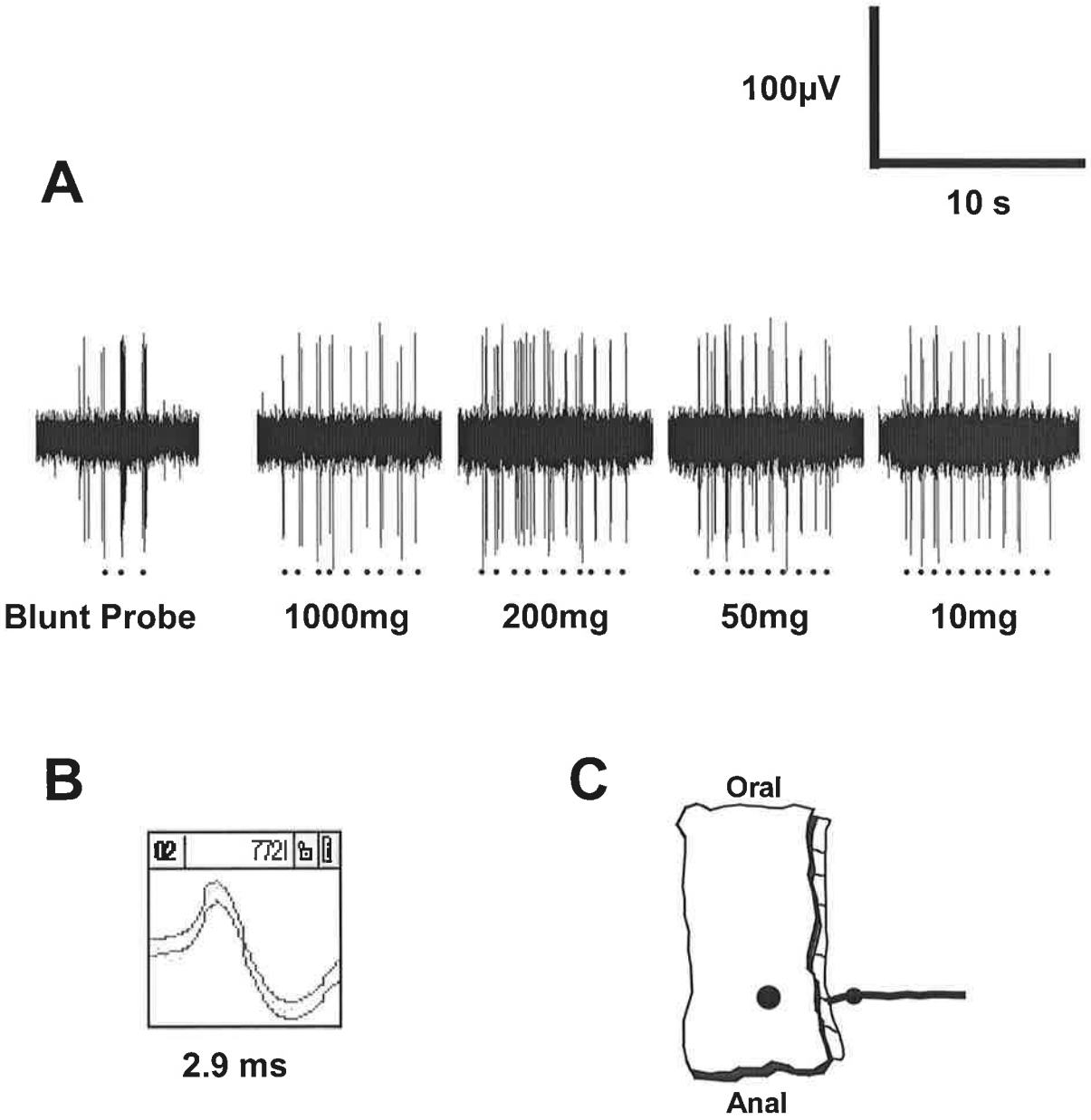

Figure 3-2. Response of mucosal fibre to repeated blunt probing and von Frey hairs. A. Raw tracing of a single unit responding to blunt probing and von Frey hairs. Each dot represents one brief stroke over the surface of the receptive field. This fibre did not respond to ungraded circumferential stretch. **B.** The wave template of the fibre. **C.** The site of the receptive field of the fibre.

sensitivity of mucosal afferent fibres was not influenced observably when tested after recovery from responses to chemical stimuli.

3.4.3 Serosal Afferent fibres

Twenty-seven fibres fitted the criteria for classification as serosal afferent fibres. Three of these were initially silent but developed activity after application of one or more chemical stimuli. All other afferent fibres had resting activity, of which approximately equal numbers increased, decreased and maintained their initial level of activity during the remainder of the study. The rate of resting activity in active fibres was 0.58±0.09 spikes s^{-1} (see Fig. 3-1 for data on all serosal afferent fibres).

Blunt probing was the most effective mechanical stimulus and all fibres demonstrated high threshold responses (e.g. Fig 3-3). Where feasible, the sensitivity of these fibres to tactile stimulation on the serosal surface rather than the mucosal surface was tested as a confirming variable and was found to show greater sensitivity. This also revealed a small (2-4mm^2) punctate receptive field. One afferent fibre responded to ungraded circumferential stretch with a short initial burst of action potentials, which was not sustained for the duration of the stretch. Four fibres fired <5 spikes at the onset of the stretch that was consistent with the serosal surface shearing against the mesh over the basement chamber. These were not reproducible responses. Of 21 fibres tested, 5 responded to a 1000mg Von Frey hair applied to the mucosal surface and one of these to a 200mg Von Frey hair. There were no responses to mucosally applied 50mg or 10mg Von Frey hairs.

3.4.4 Muscular Afferent fibres

Ten fibres were classified as muscular afferent fibres. Seven of these had spontaneous activity (see Fig. 3-1 for group data) and responded readily to ungraded circumferential stretch (e.g. Fig 3-4). The response was maintained for the duration of the stimulus but ceased as the stimulus was removed. This activity

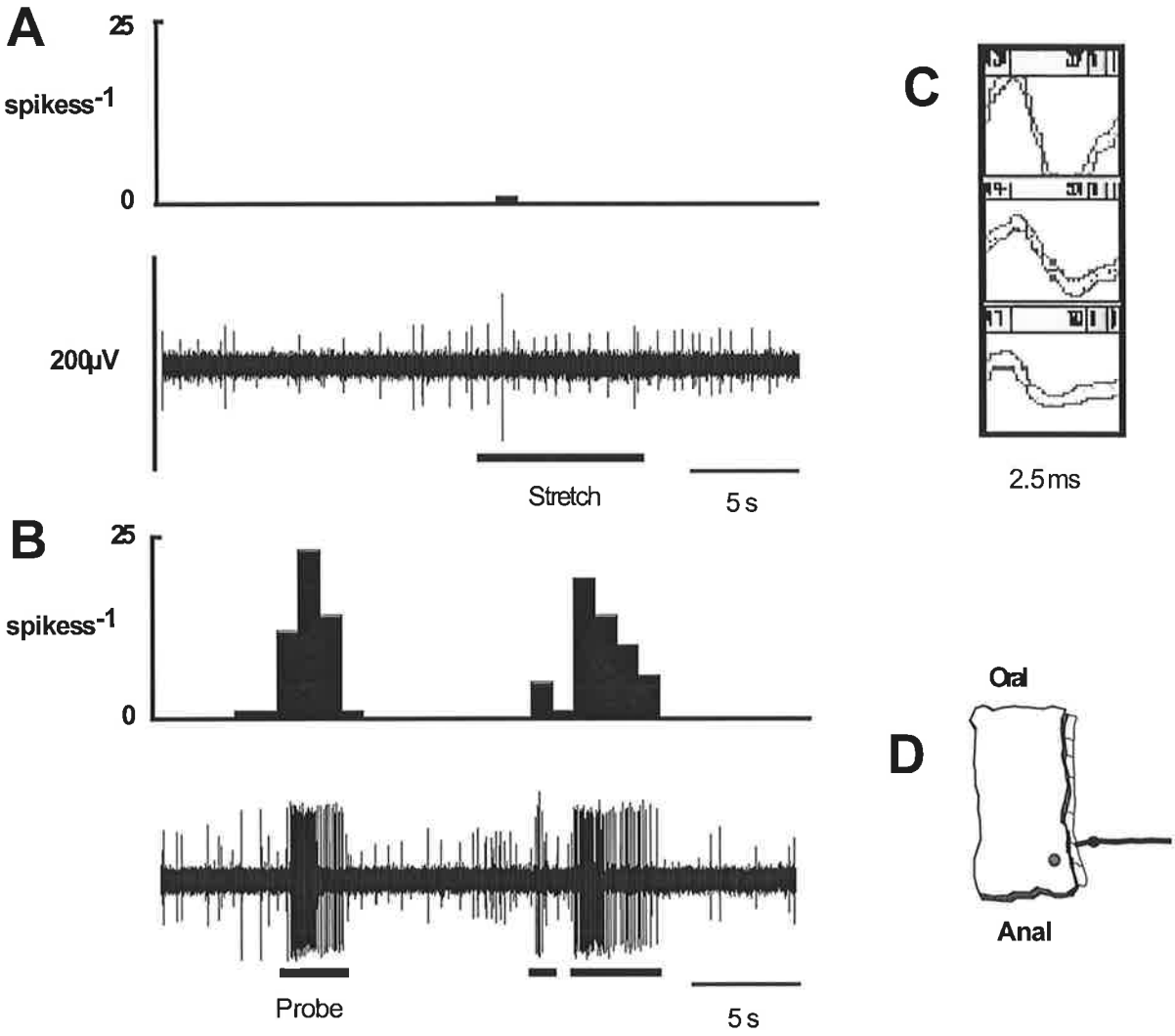

Figure 3-3. Response of a serosal fibre to circumferential stretch and blunt probing. A & B: The integrated record of the fibre's activity is directly above the raw record. Three fibres were active on this strand, the unit of interest is the one with the largest amplitude (**C**). A single action potential was all that was evoked by circumferential stretch (**A**), whereas a robust discharge was seen during probing (**B**). An illustration of the precise location of the receptive field of this fibre is shown in **D**.

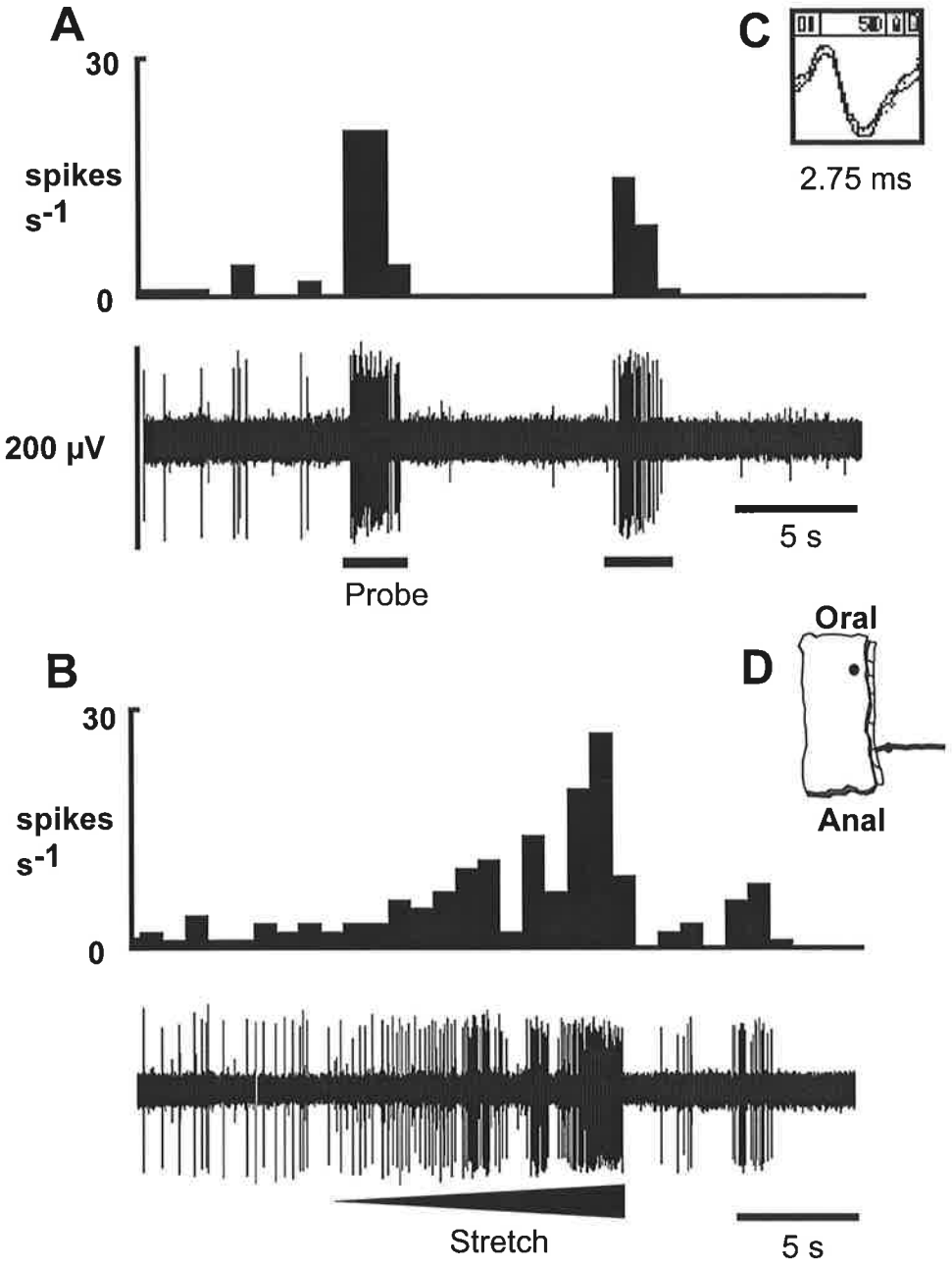

Figure 3-4. Response of a muscular fibre to blunt probing and circumferential stretch. A & B: The integrated record of the fibre's activity is directly above the raw record. Only the unit of interest is active on this strand. This unit had a low level of spontaneous activity. It responded to probing (**A**) with a burst of firing that stopped abruptly when the stimulus was removed. There was a subsequent reduction in the spontaneous activity. This fibre has a slowly adapting response to circumferential stretch (**B**). The degree of distortion is gradually increased throughout the stimulus that resulted in a gradual increase in the rate of firing. When the stretch was released, a brief reduction in activity below resting level occurred. The shape of the action potential is shown in **C**. The location of the receptive field is illustrated in **D**.

could not be correlated with muscular contractions as little or no spontaneous muscular activity was seen in this tissue.

One fibre responded to Von Frey hairs (10-1000mg), probing of the mucosal surface and ungraded circumferential stretch. This was the only fibre that responded to all three of the search mechanical stimuli. This fibre was not included in the mucosal, muscular or serosal classifications. The mechanosensitivity of this fibre is consistent with a further classification of vagal afferent fibres in the oesophagus of the ferret which are termed tension-mucosal and are sensitive to both mucosal stroking and circumferential stretch of the tissue (Page & Blackshaw, 1998).

Three afferent fibres initially had no mechanoreceptive fields and were not spontaneously active, but were recruited during application of chemicals to the receptive field and surrounding tissue of another fibre under investigation. Subsequent reinvestigation of mechanical sensitivity revealed responses to probing in all cases, and in one afferent fibre a 10mg Von Frey hair was tested and a response evoked.

One other type of fibre, not included in this chapter, with multiple receptive fields was encountered and a full description of them can be found in Chapter 6.

3.5 Discussion

This study provides the first description of primary afferent fibres with receptive fields in the colonic mucosa. These were found along with muscular and serosal afferent fibres in the lumbar splanchnic and intermesenteric nerves of female Sprague-Dawley rats. This study is also the first to investigate the functional properties of afferent fibres following this pathway in the rat. These findings were made using a novel *in vitro* technique that allowed improved access to the colon over traditional *in vivo* techniques. In small mammals *in vitro* techniques have proved invaluable in improving understanding of the functional properties of afferent fibres in the rat stomach (Wei *et al.*, 1995) and small intestine (Cervero &

Sharkey, 1988), guinea-pig ureter (Cervero & Sann, 1989) and airways (Fox *et al.*, 1993), ferret oesophagus and stomach (Page & Blackshaw, 1998) and mouse skin (Koltzenburg *et al.*, 1997). In addition to substantiating findings made *in vivo*, *in vitro* studies have revealed previously unidentified properties or even populations of afferent fibres, as was the case in the ferret oesophagus and stomach (Page & Blackshaw, 1998) and in the present study.

3.5.1 Mucosal Afferent Fibres

All of the mucosal afferent fibres in this study responded to fine mechanical stimulation with a 10mg Von Frey hair. This degree of sensitivity indicates that primary afferents from the colon are capable of sensing the movement of stool or liquid over the surface of mucosa. Recruitment of many mucosal afferent fibres would also give accurate information on the consistency of stool (inherent movement of loose stool would provide greater tactile information than a solid static stool) and the progression of pellets within the colon. Mucosal afferent fibres did not respond to circumferential stretch of the tissue - the adequate stimulus for colonic mechanosensitive afferent fibres in all previous studies, in which circumferential stretch was achieved by distension with fluid or a balloon (Blumberg *et al.*, 1983; Haupt *et al.*, 1983; Sengupta & Gebhart, 1994; Su & Gebhart, 1998). Upper gastrointestinal mucosal afferent fibres recorded *in vitro* (Page & Blackshaw, 1998) have a comparable response to Von Frey hairs as the colonic mucosal afferent fibres described in the present study and similarly show no response to circumferential stretch. Colonic mucosal afferent fibres have either no resting discharge or a low rate of resting discharge (<0.5Hz), similar to the findings of Page & Blackshaw (1998) and those of *in vivo* studies of gastroduodenal mucosal afferent fibres (Blackshaw & Grundy, 1990; Blackshaw & Grundy, 1993*a;* Clarke & Davison, 1978; Cottrell, 1984*b*). There is also agreement with regard to the property of mucosal afferent fibres to show no initial spontaneous discharge but to develop spontaneous activity over the course of the study. These dissimilarities with previously described colonic afferent fibres and similarities with upper gastrointestinal mucosal vagal afferent fibres support the classification of the new group of colonic afferent fibres described here as mucosal afferent fibres.

Mucosal afferent fibres have probably been encountered before, but their adequate stimulus not found. In reports of the rat (Sengupta & Gebhart, 1994) and cat (Janig & Koltzenburg, 1991) pelvic nerve and cat lumbar splanchnic nerves (Blumberg *et al.*, 1983), from 33 to 95% of afferent fibres identified by nerve stimulation that were otherwise silent were insensitive to colonic distension or extraluminal mechanical stimuli. In most cases these were believed to end in the colon, and it was speculated that they correspond to either the silent nociceptors described above, or with antidromically activated postganglionic sympathetic motorneurones. I found that approximately a quarter of the afferent fibres in the present study were insensitive to distension and had receptive fields in the mucosa, and therefore suggest that many of the distension-insensitive afferent fibres described previously may also belong to this class.

3.5.2 Serosal Afferent Fibres

The current findings on serosal afferent fibres are comparable with descriptions of lumbar spinal serosal afferent fibres in the literature (Blumberg *et al.*, 1983; Haupt *et al.*, 1983). In these fibres the response to circumferential stretch was either absent or seen only at the onset of the stretch, whereupon they showed no relationship to tension or length of the muscle. This short burst of firing was consistent with the initial movement of the tissue either shifting the mesentery or rubbing the receptive field against the basement mesh of the bath. The serosal fibres described in this study responded to firm probing of the luminal surface and responded at a lower threshold to probing of the reflected serosal surface, although this was not always possible to confirm and depended on the location of the receptive field being at either end of the preparation. This problem is similar to that encountered by Blumberg *et al.* (1983) in locating serosal receptive fields on the posterior aspect of the cat colon *in vivo*.

3.5.3 Muscular Afferent Fibres

Low-threshold lumbar colonic afferent fibres sensitive to small changes in intraluminal pressure are well documented (Blumberg *et al.*, 1983; Haupt *et al.*,

1983). The muscular afferent fibres recorded here showed similar properties in that they consistently responded to circumferential distortion of the colon. However, they do not respond to graded increases in circumferential strain, which is the stimulus that should most closely correlate with increased intraluminal pressure. An interesting precedent may be in guinea pig colon from which the mechanosensory input to prevertebral ganglia may be proportional to volume rather than intraluminal pressure (Anthony & Kreulen, 1990). As this matter is not resolved using the stimulus of ungraded stretch, a more vigorous investigation of the adequate stimulus in circumferential stretch-sensitive fibres is discussed in Chapter 5.

3.5.4 Distribution of Receptive Fields

Receptive fields of all three types of afferent fibre were clustered along the mesenteric border of the colon. This was partly the result of technical features of the preparation, because the colon was opened longitudinally off-centre. This procedure would inevitably sever the peripheral processes of afferent fibres as they projected circumferentially around the colon to the anterior aspect (the far left in Fig 2-1). This does not, however, account for the relatively small numbers of receptive fields found in the centre (corresponding to the antimesenteric side) of the preparation. Thus, it was concluded that there was a natural bias to the innervation of the colon by these types of fibre. This confirms an observation made by Blumberg *et al.* (1983) on muscular and serosal/mesenteric afferent fibres in the cat colon. Only fibres with one receptive field were included in this chapter. 2/3 distension-sensitive fibres in the cat lumbar splanchnic nerves have only one receptive field, the others have two (e.g. Blumberg *et al.*, 1983; Haupt *et al.*, 1983). Other studies of serosal and mesenteric fibres have described multiple receptive fields (e.g. Morrison, 1973). Because our preparation included only the distal colon with little attached mesentery, it is not possible to make direct comparison because connections to other receptive fields on adjacent organs may have been severed.

3.5.5 Origins of Fibres Recorded

The extrinsic afferent fibre innervation of the distal colon arises from the pelvic and splanchnic nerves, with central endings in the spinal cord, and from the vagal nerves with central endings in the dorsal medulla. Recordings were from fibres in the intermesenteric and lumbar splanchnic nerves, which together contain predominantly lumbar spinal afferent fibres (Baron *et al.*, 1988). Sacral afferent fibres follow a separate anatomical pathway in the pelvic nerves (Baron & Janig, 1991; Janig & Koltzenburg, 1991), and vagal fibres are relatively sparse at the level of the colon (Altschuler *et al.*, 1993; Berthoud *et al.*, 1991). The pathway taken by vagal fibres is not known. Therefore, in addition to the most likely event that our recordings are from lumbar spinal afferent fibres, other possible extrinsic origins are apparent. As well as extrinsic fibres, our recordings may have been from axons originating from second order enteric neurones projecting out of the gut (intestinofugal fibres). Intestinofugal fibres are believed to project as far as the IMG (Keef & Kreulen, 1990; Weems & Szurszewski, 1977), but not as far as the recording site used in the current study. Enteric neurones projecting through the IMG to the spinal cord were thought to exist in the rectum but not in the colon (Doerffler Melly & Neuhuber, 1988; Neuhuber *et al.*, 1993). However, more recently there has been evidence that in the rat, a small number (approximately 20 per 5mm length of colon) of intestinofugal fibres pass through the IMG and project into the intermesenteric nerves, particularly those arising from the distal colon (Luckensmeyer & Keast, 1995). Therefore, the possibility that some of the current recordings are from intestinofugal fibres cannot be excluded, but proportionally these would be a small minority.

3.5.6 Basic Properties of Colonic Afferent Fibres

Spontaneous activity was present in a subgroup of each category of afferent fibres in this study. Where spontaneous activity was present, it was of low frequency. Analysis of group data on the three categories we encountered showed that mucosal afferent fibres had significantly lower rates of resting discharge. This is similar to differences previously reported between mucosal and other classes of fibres in the vagal innervation (Blackshaw & Grundy, 1990; Blackshaw & Grundy,

1993*a;* Cottrell & Iggo, 1984*a;* Cottrell, 1984*b;* Page & Blackshaw, 1998). Muscular and serosal afferent fibres had similar resting discharge rates to those previously reported in the cat lumbar splanchnic innervation (Blumberg *et al.*, 1983). Spontaneous activity in some mucosal and serosal afferent fibres increased over the course of the study, and this was generally associated with application of chemical stimuli. A previous study of spontaneous activity that developed in this way (Blackshaw & Grundy, 1993*a*) showed that it could be abolished by administration of a 5-HT_3 receptor antagonist, indicating a role for endogenous release of 5-hydroxytryptamine in influencing mucosal afferent excitability. The mechanism for what increases spontaneous activity of these fibres *in vitro* was not a subject for investigation in this project.

For methodological reasons the conduction velocity of afferent fibres in this study was not determined. Although this data would have provided an additional classification criterion for future studies, it is unlikely to be of functional significance to the type of sensory information encoded. Previous studies of lumbar spinal afferent fibres in the cat have shown no correlation between conduction velocity, resting discharge and distension threshold (Blumberg *et al.*, 1983). Prior studies on lumbar colonic afferent fibres in the rat are lacking, but a detailed investigation of rat sacral colonic afferent fibres similarly showed no correlation between conduction velocity and functional properties (Sengupta & Gebhart, 1994). These authors did find an increased proportion of Aδ fibres in the afferent innervation of the anal mucosa compared to the colon, but these presumably belonged to the somatic sensory supply. Otherwise, 81% of colonic afferent fibres in Sengupta and Gebhart's study were unmyelinated.

The development of an *in vitro* technique for the study of colonic afferent fibres led me to the first demonstration of mucosal endings in the colon. The mucosal afferent fibres described in the present study closely resemble vagal afferent fibres in the upper gastrointestinal tract. The existence of mucosal afferent fibres in the colon suggests a complexity in sensation from the colon that is as yet unexplored. The precise physiological roles of these mucosal fibres in visceral sensation and reflexes can only be fully elucidated with further investigation such as recordings of

convergence on dorsal horn neurones *in vivo*. My descriptions of muscular and serosal fibres also concur with *in vivo* data. Thus, this novel *in vitro* method is suitable for broader investigations of colonic afferent fibre sensitivity.

Chapter 4. Chemosensitivity of Mucosal, Muscular and Serosal Colonic Afferent fibres

4.1 Abstract

1. Chemosensitivity was investigated in 24 serosal, 6 muscular and 20 mucosal fibres. 19/24 serosal, 6/6 muscular and 15/20 mucosal fibres were sensitive to the chemical stimuli applied in this study.
2. Most fibres responded to hypertonic saline. 17 fibres were tested with different concentrations of saline. Afferent fibres were not sensitive to H_20 or isotonic saline but were to hypertonic saline. 10 fibres were tested with different osmotic stimuli. At identical osmolarities, hypertonic saline was a more effective stimulus than D-mannitol.
3. 19 afferent fibres were tested with HCl 50mM. 3/10 serosal, 1/2 muscular and 3/7 mucosal fibres responded.
4. 19 afferent fibres were tested with ferret bile. 6/11 serosal, 0/1 muscular and 4/7 mucosal fibres responded.
5. 9 afferent fibres were tested with bradykinin 1µM. 0/4 serosal, 1/2 muscular and 1/3 mucosal fibres responded.
6. 36 afferent fibres were tested with capsaicin 100µM. 7/17 serosal, 2/5 muscular and 2/14 mucosal fibres responded.
7. 12 afferent fibres were tested with 5-HT 10-100µM. 4/7 serosal, 0/1 muscular and 0/4 mucosal fibres responded.
8. Chemosensitive afferent fibres were generally responsive to >1 chemical stimulus.
9. This is the first documentation of chemosensitivity in mucosal colonic afferent fibres and sensitivity of colonic serosal afferent fibres to luminally applied stimuli.

4.2 Introduction

Vagal and spinal afferent fibres have consistently been shown to be chemosensitive. Some groups maintain that afferent fibres are chemospecific, for example glucoreceptors (e.g. Mei, 1978) and others that afferent fibres are more commonly polymodal (e.g. Sengupta & Gebhart, 1994; Su & Gebhart, 1998).

Afferent fibres potentially respond to both humoral mediators and to luminal nutrients. Luminal application of chemical agents has been regularly investigated in upper gastrointestinal vagal fibres, although in spinal afferent fibres innervating the colon and other viscera it is more common to apply chemical stimuli as serosal pledgets, or by perfusion means such as intravenous or intraarterially (Haupt *et al.*, 1983; Longhurst *et al.*, 1984; Sengupta & Gebhart, 1994). The one exception is a study of the rat pelvic nerves in which chemical mediators were applied using both methods (Su & Gebhart, 1998). In the study described here, the sensitivity of primarily mucosal and serosal afferent fibres to luminally applied stimuli was investigated. A range of chemical stimuli was chosen to include mediators that would be expected in the lumen both in physiological and unphysiological situations and local humoral stimuli. For example hypertonic stimuli were included as colonic contents in semi-solid states are highly concentrated (Read, 1988); during diarrhea, bile (which is usually reabsorbed before passing the ileo-caecal junction) reaches the colon (Edwards *et al.*, 1989) and 5-HT acts on 5-HT_3 receptors to excite afferents innervating the upper gastrointestinal tract (Blackshaw & Grundy, 1993*a;* Grundy *et al.*, 1994). By choosing a range of different stimuli likely to mediate different aspects of sensation, a broader understanding of colonic afferent chemosensitivity was gained.

4.3 Methods

A full description of the methods used in this chapter can be found in Chapter 2.

4.4 Results

Many of the fibres (but not all) described in the last chapter were included in this study of chemosensitivity. Other fibres fitting the classification criteria used in the last chapter, but not described there, were specifically investigated for their chemosensitivity.

4.4.1 Hypertonicity

17 fibres (4 mucosal, 1 muscular and 12 serosal) were tested with distilled H_20, 154mM NaCl (isotonic saline) and 308mM NaCl (hypertonic saline). Of the 4

mucosal fibres, one responded to none of the stimuli and the other three responded to NaCl 308mM but not to distilled H_20 or isotonic saline. The muscular fibre responded to 154mM and 308mM but not to distilled water. These responses were unrelated to muscular activity as concurrent circumferential tension recordings revealed no muscular response to either of these stimuli. Of 12 serosal fibres, 3 responded to normal saline and 11 fibres responded to hypertonic saline. 4 serosal fibres responded to distilled water, but these individual fibres subsequently did not respond to normal saline (all four responded to hypertonic saline). These results are summarised in Table 4-1.

Ten fibres (7 serosal, 1 muscular and 2 mucosal) were tested with 308mM D-mannitol (isotonic), 616mM D-mannitol (hypertonic, equal to 308mM NaCl) and 308mM NaCl (hypertonic). All fibres responded to NaCl 308mM. Two of 7 serosal fibres responded to 616mM D-mannitol as did the muscular fibre. Mucosal fibres did not respond to 616mM D-mannitol. None of the fibres tested responded to 308mM D-mannitol. There were not significant differences between the responses to 616mM D-mannitol and 308mM NaCl in the mucosal fibres or the serosal fibres ($p=0.051$). It was not possible to apply statistical measures to the responses of the two mucosal fibres, however the responses to 308mM NaCl appear to be greater than the responses to 616mM D-mannitol. With all fibres combined, there was a difference between the responses to 616mM D-mannitol and 308mM NaCl ($p<0.05$) (Fig 4-1). The dose response curve of one serosal fibre to a range of NaCl and D-mannitol can be seen in Fig 4-2.

4.4.2 HCl

19 fibres were tested with HCl 50mM (10 serosal, 2 muscular and 7 mucosal). Of the mucosal fibres, 3 out of 7 fibres responded to 50mM HCl. The latencies for these responses varied considerably (4, 20 and 60 s). Three out of 10 serosal fibres responded to 50mM HCl, with latencies of 3, 3 and 60 s. There were no unifying characteristics in the shape or size of responses, either between serosal or mucosal fibres (Fig 4-3). There was no muscular activity during the application of HCl associated with afferent responses. One muscular fibre responded to HCl

Unit	Distilled H20	Normal Saline 154mM NaCl	Hypertonic Saline 308mM NaCl
Mucosal			
76-1	0	0	0
69-1	+	0	+
58-1	0	0	+
58-2	0	0	+
Muscular			
71-3	0	+	+
Serosal			
84-1	+	0	+
83-1	0	+	+
74-1	0	0	+
71-2	0	0	+
71-4	0	0	+
69-2	0	+	+
68-1	+	0	+
64-1	+	0	+
64-2	+	0	+
61-1	0	0	+
69-3	0	0	+
61-2	0	0	+

Table 4-1. Summary of responses to hypertonicity of individual afferent fibres. A response to a stimulus or presence of spontaneous activity is indicated with **+**, no response or activity with **0**. A blank is left when a particular stimulus was not tested in a study. Afferents are grouped in classifications of serosal, muscular, mucosal afferents (see methods for classification criteria).

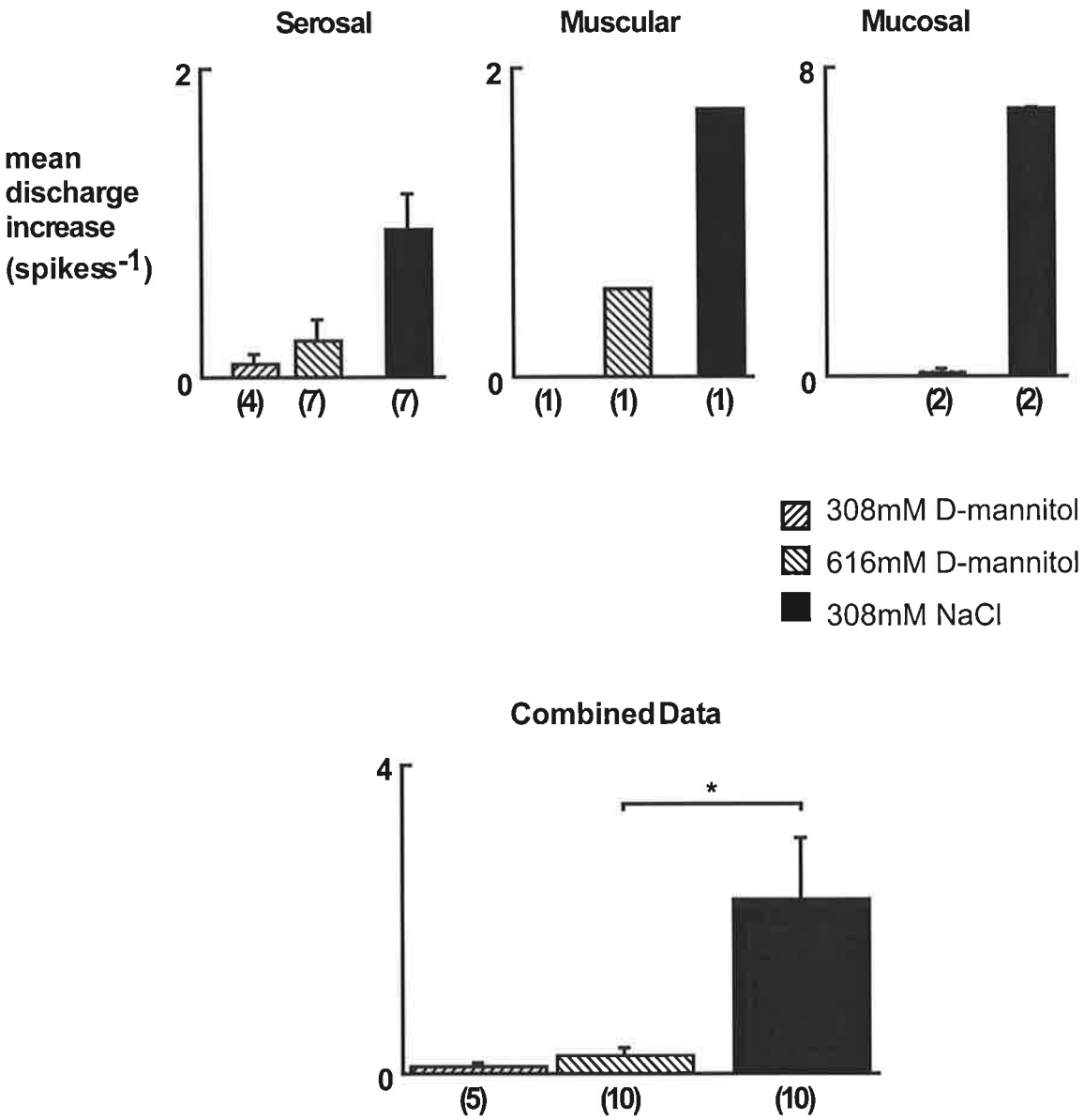

Figure 4-1. Sensitivity of afferent fibres to hypertonicity. Graphs show mean response (increase from basal discharge) of afferents to D-mannitol and NaCl 308mM. All fibres tested are included, including non-responders. Osmolarity of 616 mM D-mannitol and NaCl 308mM are identical. * signifies $p<0.05$. The response of mucosal fibres to NaCl 308mM was significantly greater than the response to 616mM D-mannitol. Serosal fibres and the muscular fibre showed responses with similar trends to that of mucosal fibres and there is a significant difference between the responses to NaCl 308mM and 616mM D-mannitol when all groups are combined. Numbers of experiments are shown in brackets.

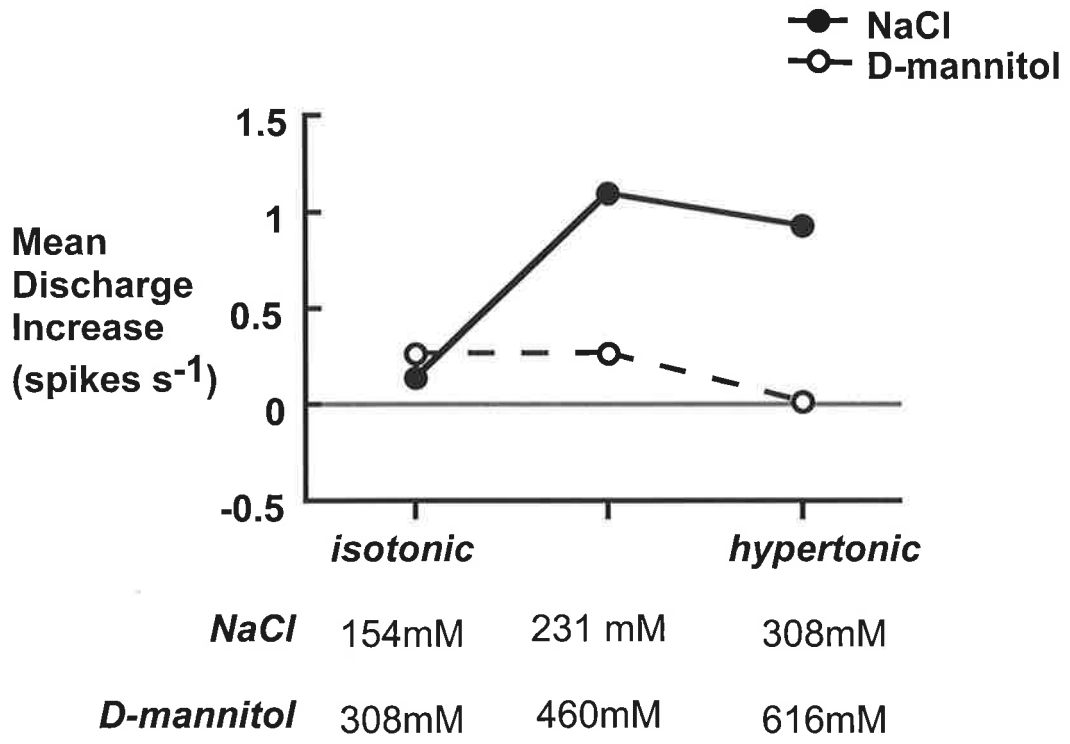

Figure 4-2. Dose response curve of one serosal fibre to equal osmolarities of NaCl and D-mannitol. Mean increase in rate of discharge from baseline is shown. Neither D-mannitol or NaCl caused a response at isotonic levels. At increasing hypertonicities this fibre responds to NaCl but not to D-mannitol.

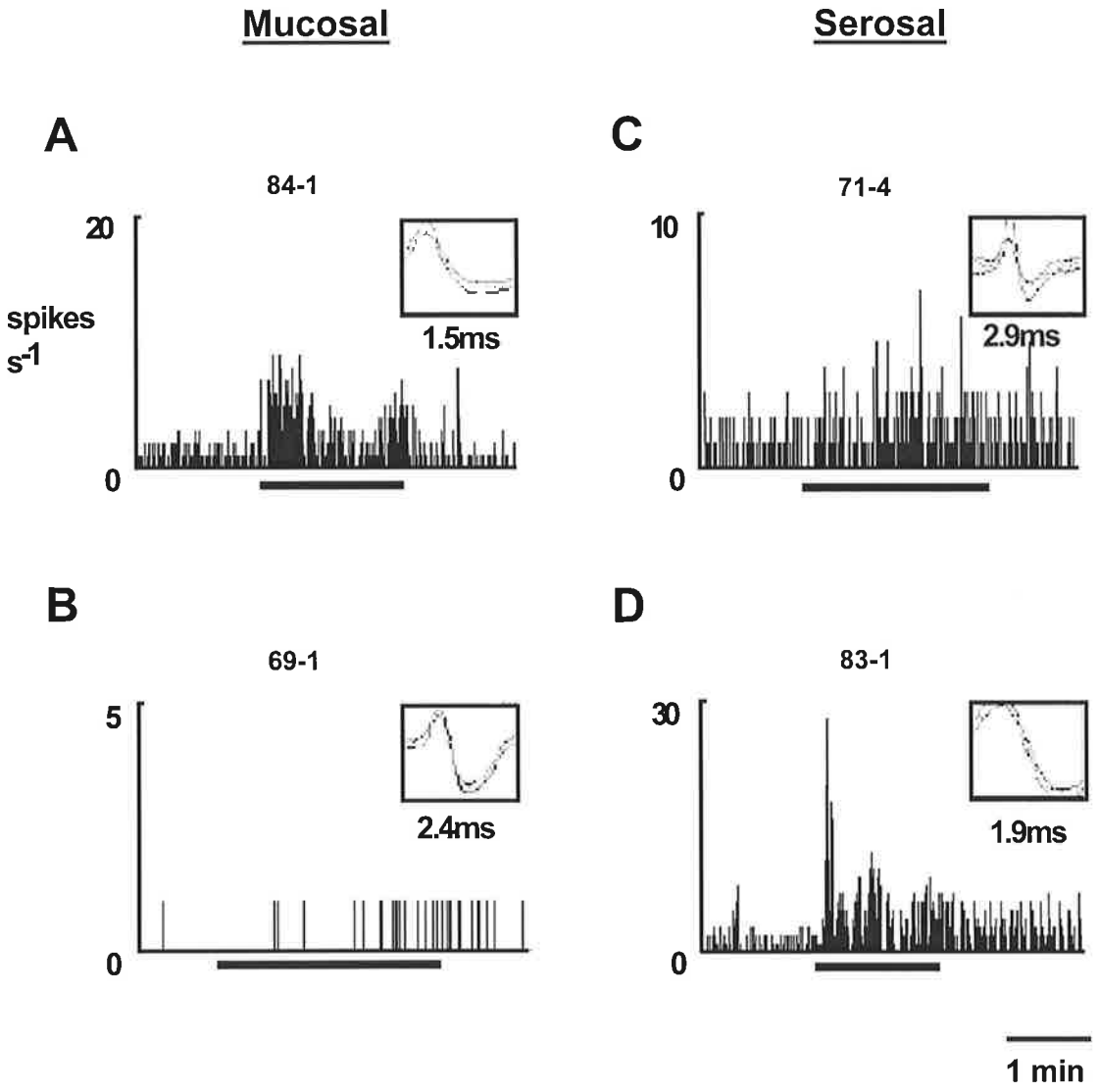

Figure 4-3. An example of two mucosal and 2 serosal afferent fibre responses to HCl 50mM. **A** and **B** are mucosal fibres. **C** and **D** are serosal fibres. Each response has the wave template displayed, the fibre identification number and the response of the fibre to HCl 50mM. **A** and **D** have short latencies with the peak of response early during chemical application. **C** also has a short latency but peak discharge occurs later during chemical application. **B** responded with only a brief, low discharge response. This response was significant because this fibre was otherwise silent.

50mM. This response was unrelated to muscular activity (Fig 4-4). Almost all fibres that responded to HCl were also responsive to other chemical stimuli. The exception was a muscular fibre that responded to HCl in isolation to the other chemicals applied.

4.4.3 Bile

Nineteen afferent fibres were tested with bile (11 serosal, 1 muscular and 7 mucosal) Four serosal afferent fibres responded to 50% ferret bile, two responded to undiluted bile, and 5 responded to neither. In all cases after the initial response to bile at either concentration, no further response to bile could be elicited even when the concentration was increased, indicating desensitization. The muscular fibre did not respond to bile. Four of 7 mucosal afferent fibres tested responded to ferret bile: 2 responded to 50% diluted bile (osmolarity not exceeding 300mOsm), and 2 responded to undiluted bile. These responses were not reproducible indicating desensitization. Bile generally induced muscular activity in the tissue (Fig 4-5).

4.4.4 Bradykinin

Nine afferent fibres (4 serosal, 3 mucosal and 2 muscular) were tested with bradykinin 1μM on the mucosal surface. One other (serosal) was tested with 189μM on the mucosal surface. A total of two afferent fibres responded to mucosally applied bradykinin, 1 mucosal afferent fibre (reproducible response) and 1 muscular fibre that responded to bradykinin in the absence of any muscular activity (Fig 4-6). To investigate the possibility that bradykinin was not penetrating the mucosal surface some fibres were tested with mucosally applied and serosally applied bradykinin. Two non-responding fibres (one serosal and one mucosal) were subsequently tested with bradykinin on the serosal surface, by perfusing the entire serosal surface with 1μM bradykinin in the Krebs solution. The mucosal fibre responded to serosally applied bradykinin (Fig 4-7), but the serosal fibre did not. One muscular fibre responded to bradykinin 1μM after the receptive field had been treated with a ten-minute application of PGE_2 100μM, but not before (Fig 4-8). The responses to bradykinin are summarised in Table 4-2.

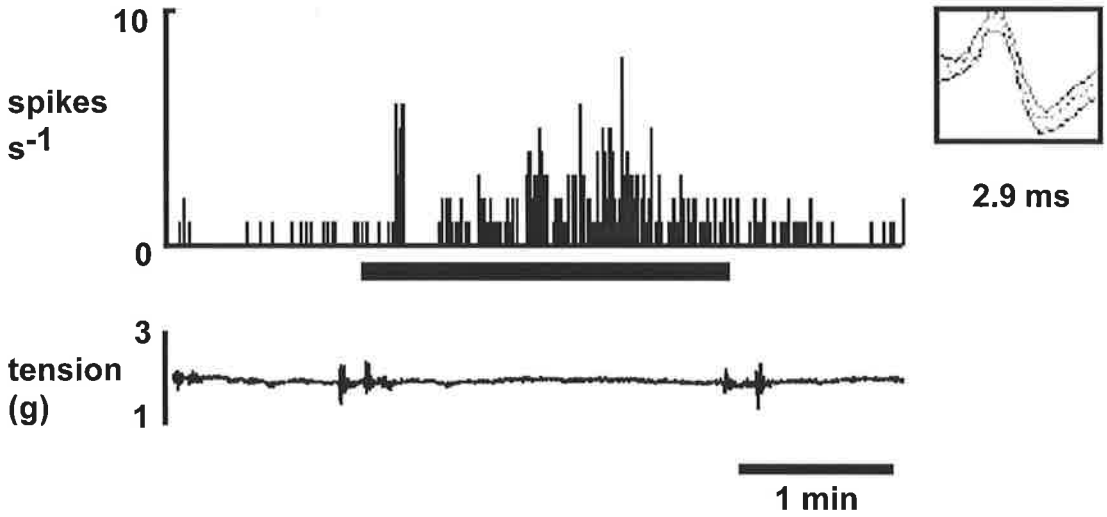

Figure 4-4. Response of a muscular fibre to HCl 50mM. The wave template is shown in the top right corner. The rate of discharge is shown above and the circumferential tension of the tissue shown below. This tissue had a 1.5g preload. The response to HCl was unrelated to activity in the circular muscle.

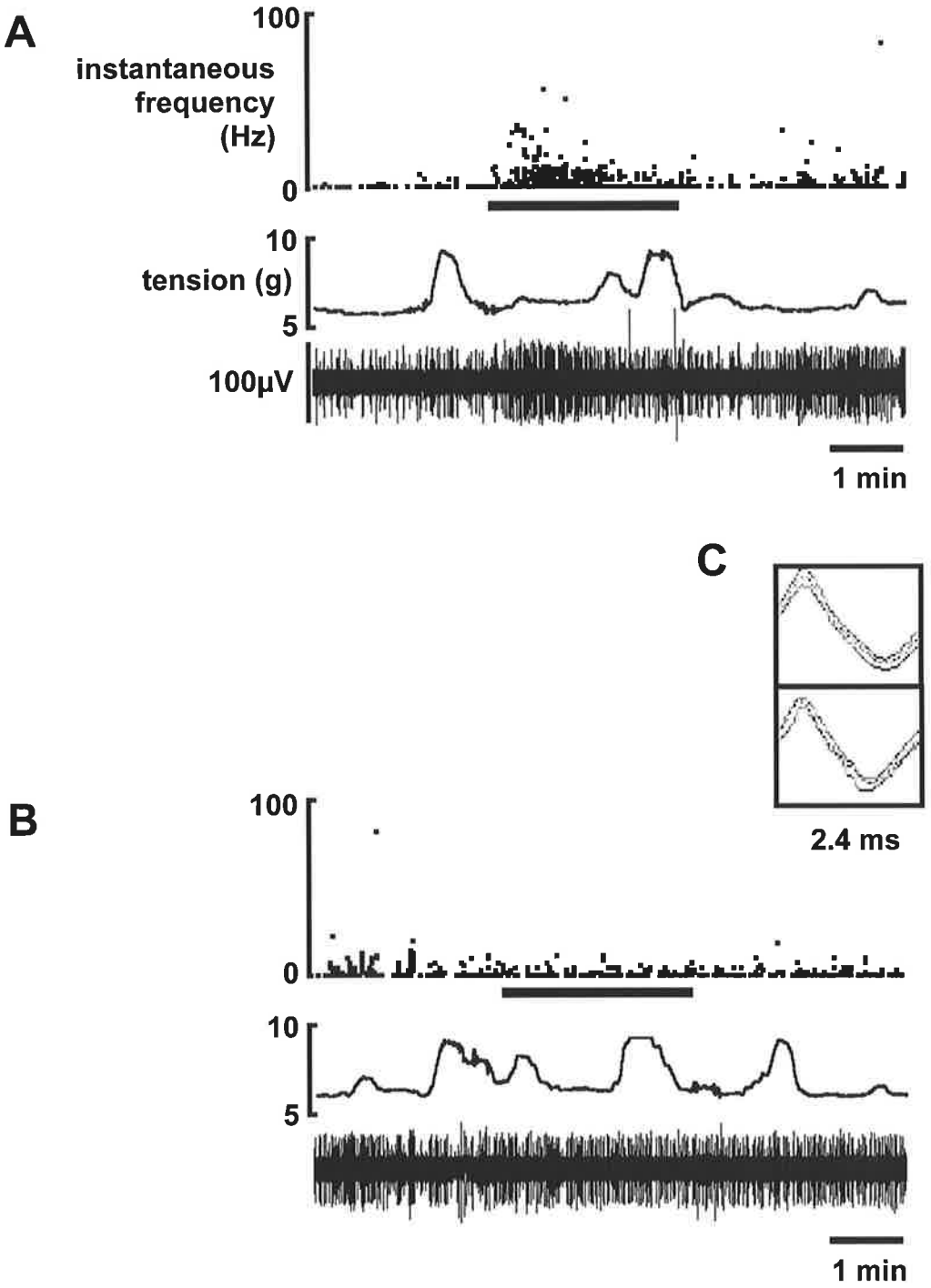

Figure 4-5. A mucosal fibre response to 50% bile and full strength bile. A. The mucosal fibre responds to 50% bile independently of muscular activity. The tissue was given a 1.5g preload. The raw tracing is shown directly below the tension recording. **B.** The fibre was subsequently tested with full strength bile and did not respond. Muscular activity still occurred in the tissue. **C.** Two mucosal fibres with similar wavetemplates , but adjacent receptive fields were recorded in this study. The responses shown here are from the fibre with the top template. The ring for drug administration surrounded receptive fields of both fibres, but only the fibre in the top template responded to bile.

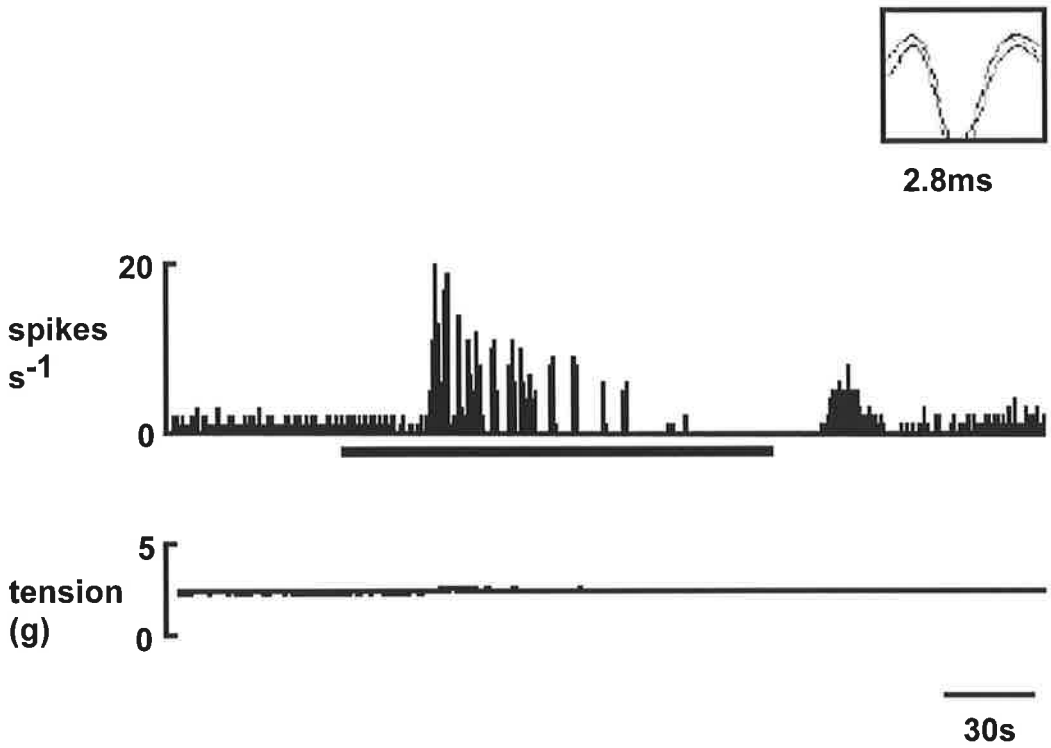

Figure 4-6. Response of a muscular fibre to 1μM bradykinin. Wave template of the fibre is in the right hand corner. Discharge rate of the fibre is shown immediately above the tension recording. The tissue was given a 1.5g preload. The fibre responded to bradykinin independently of the circular tension in the tissue surrounding the receptive field. The response had a latency of 30s and was adapting. There was a burst of activity during the washout period.

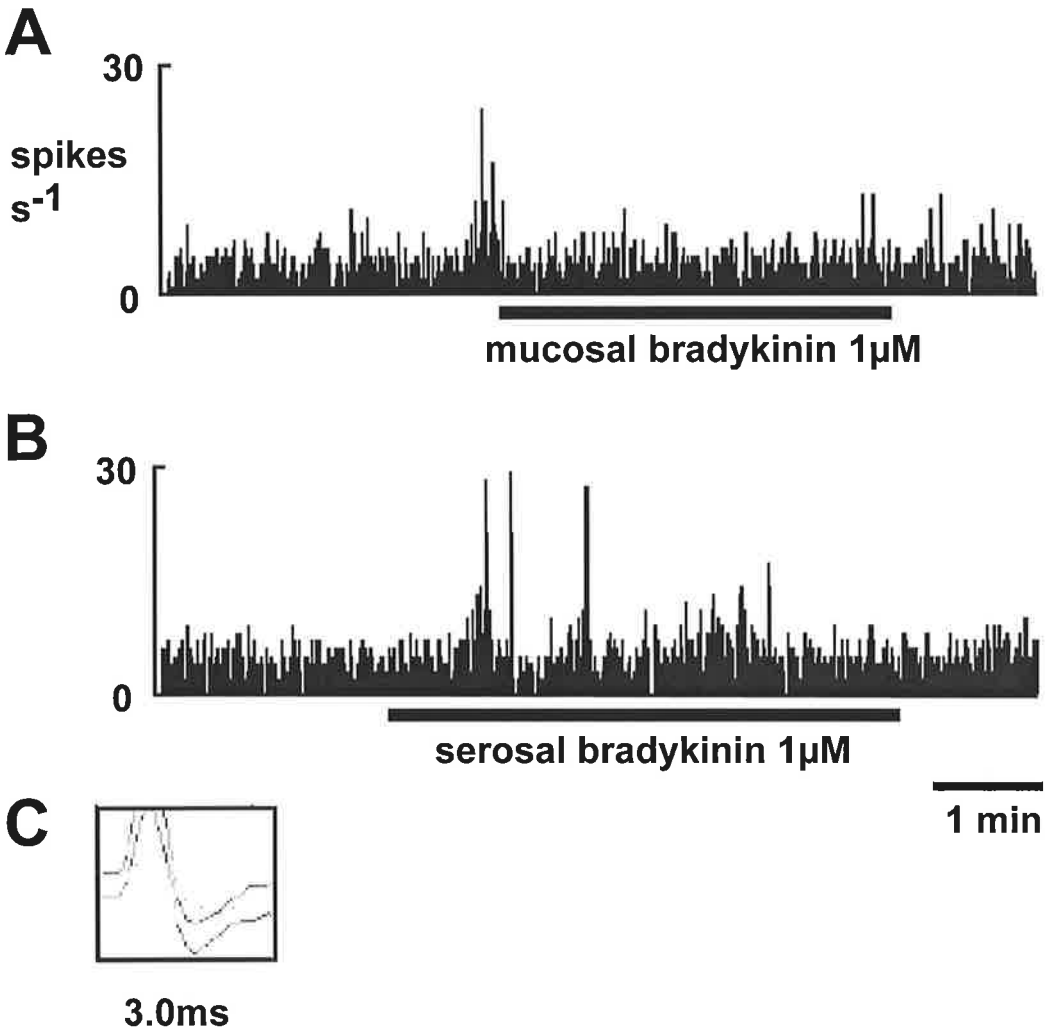

Figure 4-7. Mucosal fibre response to 1µM bradykinin on mucosal and serosal sufaces. A. This fibre does not respond to mucosally applied bradykinin. A burst of firing is observed before application of the stimulus consistent with touching the receptive field whilst placing the ring. **B.** This fibre responds to bradykinin superfused across the serosal surface of the tissue. The latency of response cannot be quantified as the concentration of bradykinin in contact with the tissue increases over 10-30 seconds as normal Krebs in the basement chamber is displaced by the bradykinin supplemented Krebs. **C.** The wave template of the fibre.

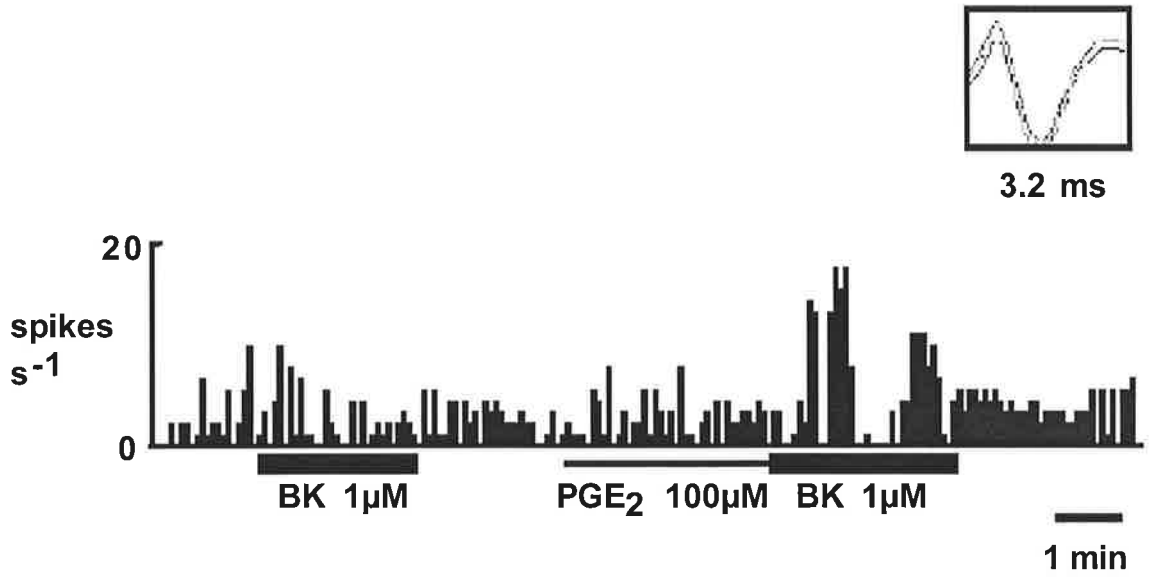

Figure 4-8. Response of a muscular fibre to bradykinin 1µM before and after application of PGE_2 100µM. The wave template for this fibre is shown in the right hand corner of the figure. This fibre did not respond to the first mucosal application of bradykinin. Neither did it respond to an application of PGE_2 100µM. The fibre responded to a subsequent application of bradykinin that immediately followed the application of PGE_2. Indomethacin was present in the bath.

Fibre	Bradykinin concentration	Response	Second mucosal application	Serosal application	Treatment-mucosal 100μM PGE_2
serosal					
200-1	1μM	0	0		
166-1	1μM	0		0	
155-1	1μM	0			
155-2	1μM	0			
122-1	189μM	0			
muscular					
181-1	1μM	0			+
148-1	1μM	+			
Mucosal					
183-1	1μM	0	0		
180-1	1μM	+	+		
168-1	1μM	0		+	

Table 4-2. Summary of responses of individual afferent fibres to bradykinin. A response to a stimulus or presence of spontaneous activity is indicated with **+**, no response or activity with **0**. A blank is left when a particular stimulus was not tested in a study. Afferents are grouped in classifications of serosal, muscular and mucosal.

4.4.5 Capsaicin

Capsaicin 100μM was applied mucosally to 36 fibres (17 serosal, 5 muscular and 14 mucosal). Seven of 17 serosal fibres (41%) responded with a mean latency of 35±11 s and increase in discharge of 2.07±0.50 spikes s^{-1}. Two of 5 muscular fibres (40%) responded with a mean latency of 3±0 s and increase in discharge of 2.2±0.76 spikes s^{-1}. Two of 14 mucosal fibres (14%) responded with a mean latency of 28.5±4.5 s and increase in discharge of 2.93±0.66 spikes s^{-1}. There was no difference in the increase in discharge between fibre type. The latency of muscular response appeared to be lower than that of the mucosal fibres but it was not possible to perform statistical analysis on small numbers. There was no statistical difference of latency between the mucosal and serosal fibres (see Fig 4-9).

4.4.6 5-hydroxytryptamine

5-HT was applied mucosally to 12 fibres (7 serosal, 1 muscular, 4 mucosal). Four serosal fibres responded to 5-HT, three to 5-HT 100μM and 1 to 5-HT 10μM. Neither mucosal nor muscular fibres responded to 5-HT 100μM. Two responsive serosal fibres were subsequently tested with 5-HT 100μM applied to the serosal surface by the process of superfusing the entire serosal surface through the basement chamber. Neither fibre responded.

4.4.7 Other Chemical Stimuli

PGE_2 was applied to 13 fibres (6 mucosal, 7 serosal). One mucosal fibre responded. A full discussion of the role of prostaglandins in chemosensitivity is found in Chapter 7. Noradrenaline (1μM), deoxycholic acid (3mM) and PYY (1μM) were also applied to some fibres (see Table 4-1). Colonic afferent fibres were generally not sensitive to these mediators. Responses were as follows; 1/3 serosal, 0/3 mucosal fibres to noradrenaline (1μM); 0/4 serosal, 1/3 serosal fibres to deoxycholic acid (3mM) and 0/1 mucosal fibres to PYY (1μM).

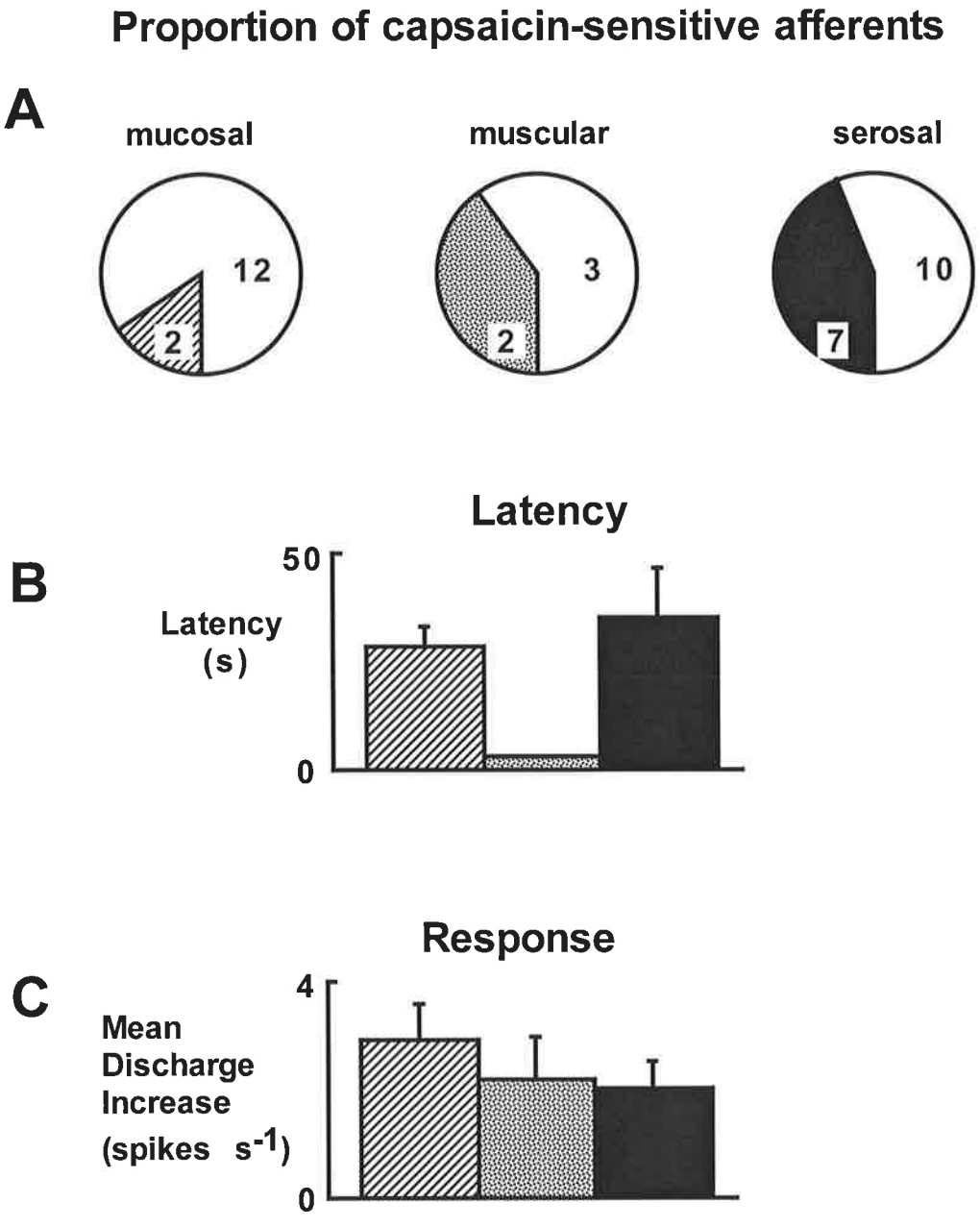

Figure 4-9. Afferent fibres sensitive to capsaicin 100μM. A. Proportions of afferents for each class of fibre sensitive to capsaicin 100μM. **B.** The mean latency of responding afferents. **C.** The mean increase in the rate of discharge from baseline for responding afferents.

Unit	Spontaneous Activity	NaCl 308mM	D-Mannitol 616mM	Bile	HCl 50mM	5-HT 100µM	Bradykinin 1µM	Capsaicin 100µM	Other drugs administered
Serosal									
130-1	+	0	0			0		0	308mM D-mann (0)
130-3	+	0	0			0		0	308mM D-mann (0)
123-1	+	+							
138-1	+	+	+			+		+	NA 1µM (+), DCA 3mM (0)
128-1	+	+	+			0		+	308mM D-mann(0), NA 1µM (0), DCA 3mM (0)
125-1	+	+						+	
114-1	+	+		+					
71-4	+	+			+			0	
74-1	0	+			0			+	
69-2	+	0		0				0	
83-1	+	+		+	+				
87-1	+	+		0				0	
48-1	+	+		0	0				CCK (0), 5-HT 10µM (+)
55-2	+	0		+	+			+	
61-1	+	0		0	0			0	
61-2	0	+		0	0			+	
64-1	+	+		+	0			0	
64-2	+	+		+	0			0	
68-1	0	0		+	0			0	
155-1	+	+	0				0		DCA 3mM (0)
155-2	+	+	0				0		DCA 3mM (0)
200-1						+	0		CCK 1µM (0), Na-butyrate 10mM (0)
166-1	+					+	0		NA 1µM (0)
122	+						0	0	
Muscular									
71-3	+	0			+			0	
57-1	0	0		0	0			0	
116-1	0								
130-2	+	+	+			0		0	308mM D-Mann (0)
181-1	+						0	+	
148-1	+						+	+	
Mucosal									
121-2	0	0				0			
137-1	0	+	0						NA 1µM (0), DCA 3mM (+)
106-2	+	+							
106-3	+	+							
47-2	+	+			+				
69-1	+	+		0	+			0	
55-1	0	+		+	0			0	
58-1	0	+		0	0			0	
58-2	0	+		+	0			0	
76-1	0	0		0	0			0	
84-1	+	+		+	+			+	
144-1	0	0	0			0		0	NA 1µM (0), DCA 3mM (0)
168-1	0	0				+	0		NA 1µM (0), PYY 1µM (0), DCA (0)
167-1	+								
151-1	0					0		0	
114-2	+	+		+					
125-6	+	+	-(?)					+	308 and 421mM D-mann(0)
180-1	0						+	0	
183-1	0	0					0	0	
121-1	0	+			+	0		+	

Table 4-3. Summary of responses of individual afferent fibres to chemical stimuli. A response to a stimulus or presence of spontaneous activity is indicated with **+**, no response or activity with **0**. A blank is left when a particular stimulus was not tested in a study. Afferents are grouped in classifications of serosal, muscular and mucosal. Spontaneous activity is shown as that present at the beginning of studies. Responses to both 50% and undiluted bile are combined.

4.4.8 Combinations of Responses

Both serosal and mucosal afferent fibres were chemosensitive to >1 mediator if they were chemosensitive at all (Table 4-3). The responses in one fibre were not consistent across the chemical mediators applied. There was no apparent pattern of activation for mucosal or serosal fibres. An example of the responses of a mucosal fibre (Fig 4-10) and serosal fibre (Fig 4-11) to a range of chemical stimuli is shown.

4.5 Discussion

This study provides the first evidence that colonic mucosal and serosal afferent fibres are directly chemosensitive to mucosally applied stimuli. Chemosensitivity has been shown in colonic afferent fibres previously (Haupt *et al.*, 1983; Sengupta & Gebhart, 1994; Su & Gebhart, 1998). Mucosal afferent fibres have not been described fully in the colon before, so this is the first formal documentation of their chemosensitivity as well as their existence. Chemosensitivity in serosal afferent fibres to luminal stimuli has not been documented previously, but rather to serosal or humoral stimuli.

With the exception of one fibre, all colonic mucosal afferent fibres showed some chemosensitivity when tested, usually to a range of different stimuli. The prevalence of chemosensitivity amongst colonic mucosal afferent fibres contrasts with that of vagal mucosal afferent fibres recorded in the ferret oesophagus *in vitro* (Page & Blackshaw, 1998) where only a small proportion of the mucosal fibres responded to luminally applied stimuli. Differences between the choice of stimuli for the present rat study and the previous ferret study may account for the differences observed, in addition to species and site along the gut. There are very few *in vitro* data from other studies with which to compare the chemosensitivity of mucosal afferent fibres. No pattern emerges from the combinations of chemical stimuli that evoked responses in mucosal afferent fibres in this study, and therefore there are no apparent distinct subpopulations of mucosal afferent fibres. It may be concluded at this stage that chemosensitivity in colonic mucosal afferent fibres is heterogeneous. The range of chemical stimuli used to investigate mucosal afferent

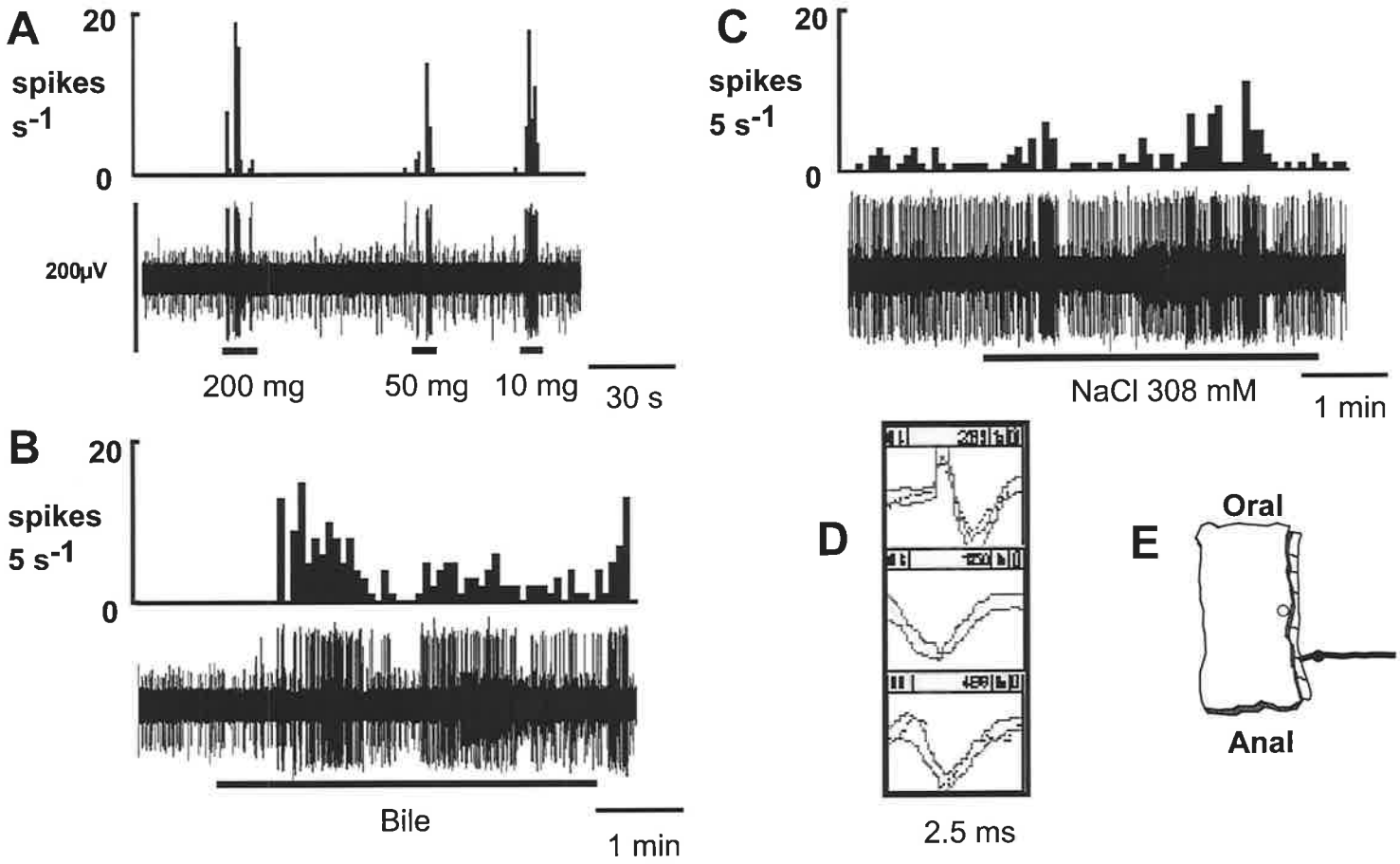

Fig ure 4-10. Response of a mucosal afferent to calibrated von Frey hairs and two chemical stimuli: 308mM NaCl and undiluted ferret bile. **A-C:** The integrated discharge in spikes/s of the fibre of interest is shown directly above the raw record of activity in the nerve strand. Three fibres were active on this strand, the discriminator templates for which are shown in **D**. The fibre of interest (top template) has the largest amplitude. One other fibre (middle template) was subsequently investigated and included in the serosal fibre data. The last fibre (bottom template) was not investigated. **A.** A brief, rapidly adapting response occurred with each application of the von Frey probe. The fibre had no spontaneous activity at this stage. **B.** The fibre responded to the application of bile with a latency of 40 s. The response was slowly adapting and was maintained after reintroduction of the normal superfusate. **C.** Hypertonic NaCl (308mM) was applied to the tissue 32 min after the application of bile. Basal resting discharge had increased after the application of bile upon which the response to hypertonic NaCl was superimposed. Hypertonic NaCl evoked a slowly adapting response with a latency of 10 s. This fibre was also investigated with 50mM HCl and 100µM capsaicin, but did not respond to these stimuli (data not shown). An illustration of the precise location of the receptive field of this fibre is shown in **E**.

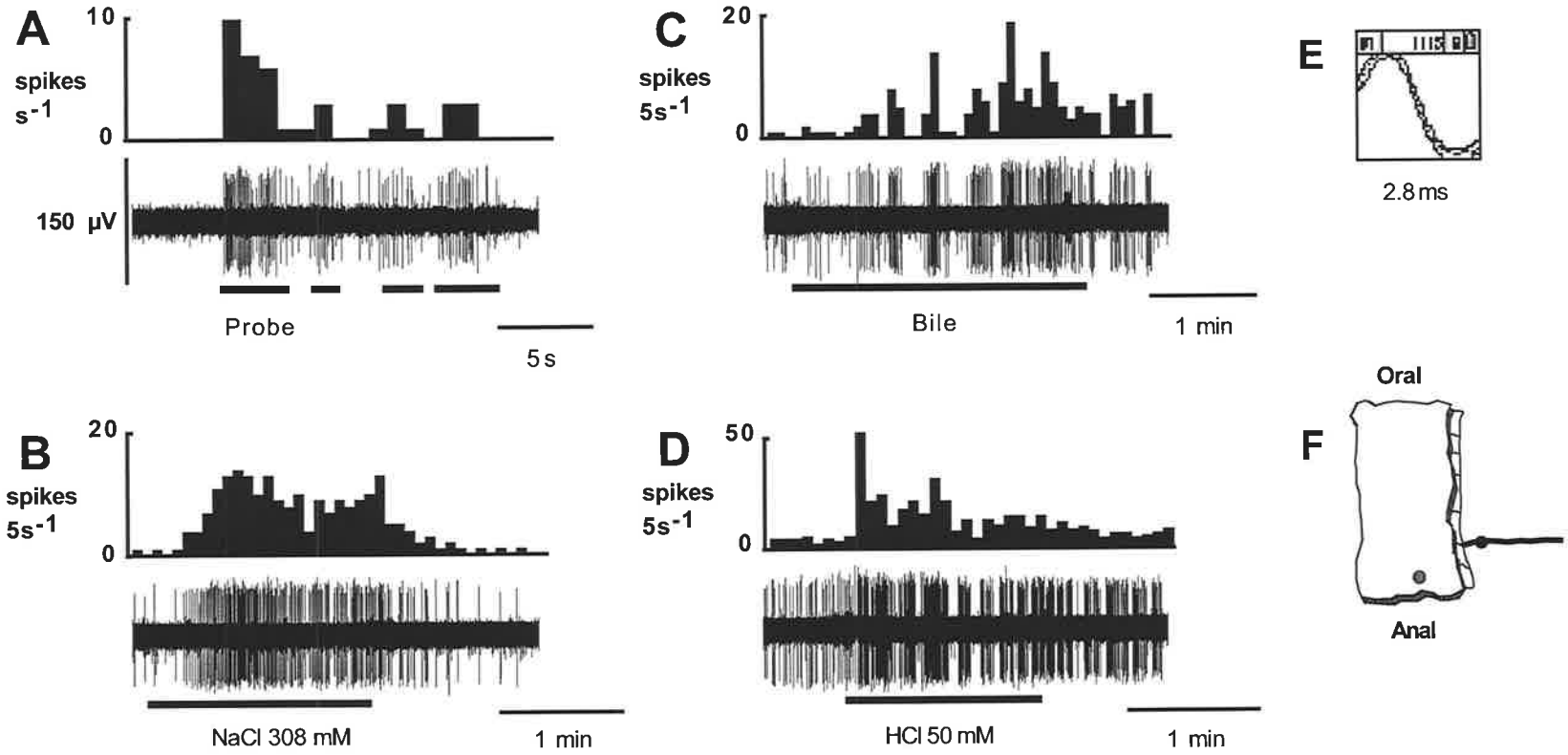

Fig 4-11. Response of a serosal afferent to one mechanical and three chemical stimuli. **A-D:** The integrated record of the fibre's activity is directly above the raw record. Only the unit of interest was active on this strand. **A.** The unit responded to probing with a burst of firing that resolved when the stimulus was removed. Subsequent responses to probing were of lower intensity than the initial response. Moving the probe in and out of the bathing solution regularly produced artefact. This can be observed where the raw record appears not to correspond with the integrated record. **B.** The fibre had a slowly adapting response to hypertonic NaCl (308mM) with a latency of 22 s. The response was not sustained after washout with Krebs' solution and the firing rate quickly returned to resting activity. **C.** The onset of the response to bile had a long latency of 52 s whereupon the firing pattern changed to short intense bursts of activity that had not been observed until this time. Washing the tissue with Krebs' caused a prompt decrease in the intensity of the firing, but the bursting pattern was maintained for a further 60 seconds. **D.** The short latency (4 seconds) response to 50mM HCl began with an intense burst of activity that was not sustained for the duration of the stimulus. Rather, a series of rapid, discrete bursts of firing was initiated that was sustained for the remaining 8 minutes of the recording period (not shown). **E:** The template for this unit. **F:** An illustration of the location of the receptive field.

fibres was chosen in order to deliver stimuli which may be encountered in the colonic lumen during physiological or mildly pathophysiological circumstances: bile is normally reabsorbed in the small intestine, but in diarrhoea it reaches most of the colon (Edwards *et al.*, 1989); acidic conditions are generated by bacterial fermentation, and hyperosmotic conditions by fluid absorption; capsaicin was used in order to determine if subpopulations of afferent fibres could be coded according to their capsaicin sensitivity, which was not evident from the data gathered. A wide range of chemical stimuli were applied to each fibre at the cost of performing dose response curves for each one. This results in a broader but less definitive understanding of the chemosensitivity of afferent fibres in the colon, but provides clear direction for appropriate and useful studies in the future.

Many serosal afferent fibres showed some chemosensitivity to stimuli applied to the luminal surface at the location of the receptive field. These responses were initiated across the wall of the tissue rather than by leakage around the sides of the preparation. This was prevented by the effective seal between compartments (confirmed using dye). It is possible that responses of serosal afferent fibres may be due to secondary effects on the tissue beyond our control, such as release of inflammatory mediators. This is on the whole unlikely as emphasis was biased towards using stimuli that could occur naturally in the lumen in the intact animal under both physiological and pathophysiological conditions. Chemosensitivity of serosal afferent fibres has been established previously *in vivo* to bradykinin, capsaicin, ischaemia and hypertonic saline administered extraluminally (Haupt *et al.*, 1983; Longhurst *et al.*, 1984), indicating a role for these afferent fibres in transmission of signals related to noxious and inflammatory events. The observation that intraluminal stimuli such as bile and low concentrations of HCl and NaCl stimulated serosal afferent fibres suggests that there may be a hitherto unexplored role for serosal afferent fibres in the sensing of the chemical composition of the luminal contents. As spinal fibres are generally thought to have an important role in nociception, this evidence that colonic afferent fibres are able to sense what are essentially non-painful luminal stimuli challenges this premise.

4.5.1 Hypertonic Saline

Hyperosmolar saline was the most consistent adequatestimulusfor both the serosal and mucosal fibres. Osmolarity as a luminal stimulus has been used before, primarily in mucosal fibres of the gastro-duodenal region. The most commonly seen response is to the extremes of osmolarity, outside the regions of normal luminal osmolarity (Clarke & Davison, 1978; Garnier & Mei, 1982) - this means to water and to hyperosmolar stimuli. The colonic afferent fibres in this study regularly responded to hypertonic stimuli and to a lesser degree to hypotonic stimuli. The distal colon in the rat is usually filled with semi-solid or solid faecal pellets, arguably neither hypertonic nor hypotonic. During diarrhoea colonic mucosa would encounter hyperosmotic stimuli and only in extreme cases would the colonic contents become hypotonic. Therefore it could also be argued that the more hypotonic the luminal contents are, the more essentially noxious the stimulus is, as very watery diarrhoea is rarely encountered. Following these arguments, one would expect a nociceptive type fibre to respond equally to the hypotonic as well as the hypertonic stimulus. Yet, in the rat distal colon these responses in afferents were not observed. *In vivo* studies have not investigated colonic sensitivity to hypertonicity. It is possible (though unlikely) that the sensitivity to this stimulus differs between *in vivo* and *in vitro* states.

The mechanism of action by which hypertonic saline exerts its effect on afferent fibres is not known. It could be argued that for mucosal fibres deformation of the mucosal surface caused by the osmolar flux could be indirectly stimulating the fibre mechanically. Indeed, hyperotonic saline has been observed to cause movement of villi in the small intestine whilst eliciting a response in mucosal afferent fibres (Paintal, 1973). This theory does not account for the responses of serosal afferent fibres or the lack of sensitivity to hypotonic stimuli - stimuli that could also cause mucosal deformation. Even more perplexing is the similarity between and variation within the latencies of mucosal and serosal fibres. A short latency (coupled with mucosal stroking) has been cited as sufficient evidence for a fibre to be classed mucosal (Garnier & Mei, 1982; Mei, 1978). Yet in this study there is a mix of both short (one or two seconds) and long (one or two minutes) latencies in response to hypertonic saline in mucosal and serosal fibres where the difference in

mechanosensitivity is distinctly different between the two groups of fibres. The only conclusion that can be made is that the response characteristics to hypertonic saline of both mucosal and serosal fibres are homogeneous. Therefore, the response is unlikely to be direct (taking into account the different depths of receptive fields and penetration rates), or mechanically mediated (again due to the different sites of the receptive fields). It is difficult to speculate further on the lack of differences in responses between serosal and mucosal fibres without a firm understanding of the mechanism of action of hypertonic saline directly on afferent fibre terminals.

D-mannitol is an osmotic stimulus that is not absorbed, in contrast to saline (Mei, 1985). Therefore D-mannitol is a true osmotic stimulus that cannot have any secondary effects after penetrating the mucosa. At identical osmolarities hypertonic D-mannitol was not an effective stimulus for colonic afferent fibres, but hypertonic saline was effective. This is consistent with another study that showed that the more absorbable the osmotic stimulus the greater the magnitude of the response (Garnier & Mei, 1982). It is likely that there is a second part of the response to hypertonic saline that is not due to osmolarity alone but to some other mechanism. One may speculate that this second part of the response results from a change in afferent fibre membrane excitability caused by increased extracellular ionic concentrations.

4.5.2 Capsaicin

Capsaicin has rarely been used in electrophysiological studies of the innervation of the gut despite its widespread use in other areas of investigation. Abdominal afferent fibres (in the mesentery or on the wall of the abdominal organs) in the spinal innervation respond to capsaicin differentially depending on their classification. 100% of C-fibres with endings in the mesentery and serosal surfaces of visceral organs respond to capsaicin, but only 38% of A-fibres (Longhurst *et al.*, 1984). This finding has recently been challenged by the observation that *in vivo* and *in vitro*, luminal or close-systemic application of capsaicin stimulates 30% of vagal afferent fibres in the gastro-oesophageal region

with no dependence on the conduction velocity or location of receptive field within the wall of the gut (Blackshaw *et al.*, 2000). In the current study 30% of all colonic spinal afferent fibres studied responded to capsaicin, consistent with that study. In contrast however, mucosal fibres were less sensitive to capsaicin than serosal fibres (only 14% compared to 41% responded). With little literature backing these results it is difficult to speculate the reason for this discrepancy in the current data. One possible explanation is that there are more serosal fibres (including mesenteric) than mucosal that are designed to signal painful stimuli. This is partially supported by the fact that serosal fibres in the gut have been thought to respond to distension only at noxious levels and therefore devoted to pain signalling. In the cutaneous innervation, only nociceptors are capsaicin-sensitive (Kress & Zeilhofer, 1999). This specificity of nociceptors extends also to heat and proton sensitivity. Indeed, it is has been proposed that protons and heat may be the natural ligands for the capsaicin receptor VR-1. However, in gastrointestinal sensation it has not been possible to assign one group of afferents to the role of a nociceptor. This is evidenced by the fact that not all serosal fibres in the current study are capsaicin-sensitive, or proton-sensitive. In addition, mucosal afferent fibres by virtue of their exquisite mechanosensitivity are not nociceptors - although they may contribute to pain – but still a proportion were capsaicin-sensitive. Thus, the physiological function of vanilloid receptors in the colon still remains to be elucidated.

4.5.3 Bradykinin

Serosal and mesenteric fibres respond to algesic mediators such as bradykinin (Haupt *et al.*, 1983; Longhurst & Dittman, 1987; Maubach & Grundy, 1999; Sengupta & Gebhart, 1994). Mucosal afferent fibres are also sensitive to bradykinin (Blackshaw & Grundy, 1993*a*). Only 2/9 afferent fibres responded to bradykinin in this study. Considering that at the same concentration as used in the current study, Maubach and Grundy (1999) had a 100% response rate, our proportion is very low. There are two primary explanations for the lack of responses seen to bradykinin in the current preparation. Firstly, the response of serosal afferent fibres is dependent on the presence of PGE_2 in the rat jejunum

(Maubach & Grundy, 1999). Therefore, the presence of indomethacin in the bath may well be inhibiting the afferent fibres ability to respond to bradykinin by preventing the production of PGE_2. On one occasion a previously unresponsive fibre to bradykinin developed sensitivity after the receptive field was treated with PGE_2. Whilst this preliminary data is hardly conclusive, it does agree with Maubach and Grundy (1999) and suggests that this is a path to pursue in future studies. The other explanation is also methodological. Bradykinin is not usually applied to the mucosal surface, but rather to the serosal surface (Longhurst *et al.*, 1984; Maubach & Grundy, 1999) or perfused into the tissue through blood vessels (Haupt *et al.*, 1983; Longhurst & Dittman, 1987). Bradykinin may not be able to penetrate the mucosal barrier effectively in order to elicit a response in an afferent ending. A new method of serosal application was being trialled at the end of the project because of concerns that the sensitivity of afferents to bradykinin along with a number of other large chemical mediators such as 5-HT, PYY and CCK was compromised by an inability of these substances to cross the mucosa. By comparing the sensitivity of all classes of afferent fibres to both mucosal and serosal application of stimuli, a better understanding of potential sites for therapeutic targets is gained.

4.5.4 HCl

Afferent fibres from the colon were not particularly sensitive to hydrochloric acid. Even on the occasion that a response was seen, the magnitude of the response was less than for some of the other stimuli. The concentration of HCl that was chosen for this study, was based on a study in the oesophagus *in vitro* (Page & Blackshaw, 1998). Stronger concentrations were not used because of concerns of damaging the mucosal surface, a concern that was well heeded in the mouse study (see chapter 8). At concentrations greater than that used in this study, the mucosa of the duodenum became ulcerated *in vivo* (Cottrell & Iggo, 1984*a*). It is likely that an *in vitro* preparation would be even more sensitive to mucosal damage. The colon is acidic under normal physiological circumstances as a result of its abundance of short chain fatty acids. Therefore, it may have been short-sighted to avoid a range of concentrations as 50mM may well be in the

physiological range and therefore not as likely to act as a stimulus. Alkaline stimuli were not included in this study. The stomach is also acidic and has afferent fibres that are sensitive to levels of acidity. The oesophagus has afferent fibres in the mucosa that are also sensitive to levels of acidity, although an acidic environment is not constant in the oesophagus.

Protons are candidates for a natural ligand for the vanilloid receptor VR-1 (Kress & Zeilhofer, 1999). Therefore, it would have been reasonable to expect that the afferent fibres in this study that were sensitive to HCl would also have been sensitive to capsaicin, but responses to HCl and capsaicin were not exclusively colocalised in the same neurons. Low pH is known to activate inward currents in the cell bodies of nerves in the dorsal root ganglion and in the central nervous system and a number of proton-gated channels have recently been cloned (Chen *et al.*, 1998). The DRASIC channel is found only in sensory neurons and is seen as the primary candidate for mediating direct responses to pH. However, the matter is not yet resolved.

Rather than a normal luminal stimulant, acid may also be seen as a potential indicator of inflammation or injury. Indeed, in the skin, the application of acid does not necessarily induce a response in a nociceptor, but does induce mechanical hypersensitivity, decreasing the threshold of mechanical sensitivity (Steen *et al.*, 1992). With this in mind, it may have been more appropriate to investigate the application of HCl in conjunction with mechanical stimuli to assess any sensitising effect it may have apart from activating the fibre directly.

4.5.5 5-Hydroxytryptamine

In the upper gastrointestinal tract mucosal afferent fibres are directly sensitive to and muscular afferent fibres indirectly sensitive to systemically applied 5-HT (Blackshaw & Grundy, 1993*b;* Blackshaw & Grundy, 1993*a*) via 5-HT_3 receptors. Luminally applied 5-HT also elicits responses in mucosal and tension receptors *in vitro* in the oesophagus but only a small proportion of afferent fibres show sensitivity (Page & Blackshaw, 1998). In the current study, serosal fibres were

sensitive to luminal 5-HT, but mucosal and muscular fibres were not. Possibly, with greater numbers, a small proportion of sensitive mucosal and muscular fibres would be revealed similar to Page and Blackshaw (1998). Concerns that 5-HT were not penetrating the mucosa to reach the receptive field are unfounded as serosal fibres showed sensitivity to 5-HT. However, it is somewhat surprising that serosal fibres were sensitive to mucosal application in the absence of responses to serosal application. Given that their mechanosensitivity suggests that serosal afferent fibres have endings much deeper within the tissue, it would be expected that drug access to the receptive field would be greater following serosal application, assuming that penetration of the stimulus through the serosal membrane was possible.

4.5.6 Bile

Bile salts have a profound effect on the colon, increasing motility *in vitro* (Squires *et al.*, 1991), and causing pain *in vivo* (Edwards *et al.*, 1989). Three main reasons exist for bile to be present in the colon; malabsorption in the small intestine, dysfunction of the ileocaecal valve and diarrhoea (Edwards *et al.*, 1989; Read, 1988). Its presence in the colon in large quantities is therefore an indication of gut dysfunction. Bile salts stimulate prostaglandin synthesis in the colon (DeRubertis *et al.*, 1984), preferentially in the submucosa. Prostaglandins have a role in chemosensitivity in the colon, a fact that is discussed in Chapter 7. In normal humans deoxycholic acid in the colonic lumen decreases thresholds of conscious sensation independently of muscular activity suggesting that colonic afferents are being activated (Edwards *et al.*, 1989). Therefore it was not surprising to observe that bile stimulates afferent fibres in the colon of the rat. However, deoxycholic acid, a major constituent of bile did not stimulate afferent fibres in this preparation. The concentration for investigation chosen here (3mM) was identical to the concentration shown to cause strong pain when perfused into the colon of humans (Edwards *et al.*, 1989). There may be species differences, but one observation of Edwards et al (1989) was that pain was more readily elicited when deoxycholic acid was exposed to a large surface area of the colon, thus exposing it to a greater number of receptive fields. This supports an intensity theory of sensation and pain

generation in the colon and may explain why this concentration did not elicit a single large afferent fibre response. Thus, a similar magnitude of response to deoxycholic acid might be observed if concentrations were matched with that in the ferret bile, or alternatively another constituent of bile might be the adequate stimulus. Bile was the only stimulus observed to cause desensitisation (with the exception of capsaicin). The mechanism of action of bile on colonic afferent fibres is unclear, but the nature of desensitisation observed leads to speculation for a role for mast cell degranulation. Bile malabsorption has been associated with increased levels of mast cells at the ileocaecal junction, and in diarrhoea predominant irritable bowel syndrome (IBS) and other diseases of malabsorption increased levels of mast cells and their degranulation have been implicated in pain and abnormal sensation (Miner, 1991; Weston *et al.*, 1993).

The data gathered on chemosensitivity covers a broad range of different chemical stimuli. There are interesting similarities between serosal and mucosal fibres with respect to their chemosensitivity. These results however, may pose more questions than they answer. From this work it is becoming apparent that there is great heterogeneity in colonic afferent chemosensitivity. Most chemosensitive fibres are not specific and those that appeared to be so probably were not tested with other stimuli that would elicit responses. Clearly both mucosal and serosal fibres are capable of mediating information about luminal contents. It is not yet clear whether the information reaching the central nervous system is interpreted in the same way for both classes of fibres. It is clear that the complexity of information that is reaching the central nervous system through this neural pathway is far greater than previously thought.

There were no observable patterns of chemosensitivity in the fibres described here. This is in contrast to the available literature on skin chemosensitivity. As discussed, sensitivity to protons is closely related to sensitivity to capsaicin (Kress & Zeilhofer, 1999), indeed protons are believed to activate the capsaicin receptor VR-1 and sensitise VR-1 receptors to capsaicin. Sensitivity to protons and capsaicin were not colocalised in colonic gastrointestinal afferents. In addition sensitivity to bradykinin is also colocalised in the skin with heat sensitivity (and

therefore capsaicin sensitivity) but not all heat sensitive afferents show bradykinin sensitivity (Kress & Reeh, 1996). One explanation for the differences in patterns of chemosensitivity between cutaneous and gastrointestinal afferents with respect to the vanilloid receptor is that a different vanilloid receptor subtype may be expressed in the skin than is expressed in the gastrointestinal system.

Three paths of future investigation in colonic chemosensitivity are immediately obvious. The first is a broader exploration of the effects of normal and pathophysiological luminal contents. This includes the potential role of short chain fatty acid's, various constituents of bile, and possibly different products of the bacteria present in the colon. Perhaps the simplest experiment but easiest to overlook would be to prepare different concentrations of the animal's own faeces, apply it to the receptive field and investigate whether these afferent fibres truly do respond to normal luminal contents. The second is a more detailed study of the mediators that have been shown to be influential in this system already, including dose-response curves and investigating the mechanisms by which these mediators exert their effects. The third is an investigation of local humoral agents present in the tissue of the distal colon. For example in the distal colon, PYY - like 5-HT - is found mainly in the enterochromaffin cells in the mucosa. It has a role in regulating secretion postprandially (Whang *et al.*, 1997) and is released after intracolonic administration of nutrients (Fe-Cheng *et al.*, 1995). The ileal brake – a functional fat-induced slowing of intestinal transit - is also dependent on PYY (Lin *et al.*, 1996), thus demonstrating a role in neural sensitivity for PYY.

Chapter 5. The Nature of Mechanical Sensitivity

5.1 Abstract

1. Colonic afferent fibres were investigated to elucidate the adequate mechanical stimulus for stretch-sensitive fibres.
2. Three different circumferential stretch stimuli were applied to colonic afferent fibres. 1) Graded static tension. 2) Graded static length. 3) Oscillating change in length.
3. 14 afferent fibres in total were identified as being muscular according to classification criteria described in Chapter 2, responding to ungraded circumferential stretch with an adapting response. Not all of these fibres underwent further investigation.
4. 21 afferent fibres were tested to graded static tension. 0/13 serosal, 0/4 muscular and 0/4 mucosal fibres responded.
5. 12 afferent fibres were tested to graded static length. 1/6 serosal, 0/4 muscular, 0/ mucosal fibres responded.
6. 2 muscular afferent fibres were tested with an oscillating stimulus. Displacement correlations between afferent fibre discharge and recorded length in the tissue demonstrated that both fibres showed peak discharge at a time between the peak velocity of stretch and the peak length, but was not strongly correlated with either.
7. Previously unidentified qualities of stretch-sensitive lumbar splanchnic afferent fibres from the colon of the rat have been identified.

5.2 Introduction

Distension-sensitive afferent fibres in the wall of the gut have been described *in vivo* for most of the length of the gut and many of the neural pathways. It is generally accepted that the adequate stimulus is tension of the gastrointestinal wall. In-series tension receptors are found in both the longitudinal and circular muscle of the gut and respond to both distension and to muscular contraction (Iggo, 1986). In the duodenum, 80% of in-series tension receptors are found in the longitudinal muscle and 20% in the circular muscle (Cottrell, 1984*b*). There are regional differences along the length of the gut in response characteristics related

to musculature differences between regions (Grundy & Scratcherd, 1989). Classification criteria for spinal in-series tension receptors differ from that used for vagal in-series tension receptors (Grundy & Scratcherd, 1989) but basic characteristics of in-series tension receptors are consistent throughout the gastrointestinal tract. However, in the light of the recent documentation of volume sensitive inputs into the IMG by intestinofugal fibres (Anthony & Kreulen, 1990), and the necessity for a comparison between the previous characterisation of distension-sensitive afferent fibres *in vivo* and this novel *in vitro* preparation, a comprehensive study was undertaken to characterise these fibres with respect to tension and length.

5.2.1 Static versus Dynamic Stimuli

A static stimulus, such as that applied when a weight is attached to the end of the tissue, is useful for investigating the relationship between fibre mechanosensitivity to the unitary value of a stimulus. A dynamic stimulus tests sensitivity to the change of a stimulus. The use of both types of stimuli was pursued to give a greater understanding of the adequate stimulus of distension-sensitive afferent fibres.

5.3 Methods

Tissue was prepared as specified in Chapter 2.

Serosal, mucosal and muscular fibres were identified as specified in chapter 2. To clarify, muscular fibres were termed stretch-sensitive fibres. They responded to an ungraded circumferential stretch applied to the edge of the tissue, probing of the receptive field with a blunt probe and did not respond to stroking.

A summary diagram of the methods subsequently used on a proportion of mucosal, serosal and muscular afferent fibres are found in Fig 5-1.

A thread was attached to the edge of the tissue closest to the receptive field and passed through a pulley such that the tissue could be pulled flat in a circumferential

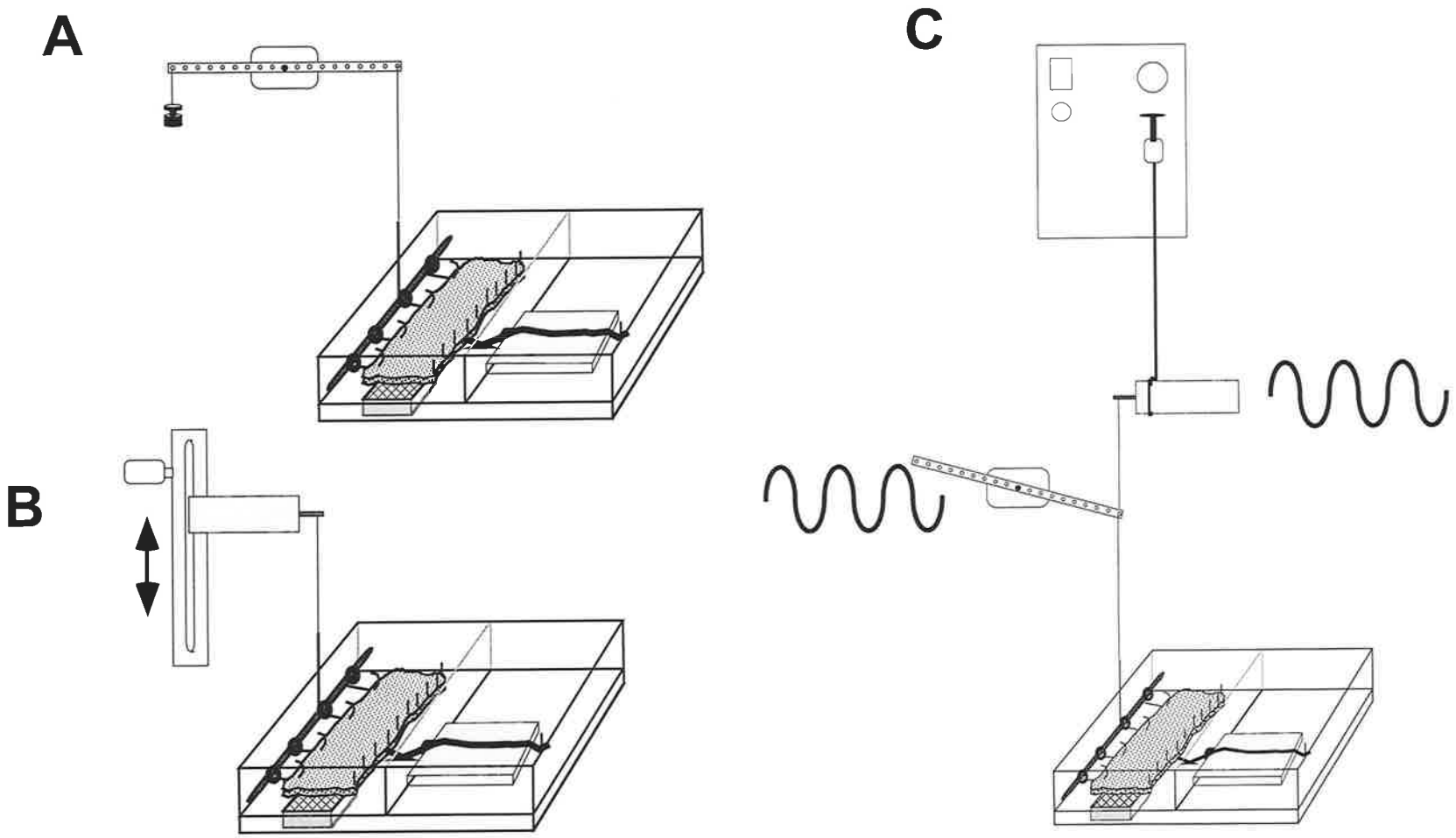

Figure 5-1. The three methods of stretch applied to the tissue. A. Graded tension by changing weights. **B.** Graded length with the use of a micromanipulator. **C.** Oscillating stimulus using a rat ventilator and concurrently recording length and tension.

manner. The thread was then attached either to a cantilever, an isotonic or isometric transducer.

The cantilever method included a free-moving cantilever with the thread attached to one side. Weights (1g, 2g, 5g, and 8g) were hung from the other side. They were added in a step-wise manner with no recovery period between.

An isotonic transducer was also used in place of the cantilever such that length of stretch could be monitored as the weights were changed. This protocol was identical to the cantilever method.

An isometric transducer was attached to the thread passing from the tissue. The circumferential length was increased in 2mm (0-12mm) increments from the resting length. Each length was held for two minutes. 12mm length effectively doubled the circumferential length of the tissue. If the tissue was visibly damaged by a lesser length, the full protocol was not completed.

In 2 studies, the isotonic and isometric transducer were attached in series to a rat ventilator, such that sigmoidal oscillating stretch could be applied to the tissue whilst measuring the length and the tension in the tissue concurrently.

Analysis for the oscillating stretch was unique to these two studies and is fully described here. The purpose of this analysis was to determine whether there was any point along the oscillating length recording where there was a stronger chance of encountering a spike occurrence. It had been previously observed that the generation of spikes failed when a purely static load or displacement stimulus (that caused a constant application of either length or tension) was applied. In contrast, spikes were generated with ungraded circumferential stretch - a varying load stimulus. This strongly suggested that a varying load/displacement was required for spike generation. It was considered that an auto correlation method of analysis between displacement stimulus and the generated spike signal would not only confirm the correlation between the signal and spike signal generation, but also identify at what phase of the displacement cycle the spikes were produced. It was

expected that the spikes would not occur at the peaks of the displacement (when momentarily there was a zero rate of change in the displacement - a state of static displacement). This in fact turned out to be the case as described in the following. The peak of the length stimulus was generated at a constant frequency. If the discharge rate of the fibre were bound to length displacement, then the correlation of spike events would be highest at the peak of displacement length, regardless of the value of length. To determine whether this was the case the displacement correlation was performed against the normalised spike signal (0 = no signal, 1 = spike signal). Thus, by moving the entire normalised spike event trace by increments over a whole length displacement cycle and performing a correlation, it was possible to observe whether the original spike trace was most closely correlated with peak of the length displacement, or at a phase/time shift from the peak displacement. The length recording was averaged around 0. Each spike was recorded as a marker (0 or 1), from here termed a spike event. Each spike event was assigned to the closest unit of length displacement measurement as the rate of acquisition for raw spike recordings was greater than that for length and a synchronised rate of acquisition for both displacement and spike events was required for this analysis. The unit of time (0.1s) was determined by the rate of acquisition of length data (10Hz). A displacement correlation was then performed such that spike event data was shifted along one time unit (time unit of length 0.1s) at a time and a new correlation of spike events and normalised length was carried out. Each correlation contained 1,505 data points for Rat 181 and 2,626 for Rat 177. Displacement correlations were performed for one full cycle of the oscillating stimulus. Values of the displacement correlations (y-axis) were then graphed against each shift of a time unit (0.1 s) (x-axis) including shifts forward and backward in time such that x=0 gives a correlation value (y) for occurrence of spike events at peak length relevant to the original record. The peak of the resulting graph demonstrates the timepoint on the whole length cycle where a spike event is most likely to occur. Thus it is possible to determine at what phase of the length displacement recording (acceleration, peak, deceleration) the spikes correlate with best.

5.4 Results

5.4.1 Ungraded Stretch

Fourteen fibres were identified as muscular fibres using the criteria discussed in chapter 2. They did not respond to stroking, but did respond to probing of a discrete receptive field. All fibres described had one punctate receptive field. Fourteen fibres responded with an adapting response to ungraded circumferential stretch at the level of the receptive field. A typical response of a muscular fibre is shown in Fig 5-2. Responses to a static ungraded circumferential stretch when the onset of the stretch was rapid and then held constant at approximately twice the *in situ* circumferential length were generally adapting. When the stretch was developed slowly, then released when approximately twice *in situ* length was reached, the rate of discharge of the fibre increased over the course of the stretch then returned to baseline when the stretch was released or a silent period was then observed. An example of a more rapidly adapting response is shown in Fig 5-3. This fibre was classified as muscular because the response to stretch was a distinct burst of activity. This is in contrast to the few (1 – 5) spikes that were occasionally observed in serosal fibres in response to stretch. The magnitude of this response was not related to the degree of stretch and therefore cannot be termed rapidly adapting with respect to other muscular fibres documented in the literature. Muscular fibres did not respond to spontaneous contractions of the tissue on the occasion that that occurred.

5.4.2 Length versus Tension

Twenty-one fibres were tested with graded tension that was applied circumferentially at the site of the receptive fields. This group was a mixture of serosal (n=13), mucosal (n=4) and muscular (n=4) fibres. None of these fibres responded to graded tension – including the muscular fibres. Twelve of these fibres (6 serosal, 2 mucosal and 4 muscular) were subsequently tested with graded length. Of these, 1 serosal fibre responded. The response to length was not linear. The stimulus was not repeated so reproducibility cannot be verified. In the serosal fibre study where tension was recorded concurrently with the length

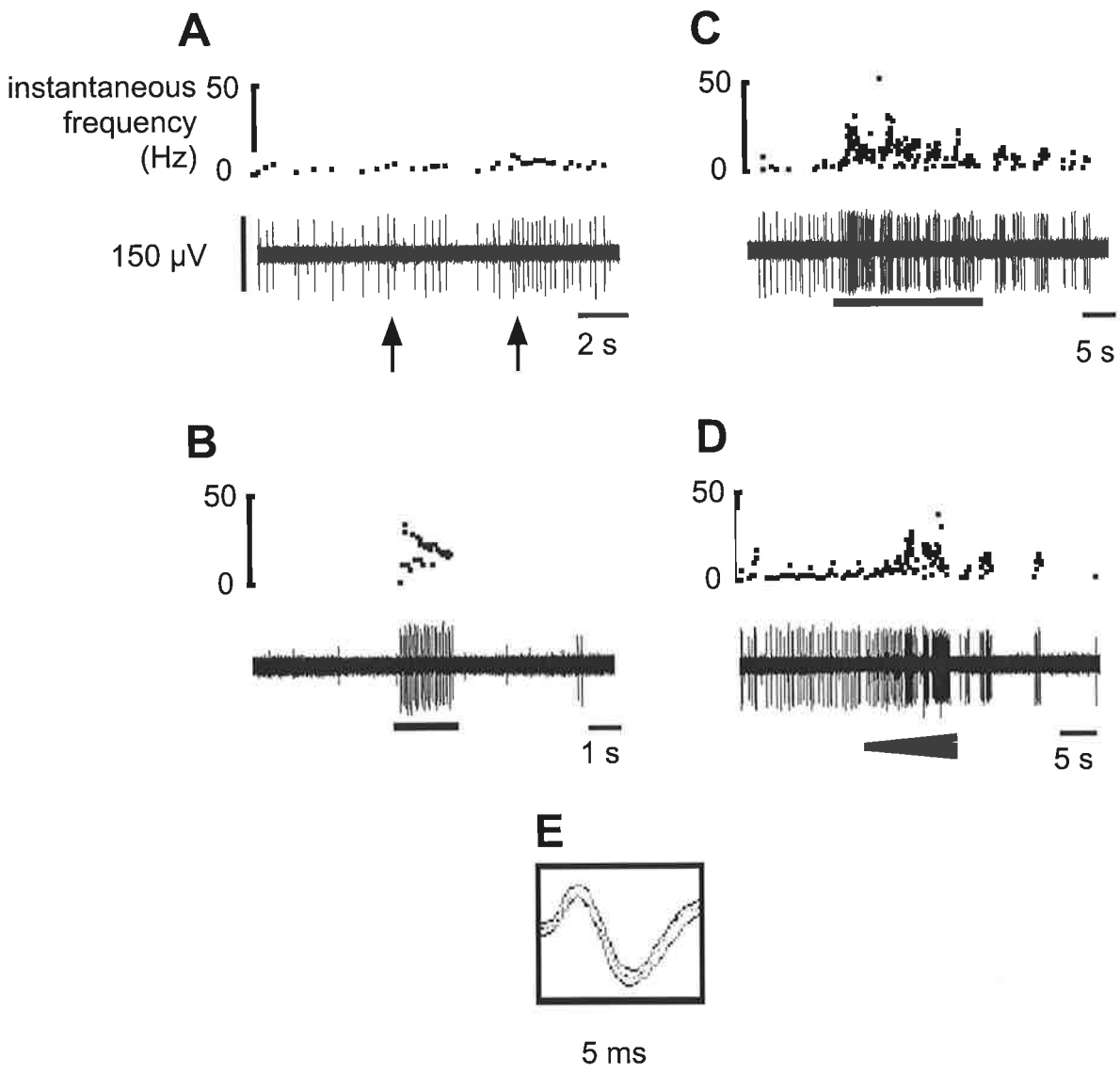

Figure 5-2. Mechanosensitivity of a single muscular fibre. A. The mucosal surface was stroked with a fine paint brush. There was no response to stroking. This fibre was spontaneously active as is demonstrated in A, C and D. **B.** A glass probe applied to the mucosal surface of the receptive field of this fibre. The response was slowly adapting and did not persist beyond the cessation of the stimulus. This was the third probe in a series of 5 probes. There was a silent period after each application of the stimulus, therefore the spontaneous activity of this fibre was not evident in this figure. **C.** This was a hand held circumferential stretch at the level of the receptive field. The tissue was quickly stretched so that it was twice the original circumference and held at that length for the duration of the stretch. The response was slowly adapting and the fibre developed a bursting pattern that persisted beyond the cessation of the stimulus for 56 s. **D.** This was a hand held circumferential stretch at the level of the receptive field. The tissue was gradually stretched over the duration of the stretch until it reached twice the at-rest width, when the stretch was released. The rate of discharge gradually increased with the increase in stretch until it developed a bursting pattern which then persisted beyond the cessation of the stimulus for 50 s. **E.** The wave template of the fibre demonstrated.

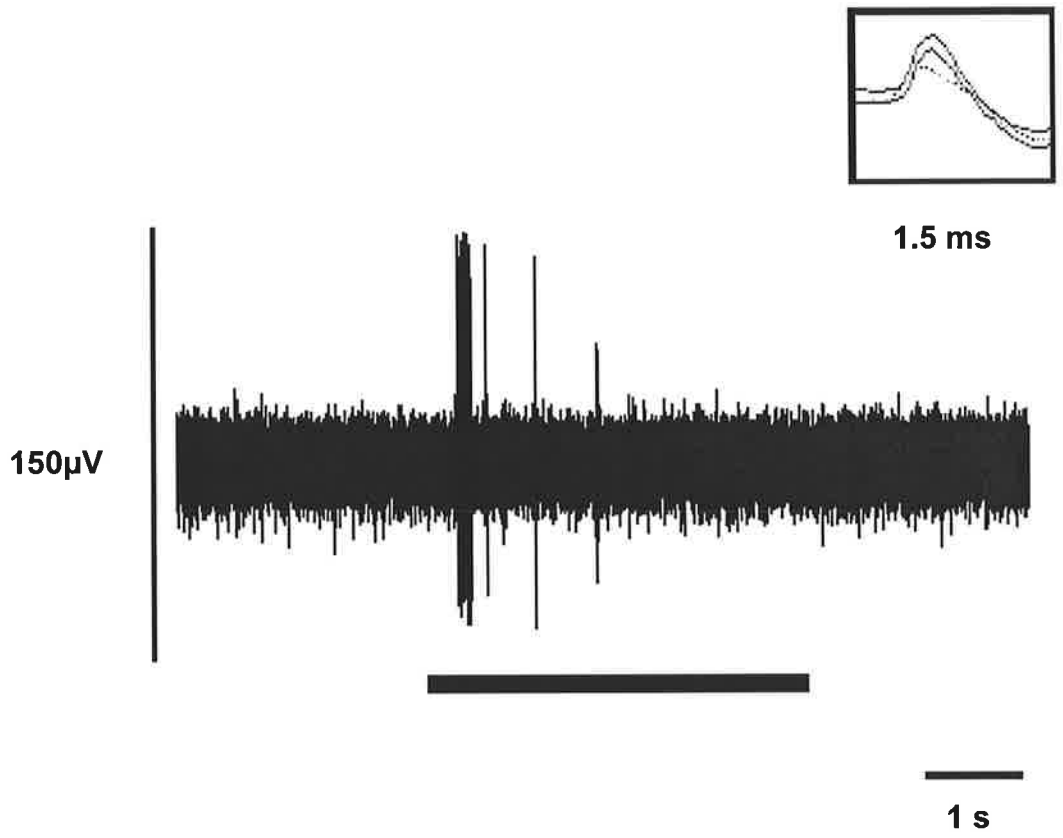

Figure 5-3. A rapidly adapting response to circumferential stretch. The raw tracing is displayed and the wavetemplate is shown in the top right corner. This fibre responded to circumferential stretch with a rapidly adapting response.

stimulus, the response to length was seen at a level of tension in the tissue that did not elicit a response when tension was applied as the controlled stimulus (Fig 5-4).

5.4.3 Dynamic Stimuli

Two studies of muscular fibres employed the oscillating length-tension stimulus described previously. This can be viewed in Fig 5-5. Both fibres were spontaneously active with no evident rhythmicity. Both fibres responded to ungraded circumferential stretch. Static length and tension stimuli were not tested on these fibres. The onset of the oscillating stimulus resulted in a bursting pattern that was loosely related to the peaks of both tension and length, but was not elicited by every oscillation (Fig 5-5). There was no relationship between the number of spikes elicited with each oscillation and the peak of length or tension. This was also true for the instantaneous frequency of discharge of the spikes. Displacement correlations showed that spikes for both studies were correlated most closely at 0.5 s for rat 181 (Fig 5-6) and 0.8 s for rat 177 (Fig 5-7) prior to the peak of the length stimulus. This means that the acceleration phase of the stimulus had just finished but the peak had not yet been reached. Thus these spikes were not correlated with the peak of the stimulus, or the acceleration of the tissue, but an area in between. This could possibly be either beginning point of deceleration or at the point where acceleration and length provide the greatest combined stimulus.

5.5 Discussion

5.5.1 Ungraded Stretch

Muscular, serosal and mucosal fibres arising from the distal colon and passing through the inferior mesenteric bundle have been describred. Muscular fibres demonstrated adapting responses to an ungraded stretch of the tissue but did not respond to the application of graded static tension, which - given the current literature on the subject - was unexpected. In comparison with other types of fibres encountered in this project, muscular fibres were encountered rarely. With only fourteen fibres to work with over the course of this project, comparing these fibres

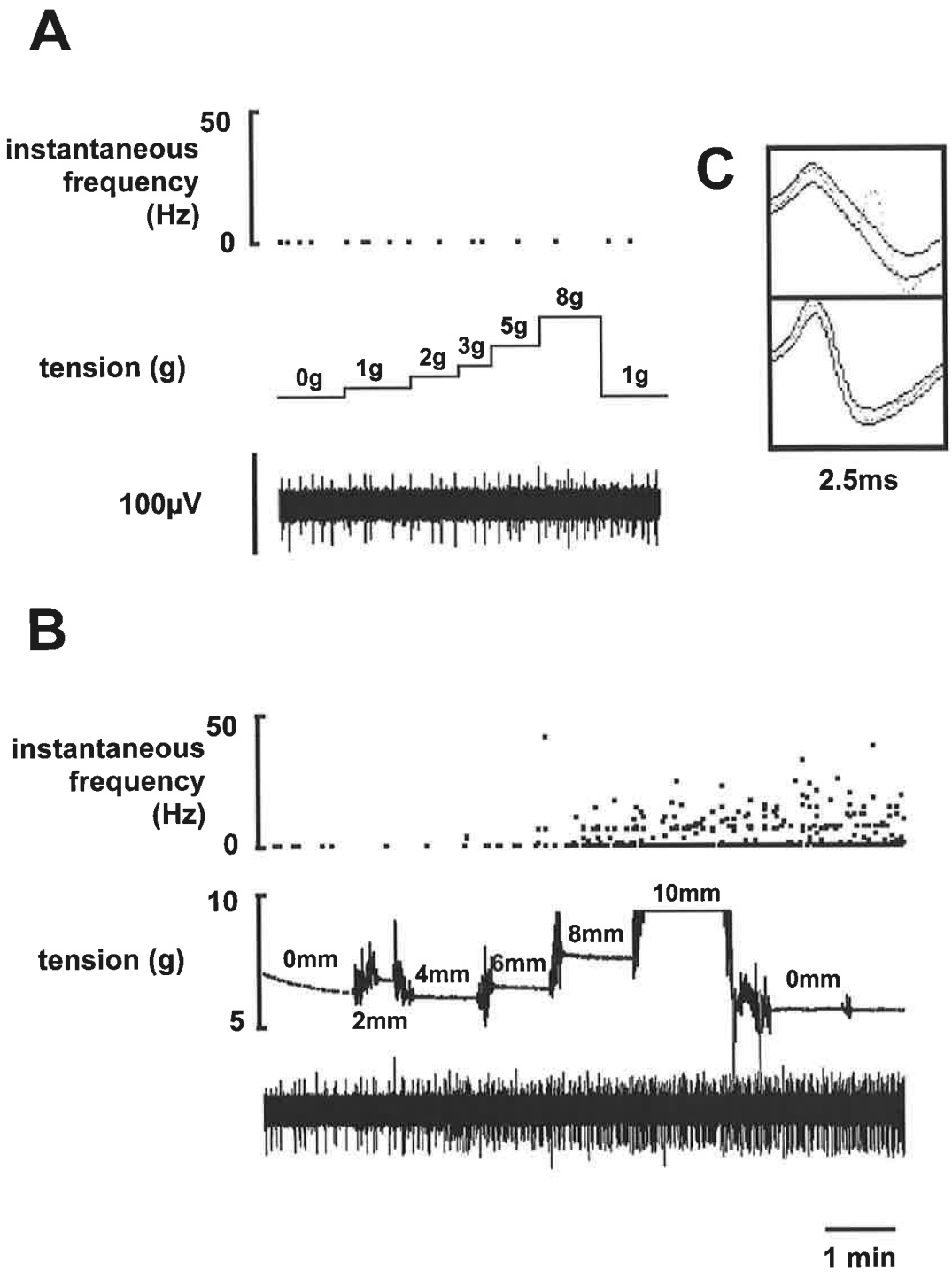

Figure 5-4. The response of a serosal fibre to length. This fibre did not respond to ungraded stretch at the beginning of the study, only blunt probing (not shown). **A.** The fibre did not respond to graded tension (tension increases shown as schematic). **B.** The fibre did respond to graded length, but was not stimulus bound. It did not decrease its discharge rate after the removal of the stimulus. **C.** Two fibres were recorded in this strand. Both were serosal and had adjacent receptive fields. The fibre demonstrated here is shown in the top wavemark template. The bottom fibre did not respond to either tension or length.

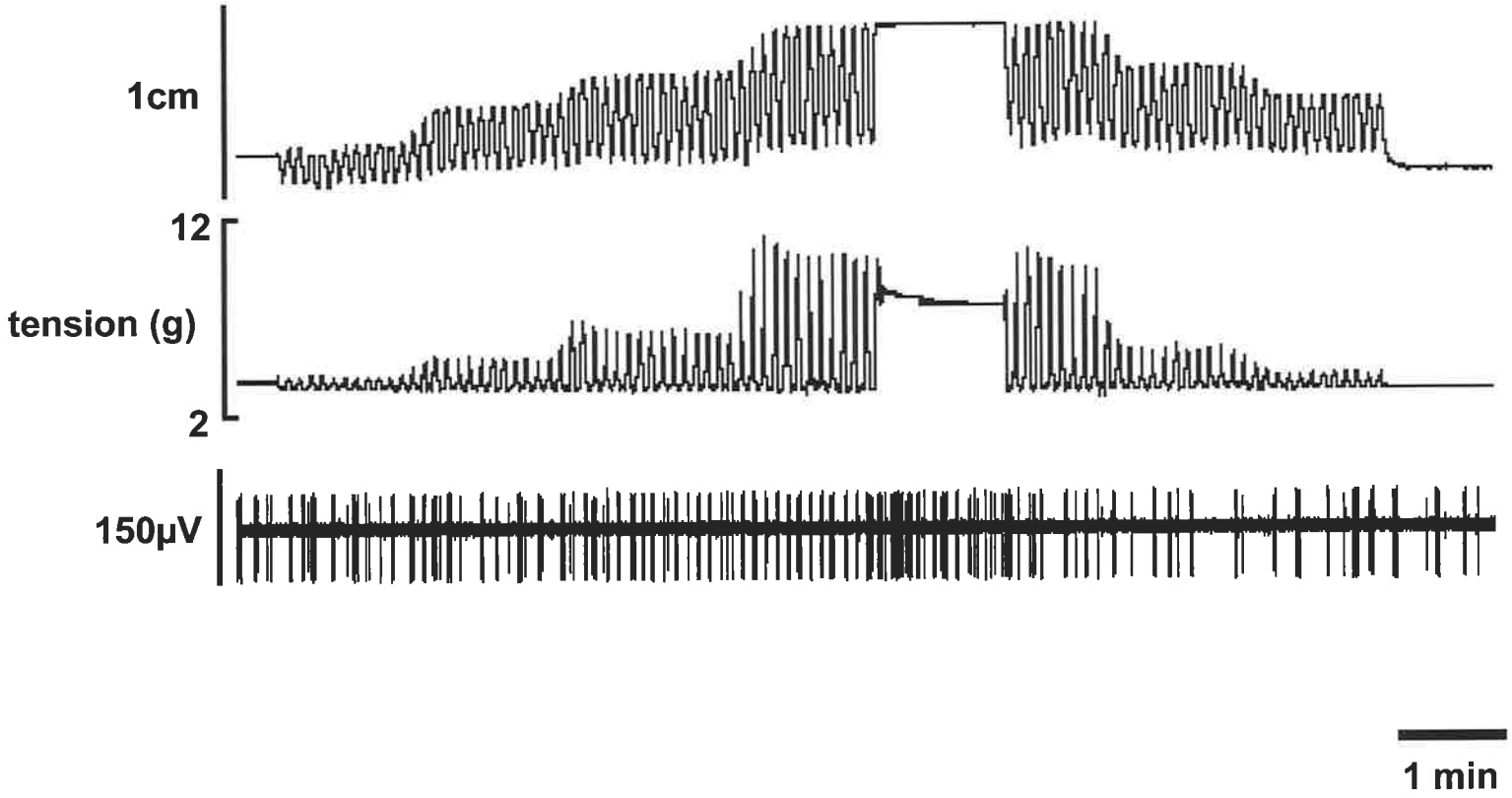

Figure 5-5. Demonstration of results of an oscillating stimulus. The lowest trace is the raw signal of afferent discharge. Immediately above that is the recording of tension. The top trace is a record of length. 7-10 oscillations per minute were used. The amplitude of the stimulus was increased until the circumferential length was double the *in situ* length. The tissue was held at a static stretch before the stimulus was gradually decreased in amplitude.

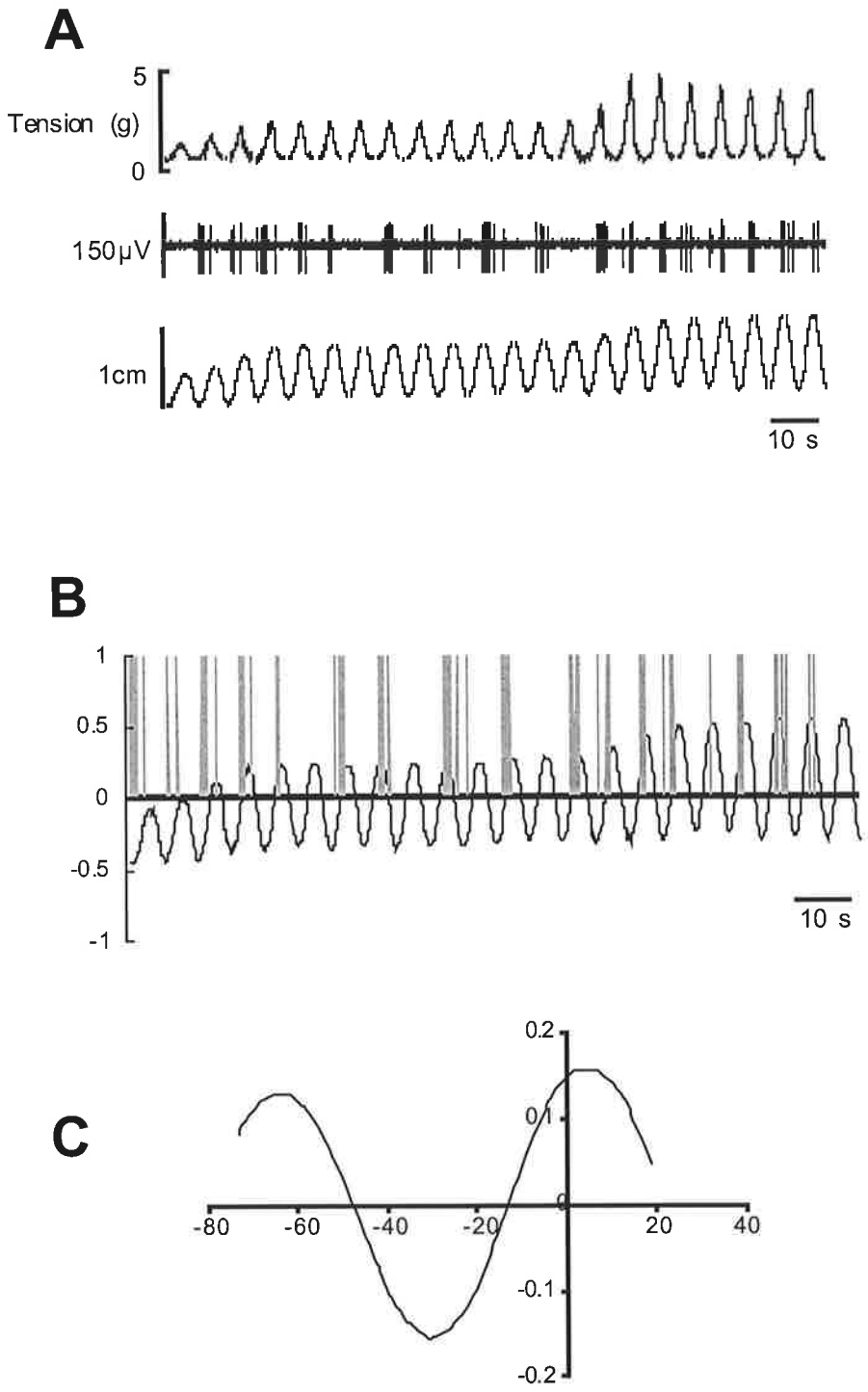

Figure 5-6. Displacement correlation analysis of a muscular fibre (Rat 181) between spike events and length. A. Raw tracing of data analysed. Raw tracing is shown above the length recording. **B.** The length data was averaged around 0, and spikes were expressed as an event (grey lines). Analysis was performed by moving the spike event along the X axis in both directions one time unit (0.1s) at a time and correlating all 1505 data points in both spike and length data. **C.** Correlation value (Y axis) graphed against time unit (0.1s) shifts (x-axis). The graph shows that the greatest correlation between spikes occuring at the peak is at the 5th time unit to the right. As the y-axis indicates the point in time for the original record this indicated that the spikes were most likely to occur 0.5s before length peak is reached.

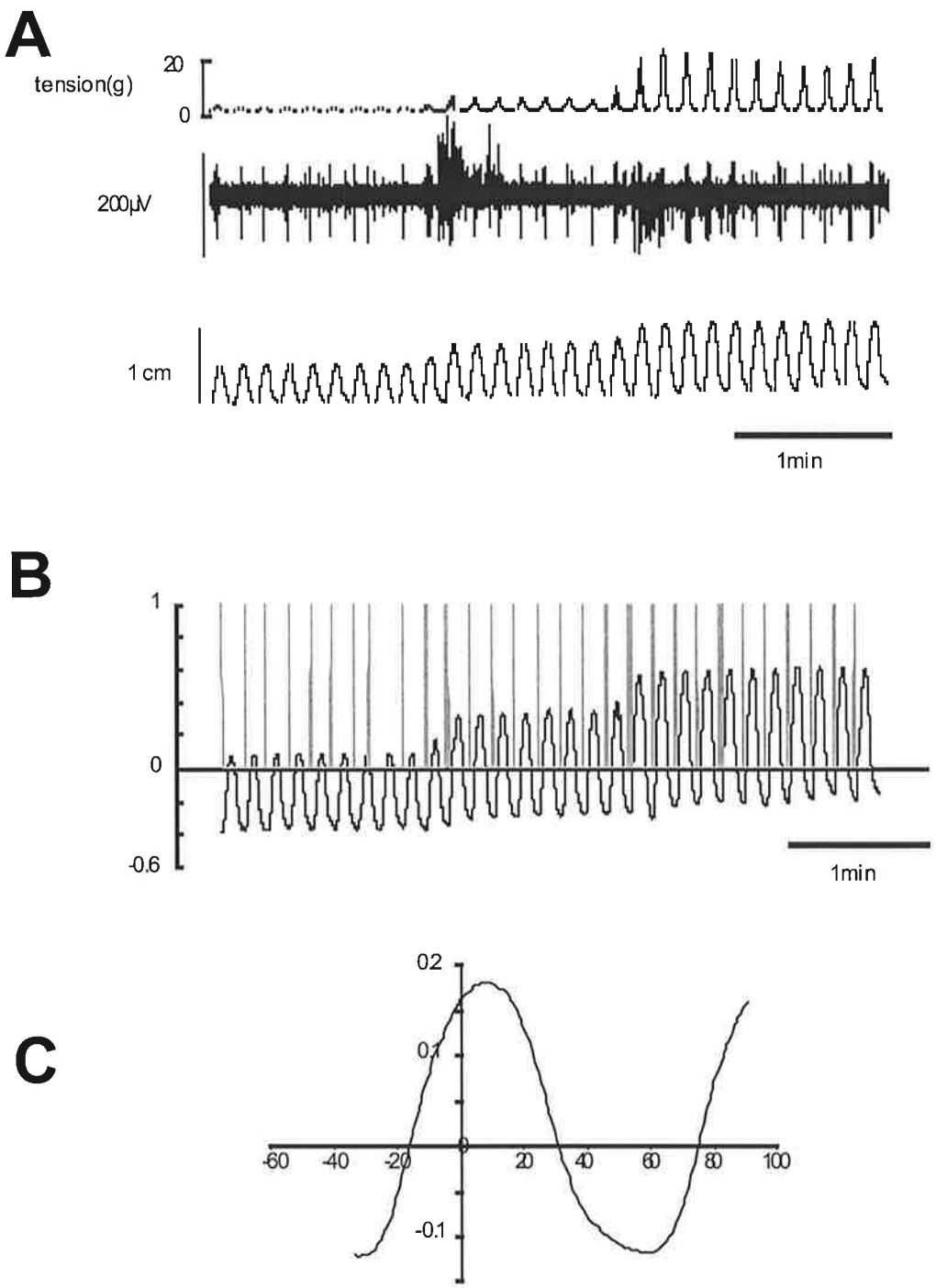

Figure 5-7. Displacement correlation analysis of a muscular fibre (Rat 177) between spike events and length. A. Raw tracing of data analysed. Raw tracing is shown above the length recording. **B.** The length data was averaged around 0, and spikes were expressed as an event (grey lines). Analysis was performed by moving the spike event along the X axis in both directions one time unit (0.1s) at a time and correlating all 2626 data points in both spike and length data. **C.** Correlation value (Y axis) graphed against time unit (0.1s) shifts (x-axis). The graph shows that the greatest correlation between spikes occuring at the peak is at the 8th time unit to the right. As the y-axis indicates the point in time for the original record this indicated that the spikes were most likely to occur 0.8s before length peak is reached.

with those described in the literature is subjective. Nonetheless, the fact that the methodology used for investigating muscular afferent fibres in this project evolved very quickly with respect to the number of fibres investigated, I have attempted to give as complete an account as possible of their sensitivity.

There was clearly a discrepancy between the responses to ungraded circumferential stretch in comparison to graded tension. But there were also obvious differences between the two stimuli. The hand held stretch probably gave a constantly changing stimulus as it is difficult to overcome hand tremors and other inconsistencies. The graded tension however, gave only a very short period of acceleration and then remained a constant static stimulus. Great care was taken during the ungraded stretch not to stretch the tissue any further than during the controlled stimuli. It is therefore unlikely that the degree of stimulus is a defining issue in this discrepancy.

This study did not encounter similar muscular fibres as those described in the same nerve in the cat *in vivo* (Blumberg *et al.*, 1983). In this study 4 classes of distension-sensitive afferent fibres were described in response to an increase of intraluminal pressure held static for 60s. The muscular fibres with either a steady state discharge, an initial transient response followed by steady state discharge, an adapting discharge or a rapidly adapting discharge. They also responded to spontaneous or induced contractions of the colon. Many specifically responded to contraction of the longitudinal muscle. There is discrepancy between their study and the current study with the technique used, but it is unlikely that the techniques used in the current study for applying stretch and tension are incompatible with those used for other *in vivo* studies of colonic muscular fibres. Indeed, the oesophagus also has tension receptors that have been identified *in vivo* in the same way as those for the colon, and have been confirmed *in vitro* with an identical method of tension application as that used in the current study (Page & Blackshaw, 1998). In this study, muscular vagal oesophageal afferent fibres responded to graded application of circumferential stretch with a sigmoidal response curve. Possibly, the cat and rat have differences in the type of activity muscular fibres in the lumbar splanchnic nerves convey, although there are few functional differences

in the pelvic innervation between the cat (Janig & Koltzenburg, 1991) and the rat (Sengupta & Gebhart, 1994). Using the current technique longitudinal stretch was not compared with circumferential stretch as it was impractical to apply longitudinal stretch in an identical manner to that used for circumferential stretch. Therefore it is unknown whether the muscular fibres described here in the current study would respond preferentially to longitudinal stretch. Two other factors may have contributed to the differences observed in this study to others, both of them methodological. The first was the use of indomethacin in the bath which would probably have decreased the normal muscular activity of the colon (Bennett *et al.*, 1976) and also change the compliance of the tissue. In addition, it has been suggested that distension of the gut causes a submucosal release of prostaglandins (Diener & Rummel, 1990), and given that they cause localised direct changes on the tone of the muscle (Bennett *et al.*, 1976), are likely to be necessary for a functionally normal response to distension. The second factor was the temperature at which the bath was maintained. It was observed from preliminary studies that there was less spontaneous muscle activity in the colon when the preparation was run at 32°C rather than 37°C. Temperature has also been shown to affect the response to distension of pelvic distension-sensitive afferent fibres in the rat (Su & Gebhart, 1998). The response in cold-sensitive fibres to distension decreases at a threshold temperature of about 30°C, close to the temperature used in this bath. Nonetheless, consistent with distension sensitive afferent fibres in the lumbar splanchnic nerves supplying the distal colon of the cat (Blumberg *et al.*, 1983), the muscular fibres described here were generally spontaneously active.

5.5.2 Length versus Tension

Volume-sensitive inputs to the IMG from intestinofugal fibres have been demonstrated in the guinea pig (Anthony & Kreulen, 1990). At low pressures, the IMG neurones received inputs that were sensitive to changes in volume and at high pressures became increasingly sensitive to pressure. This may be indirectly supported by an older study (Weems & Szurszewski, 1977) where it was found that IMG neurons in the guinea pig responded to intraluminal pressure only at higher

pressures and were insensitive at lower physiological pressures. Rather than applying stepwise stimuli the Anthony study held either the volume or the pressure in the colon constant and observed whether the IMG cell responded to continuing changes in the other parameter. In this way, it was easily observed that there was a constant discharge rate whilst the volume was held constant despite changing pressures, and that the discharge rate changed with changing volumes when the pressure was held constant. It was hoped that the methods used here employing both length and tension (whilst recording the other concurrently) would be as fruitful in elucidating the encoding of responses to ungraded stretch. Few muscular fibres were encountered that could be investigated after this new protocol was set up. Confounding the final interpretation, the fibre that did show a greater sensitivity to the controlled length stimulus did not show a linear relationship to length. Nor did it show a relationship with the changing tension during a static length. In this fibre the response extended long after the cessation of the stimulus indicating that the length itself was not the sole stimulus effecting the fibres' response. Given that this fibre did not respond to ungraded stretch and was initially classified as being serosal, these observations become increasingly difficult to explain.

5.5.3 Dynamic Stimuli

The one explanation that seemed to unite the results discussed so far was that these fibres were sensitive to neither length nor tension specifically, but rather to acceleration, movement or either of these in combination. Thus, the final protocol was employed. Only two muscular fibres could be used for this protocol. The results however, were surprisingly convincing. By using displacement correlations over 9-20 cycles of data in each experiment, it became obvious why my intensive but unfruitful investigation of spontaneous discharge, number of spikes per oscillation and mean discharge rate over a number of cycles did not show linear relationships with either the degree of peak tension or the degree of length. It was observed that the frequency of spikes was unrelated to the peak of the cycle. The results of this analysis showed conclusively that in two muscular fibres the peak discharge occurs just after the highest velocity of stretch and just prior to the peak of the stimulus. I suspect that this peak discharge in the fibre occurred at the point

where velocity of the stimulus and the degree of length combined together to give the greatest intensity of stimulus affecting the tissue. This could be investigated by multiplying the velocity of the stretch with the degree of length to see whether peak discharge of the fibre is most closely correlated with the resulting value. Displacement correlations were used only with the length recordings and not with the tension recordings because the tension recording was slightly out of phase with the movement of the muscle. This was because the isometric (attached to ventilator) and isotonic (attached to the tissue) were linked by a silk thread that became slack on the downward movement of the oscillation. The length recording however, was in phase with the movement of the muscle because the tissue had enough intrinsic tone to keep the silk thread taut on the downward phase. This accounts for the rather jerky recording of tension seen on the trace (despite the fact that its movement was identical to the sinusoidal stimulus from the ventilator) and the smooth, relatively sinusoidal recording of length (movement identical to the movement of the tissue). Because of the jerky recording of tension, a similar analysis using displacement correlations would only yield useful information if the spikes were correlated with the acceleration phase, the only part of the recording that reflects accurately the movement of the colon itself. This analysis was time-consuming at the time of writing and so analysis with tension has not been carried out, but there is an option for making this analysis considerably more automated, and thus more efficient to use.

5.5.4 Comparison between In Vitro and In Vivo Preparations

The methods described here will be beneficial for future use. The lack of the traditional correlation of tension with discharge rate in these stretch-sensitive fibres forced alternative approaches to investigation of the adequate stimulus. The adequate stimulus for the muscular fibres described here has not yet been determined categorically, but the methods that have been established show promise for being able to elucidate this effectively. An oscillating stretch stimulus with constantly changing acceleration, length and tension has not been adequately investigated. However, this form of analysis may prove to be a powerful tool. Modifying the setup to accommodate the needs of muscular fibres (correct

temperature and pharmacological additions) should further increase the usefulness of this tool. A flat *in vitro* preparation eliminates assumptions relating to intraluminal pressure/tension and volume/length of the tissue. The Laplace theory states that in a round hollow organ, the filling pressure is directly proportional to the tension generated in the wall. This has been translated for use in the distension of the gut. Thus, the increasing pressure of the filling balloon is assumed to be directly related to the tension being exerted onto the receptive field of a distension sensitive afferent fibre. A number of false assumptions are made in this situation. The first is that the gut is not a spherical hollow organ but a cylindrical one, thus distorting the potential for a linear relationship between pressure and wall tension. The LaPlace theory is also unable to accommodate for compliance of the tissue, which changes under various physiological conditions, further confounding the relationship between wall tension and luminal pressure. These problems have been accommodated to a certain extent by the use of barostats, measuring tension and volume concurrently. With a technique such as the one used in this thesis, one can bypass the necessary use of the LaPlace theory and apply tension directly to the wall of the tissue. In addition, changes in length are more easily applied. Thus, the graded tension (already confirmed in the ferret oesophagus (Page & Blackshaw, 1998)) and length stimuli give advantages over *in vivo* preparations. Thus, despite the initial negative results seen in this study, the data from using the oscillating stimulus has begun to define the adequate stimulus for stretch-sensitive afferent fibres described in this thesis.

Chapter 6. Colonic Afferent fibres with Multiple Receptive Fields

6.1 Abstract

1. Colonic afferents with multiple receptive fields were investigated for response characteristics to mechanical stimuli. Chemosensitivity of these fibres was also investigated.
2. 4 afferent fibres had multiple receptive fields. 2 fibres each had two punctate receptive fields and 2 each had three punctate receptive fields.
3. All fibres showed mechanosensitivity consistent with having endings in the serosa. Mechanosensitivity between receptive fields was consistent for each fibre investigated.
4. Hexamethonium (3μM) applied to the superfusate of two fibres had no effect, indicating that these fibres are primary afferent fibres with no cell body in the gut wall.
5. Chemosensitivity was investigated in two fibres. Chemosensitivity between receptive fields was not consistent for each fibre investigated.

6.2 Introduction

Gastrointestinal afferent fibres have been reported to have only one punctate receptive field (Cervero, 1994; Grundy & Scratcherd, 1989). The exception are afferent fibres from viscera that have been reported to have up to seven punctate receptive fields (Morrison, 1973). These multiple receptive field fibres are associated with divisions of the blood vessels supplying the viscera (Morrison, 1973). They have been described in the hypogastric (Floyd *et al.*, 1976) and splanchnic nerve of the cat (Morrison, 1973) and the splanchnic nerve of the dog (Floyd & Morrison, 1974). Interestingly, work by the same group showed that similar fibres in the cat pelvic nerves never had more than two punctate receptive fields when recording from the colon or the bladder (Floyd & Lawrenson, 1979).

Gastrointestinal afferent fibres have not been described with multiple receptive fields apart from those described on the mesentery and serosa. This part of the

project sought to investigate the existence and the nature of fibres with multiple receptive fields in rat colon.

6.3 Methods

Tissue preparation and fibre identification were carried out as specified in Chapter 2.

Two criteria were employed for confirming that differing receptive fields belonged to the same unit. These fibres were visually confirmed on an oscilloscope as single units with multiple receptive fields by an experienced independent observer unaware that more than one receptive field was being manipulated. Subsequently, off-line analysis was performed using Spike2. Spike templates were generated and the afferent fibre only accepted as having multiple receptive fields if only one template was produced for all data combined from all receptive fields. Slightly different protocols were carried out for each experiment in this study as they were given the protocol being used in an experimental series on the day of encounter.

Rat 119. After identifying the fibre and locating the receptive fields, NaCl 308mM was applied to each receptive field in sequence for 2min. The stimulus to the receptive field furthest oral was applied first, followed 3min after washout by the receptive field furthest anally. Hexamethonium 3μM was then added to the superfusates of both chambers and the tissue was superfused for a further 10 minutes to ascertain whether the fibre being recorded from was a second order neuron with its cell body in the intestinal wall. NaCl 308mM was then applied to both receptive fields - as previously described - in the presence of hexamethonium 3μM. The normal superfusate was then replaced and the tissue was superfused for a further 10 minutes. NaCl 308mM was then applied to both receptive fields as previously described. Subsequently, capsaicin 100μM was applied to both receptive fields.

Rat 135. After identifying the fibre and locating the receptive fields, hexamethonium 3μM was added to the superfusates of both chambers and the tissue was superfused for 10 minutes to test effect on spontaneous activity.

Rat 176. After identifying the fibre and locating the receptive fields, NaCl 308mM was applied at each receptive field, separated by a time of approximately 3min. All chemical stimuli were applied systematically from the most oral receptive field to the most distal receptive field. Chemical stimuli were applied in this order; noradrenaline 1μM, 5-hydroxytryptamine 100μM, bradykinin 1μM, capsaicin 100μM and finally, NaCl 308mM was applied again.

Rat 196. After identifying the fibre and locating the receptive fields, NaCl 308mM was applied to the two most proximal receptive fields simultaneously. This stimulus was repeated, along with all mechanical stimuli 50 min later. The tissue was turned over, exposing the serosal surface but still maintaining nerve recordings and the precise receptive fields were located on the serosal surface under the dissecting microscope.

6.4 Results

Four fibres with multiple receptive fields will be described. Many such fibres were encountered during the project but were discarded and not investigated. There were two reasons for this. Firstly, because there is not a depth of literature supporting gastrointestinal afferent fibres with multiple receptive fields it was assumed at the beginning of the project that two receptive fields meant two fibres with identical waveforms. Secondly, after being convinced that multiple receptive fields were a true phenomenon it was then necessary to discard any fibres about which there was the remotest doubt of interference from multiple afferent fibres. The criteria used for inclusion of these fibres in this study have been described.

Four afferent fibres were investigated, two fibres had three receptive fields and two fibres had two receptive fields. The receptive fields were punctate and were not grouped, rather each fibre showed individual patterns of distribution. In two of four

of these fibres, the receptive fields lay directly above the nerve and blood vessel bundle that lies directly beneath the surface of this preparation. In the other two studies, one had a receptive field directly over the bundle and another away from the bundle, more central on the tissue and the other had 2/3 three receptive fields away from the bundle. One fibre had two receptive fields above and one below the main insertion of the neurovascular bundle into the colon indicating that processes can travel in both oral and anal direction. All fibres showed characteristics of serosal fibre responses to mechanical stimuli. Despite the consistency of mechanosensitivity observed between receptive fields, chemosensitivity was inconsistent between different receptive fields in the same afferent fibre. Each fibre is described in detail below.

In rat 119, the fibre had two punctate receptive fields. Both responded to probing, to Von Frey hairs down to 200mg, but not to stretch. They were 16mm apart and both receptive fields lay adjacent to the nerve bundle lying underneath the tissue (Fig 6-1). There was no apparent difference between the shape, size or mechanical sensitivity between the two receptive fields. The fibre responded to NaCl 308mM at both receptive fields (Fig 6-2) (A - latency 2s, mean discharge rate 2.40spikes s^{-1}; B - latency 2.9s, mean discharge rate 3.50spikes s^{-1}). This larger mean response is not reflected in the figure because the bin widths of 5s enhance the initial intensity of the response at the oral receptive field (A), whilst the second response (B) is less intense but lasts for a longer period of time. There was essentially no change in the response of this fibre at either receptive field in the presence of hexamethonium (A - latency 3.6s, mean discharge rate 2.32spikes s^{-1}; B - latency 13s, mean discharge rate 1.52 spikes s^{-1}). This was not confirmed with a nicotinic agonist. The recovery period showed no difference in the response of this fibre to NaCl 308mM at either receptive field (A - latency 5s, mean discharge rate 2.15 spikes s^{-1}; B - latency 6.8s, mean discharge rate 2.58 spikes s^{-1}). This fibre subsequently responded to capsaicin 100μM at both receptive fields (Fig 6-3) (A - latency 8s, mean discharge rate 1.97 spikes s^{-1}; B - latency 9.7s, mean discharge rate 1.81 spikes s^{-1}). The response at the second receptive field (B) demonstrated a bursting pattern not present in the response at the first receptive field.

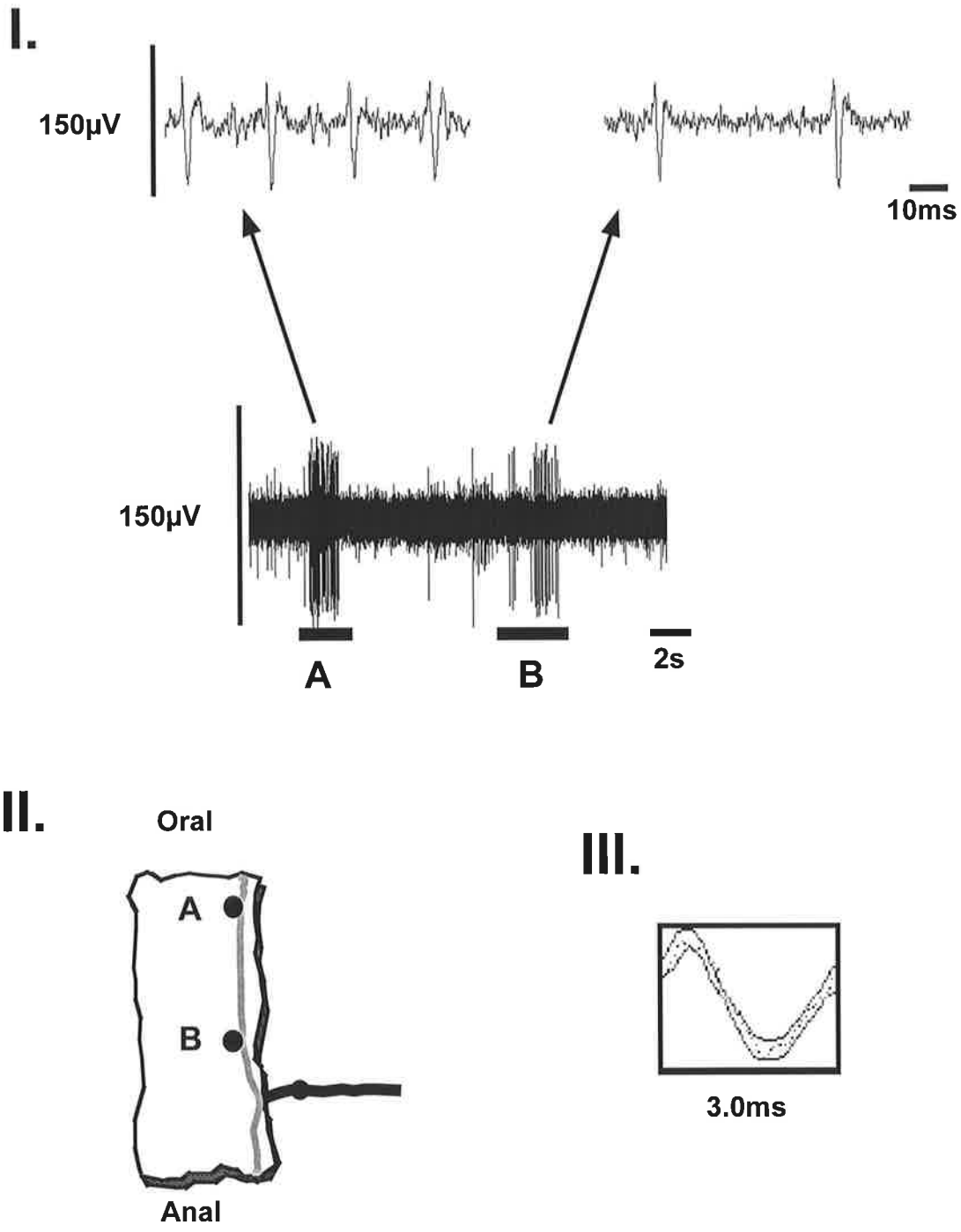

Figure 6-1. The response of one fibre to blunt probing at two receptive fields. I. The raw record of the response to blunt probing is shown at the bottom and expanded above to demonstrate the identical waveform at each receptive field. **II.** The site of the receptive fields. Two punctate receptive fields were identified. **III.** The template of the fibre.

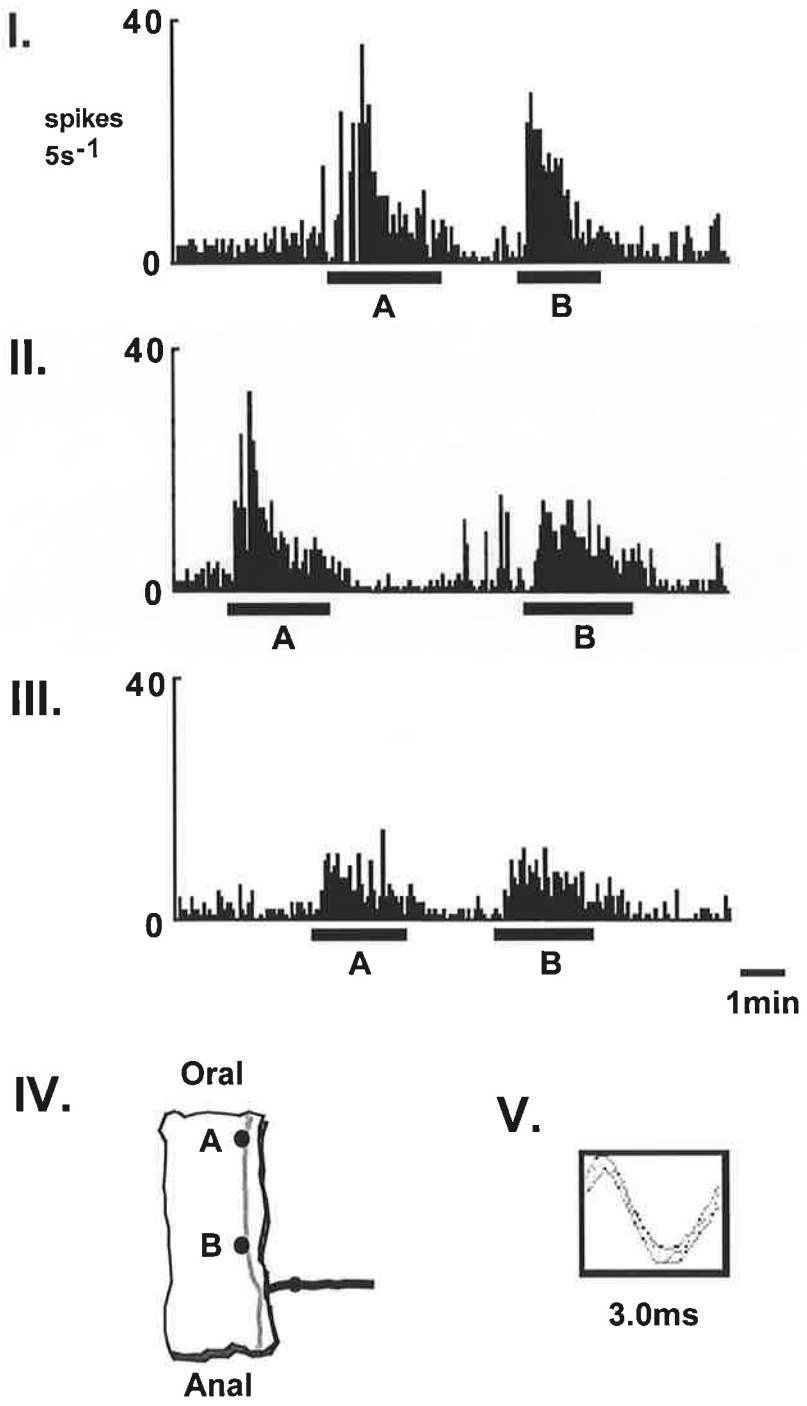

Figure 6-2. The response of one fibre to NaCl 308mM in the absence and presence of hexamethonium 3μM at two receptive fields. I.-III. NaCl 308mM was applied to the receptive field at A and then B. **I.** The control application. **II.** Application after Hexamethonium 3μM-supplemented Krebs was superfused for 12 minutes. Hexamethonium did not abolish the response of the fibre to NaCl 308mM at either receptive field. **III.** The recovery application in the presence of normal Krebs. **IV.** A schematic of the sites of the receptive fields. **V.** The wave template of the fibre.

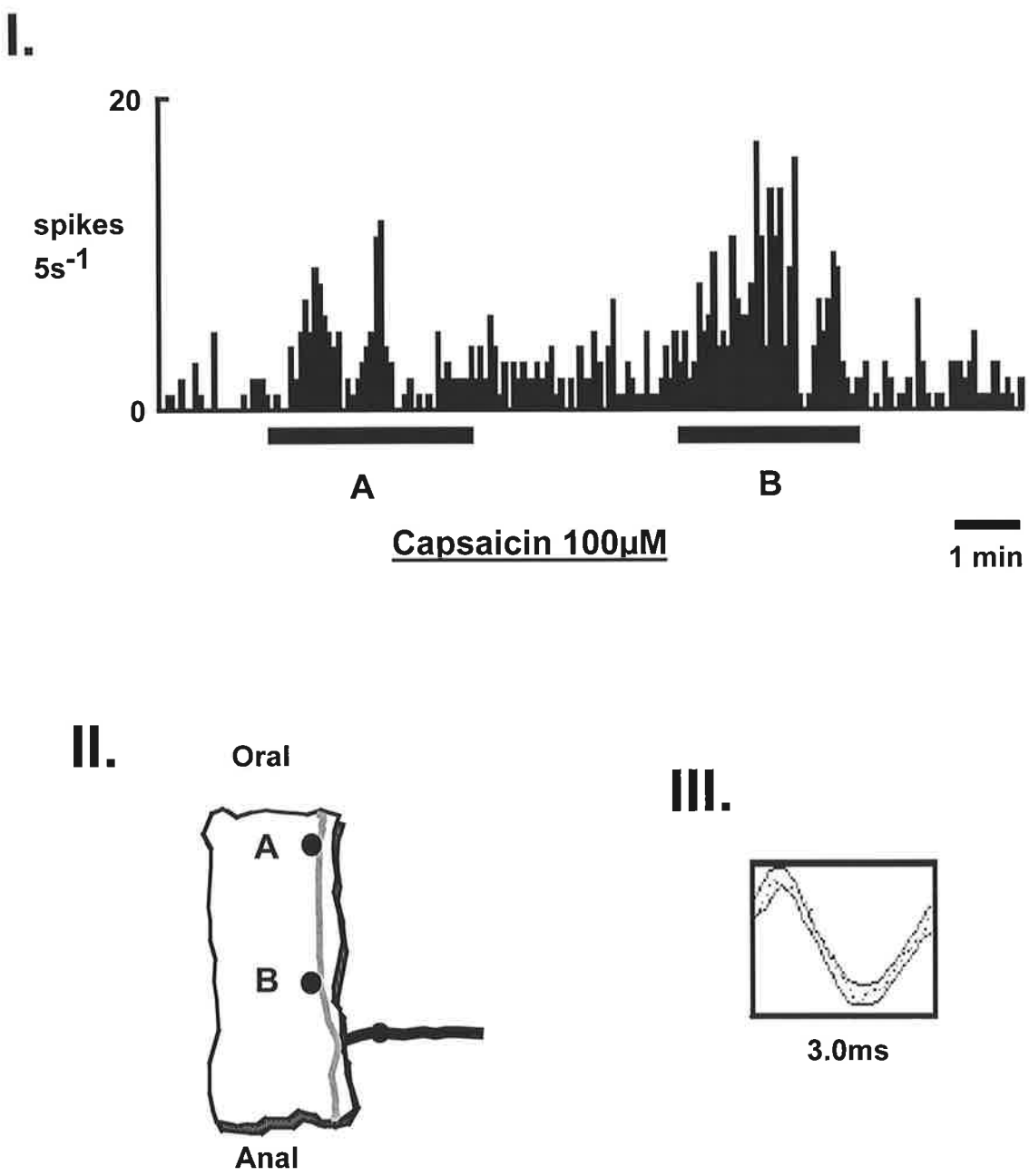

Figure 6-3. The response of one fibre to capsaicin 100µM applied at two reptive fields. I. The fibre responds to capsaicin at both receptive fields. The application at A elicits a smaller response to capsaicin 100µM than the application at B, despite the order of application. **II.** A schematic of the sites of the receptive fields. **III.** The wave template of the fibre.

Rat 135, also had two punctate receptive fields that were separated by 40mm. The proximal receptive field lay over the nerve bundle lying underneath the tissue. The distal receptive field was 2mm to the central side of the nerve bundle. Both receptive fields responded to probing but not to stretch or stroking. No conclusive statements can be made on the effects of hexamethonium 3μM being present in the superfusate. This concentration was not investigated for efficacy with a nicotinic agonist. This fibre is not illustrated.

Rat 176. This fibre had three punctate receptive fields. Receptive field A was large (3mm diameter) and lay adjacent to the nerve bundle lying underneath the tissue (Fig 6-4). This receptive field had a sensitive centre and a more diffuse outer ring. Receptive field B was 21mm distal to receptive field A and 3mm from the nerve bundle, closer to the centre of the preparation. Receptive field C was 6mm distal to receptive field B and was 4mm from the nerve bundle. All receptive fields were responsive to blunt probing but not to stretch or stroking. There was no response to NaCl 308mM at any of the three receptive fields (not illustrated), or noradrenaline 1μM (Fig 6-5). Receptive field A and B responded to 5-hydroxytryptamine 100μM (A – latency, 30s mean discharge rate 0.38 spikes s^{-1}; B - latency 36s, mean discharge rate 0.01 spikes s^{-1}) but receptive field C did not. Bradykinin 1μM caused a response at receptive field A but not B or C (A - latency 15s, mean discharge rate 0.1 spikes s^{-1}). There were responses to capsaicin 100μM at all three receptive fields (A - latency 15s, mean discharge rate 4.98 spikes s^{-1}; B - latency 20s, mean discharge rate 4.76 spikes s^{-1}; C - latency 13s, mean discharge rate 0.38 spikes s^{-1}). Reapplication of NaCl 308mM at all receptive fields did not elicit responses.

Rat 196. Three punctate receptive fields were identified for this fibre (Fig 6-6). The two most proximal receptive fields were situated over the nerve bundle lying underneath the tissue and were 4mm distant from each other. They were responsive to probing but not to stroking or stretch. The third receptive field was not mechanically sensitive at the onset of the protocol, but demonstrated sensitivity to probing after 50 mins when the stimuli were reapplied. This third receptive field

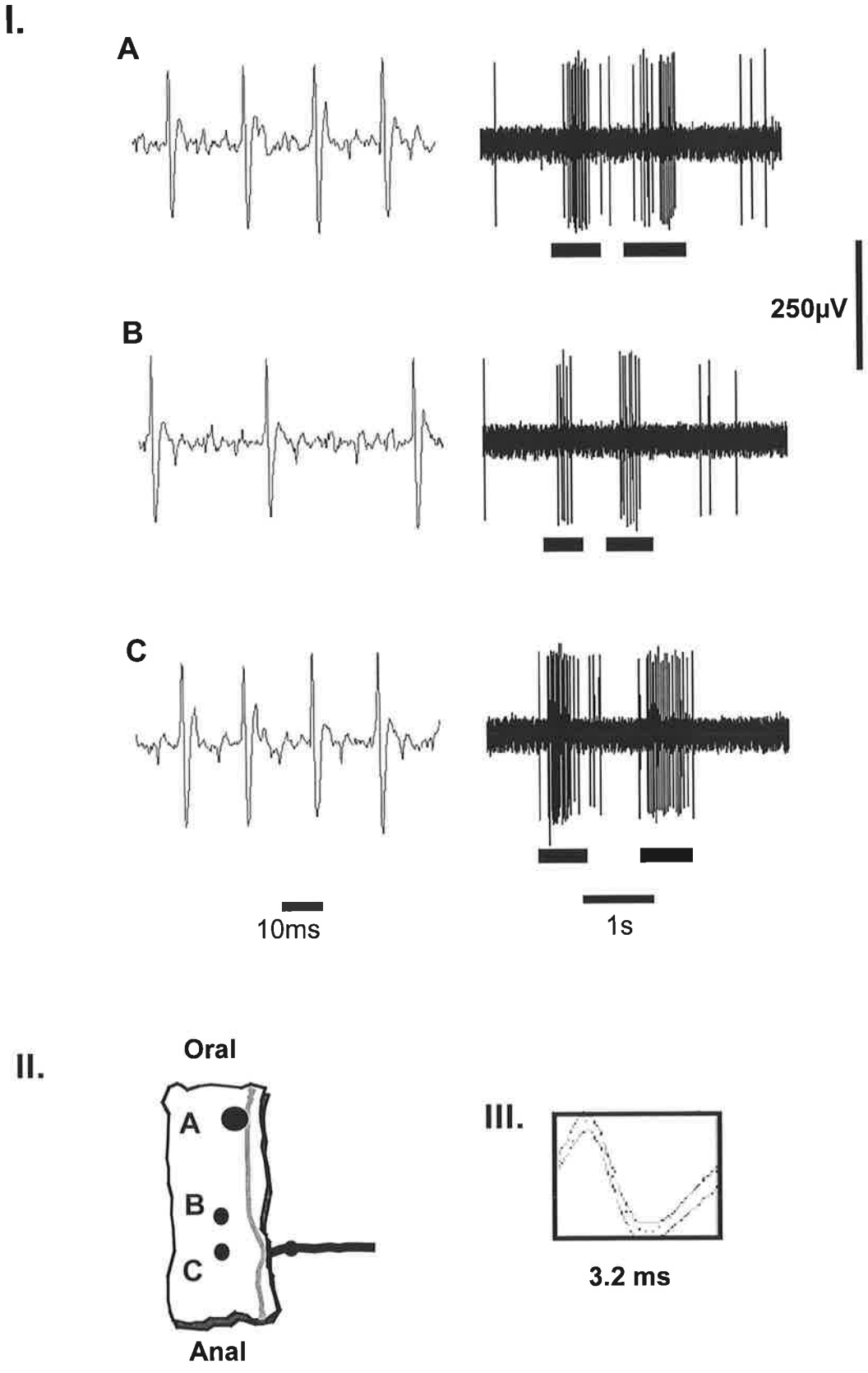

Figure 6-4. The response of one fibre to blunt probing at three punctate receptive fields. I. The raw record of the response to probing for each receptive field is shown in the right panel. A sample of the response to probing at each site was expanded and is shown in the left panel to demonstrate the identical waveforms at each receptive field. **II.** The sites of the receptive fields. Three punctate receptive fields were identified. **III.** The wave template of the fibre.

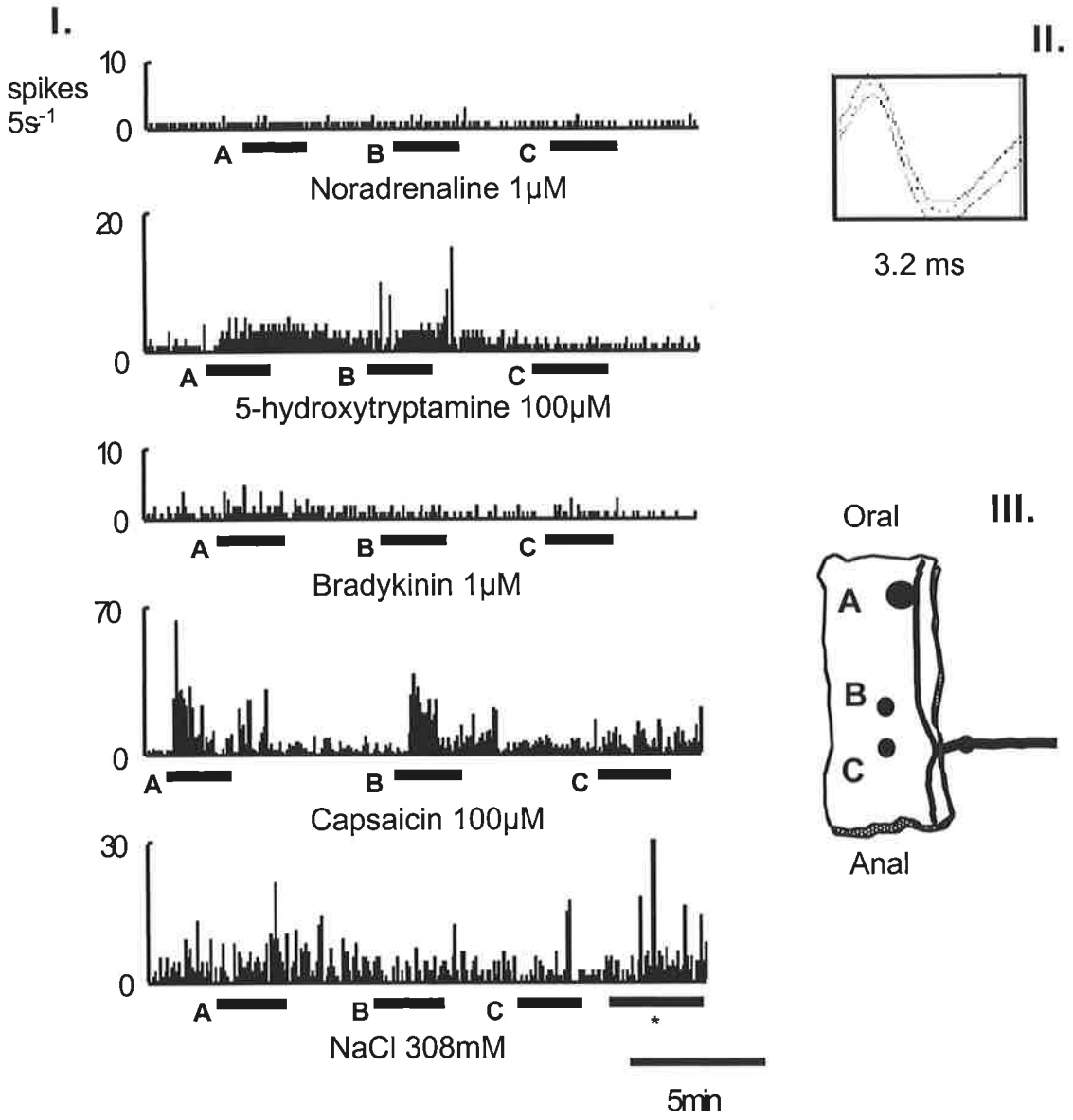

Figure 6-5. Response of a single fibre with three punctate receptive fields to chemical stimuli. I. Stimuli applied included noradrenaline, 5-HT, bradykinin, capsaicin and hypertonic NaCl - in that order. Responses were seen to all chemical stimuli except for noradrenaline. Receptive field A was more sensitive to chemical stimuli than B or C. * indicates scraping of the mucosa of the whole tissue, thus providing mechanical stimuli to the receptive fields. **II.** The wave template of the fibre investigated. **III.** A schematic of the relative site of the receptive fields of this fibre.

The order of application to receptive fields of chemical stimuli was consistently A, B and then C. Decreased chemosensitivity at B and C may be due to desensitisation to the chemical stimuli.

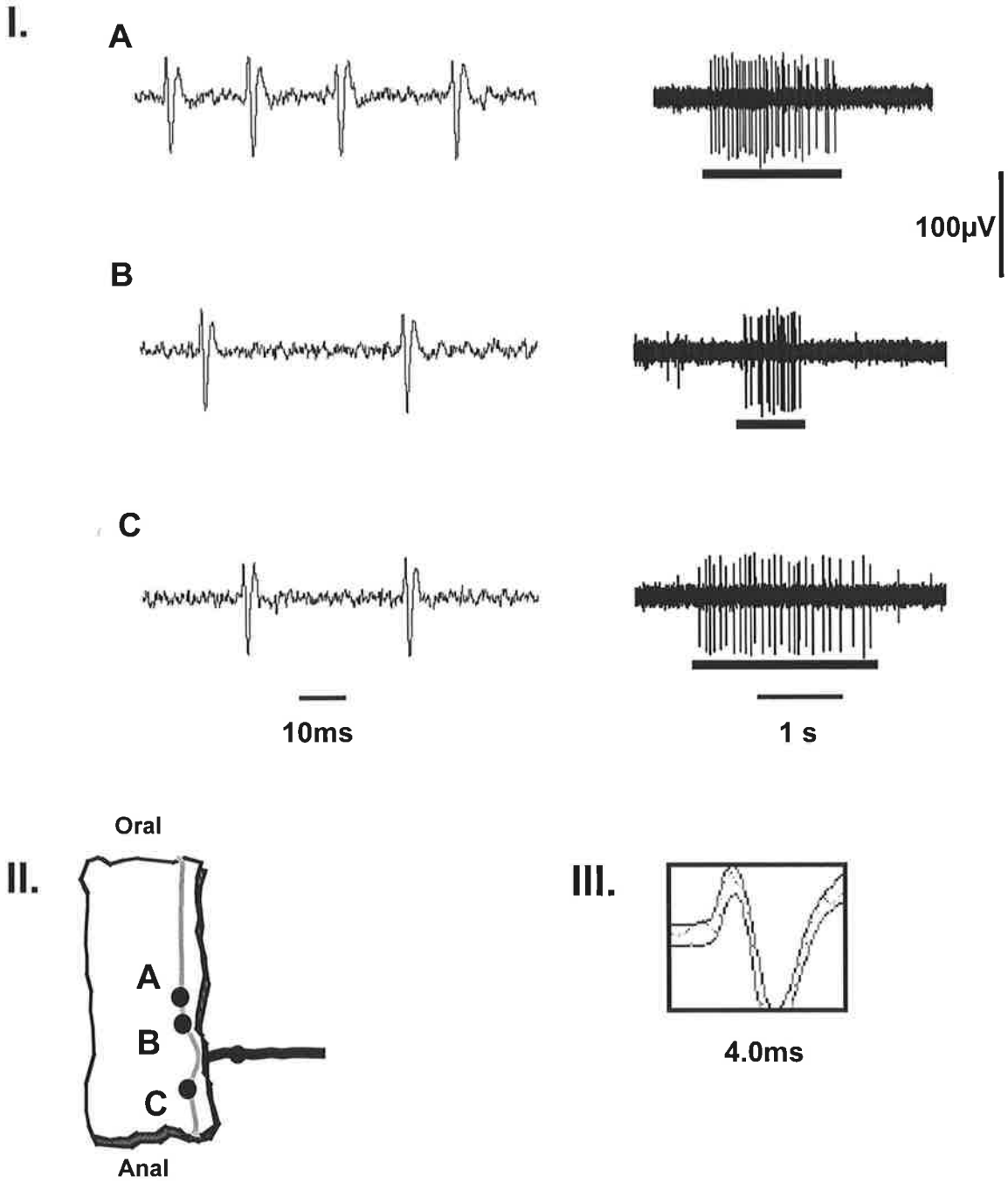

Figure 6-6. Response of a fibre with three receptive fields to probing of the mucosa at each receptive field. This fibre was spontaneously silent and all spikes are a result of probing the mucosal surface. **I.** The right panel shows the response to probiing at each receptive field. The left panel shows an expanded portion of the response to probing demonstrating the identical nature of the action potentials generated at each receptive field. **II.** A schematic demonstrating the position of each of the three punctate receptive fields identified as receptive field A, B and C. **III.** The wave template of the fibre.

lay below the insertion of the nerves into the colon, and 10mm below the other two receptive fields. This fibre did not respond to the application of NaCl 308mM.

On reflection of the tissue, the precise location of the receptive fields was determined. The most proximal receptive field (A) was located 1.5mm away from the main arterial trunk adjacent to a branch artery on the serosal surface. It responded to light pinching with a pair of forceps and to a 10mg Von Frey hair. Probing or Von Frey hairs heavier than 10mg were not effective and initiated a silent period after the stimulus. Only the outermost layer of connective tissue and serosa had to be pinched for a response to be elicited. The second receptive (B) field was not located. The most distal receptive field (C) was not adjacent to any blood vessels visible under the dissecting microscope. It responded readily to light probing and pinching. The response to probing was destroyed when the layer of connective tissue was removed over the site of the receptive field.

6.5 Discussion

6.5.1 Fibre Characteristics

All of the fibres with multiple receptive fields in this study had characteristics of being serosal fibres using the characterisation criteria discussed in chapter three. They responded to probing of the mucosal surface but not to stretch of the tissue or stroking of the mucosa. This was consistent between the different receptive fields of each fibre. The fibres with multiple receptive fields reported by the Morrison group (Floyd *et al.*, 1976; Floyd & Morrison, 1974; Morrison, 1973), were all on the serosal surface of the gastrointestinal tract, indeed on the serosal surface of all visceral organs described. Mucosal fibres and muscular fibres have not been reported to have more than one receptive field. Therefore, it is perhaps not surprising that the fibres described in this chapter fit the criteria for serosal but not mucosal or muscular receptive fields.

6.5.2 Site of Receptive Fields

The lumbar colonic nerve bundle lies directly under the colonic tissue and so includes the mesentery that attaches the bundle to the colon. Therefore, if the fibres do compare to those described by the Morrison group, a concentration of fibres innervating the mesentery, and its blood vessels would be expected in the area surrounding the site of this bundle. Not all fibres described in the current study showed this pattern of distribution. However, if the concentration of the fibres were primarily associated with blood vessels, then it would be expected that a proportion of the receptive fields would be associated with branches of arteries and so be away from the main branch. Therefore, the small numbers of receptive fields away from the main branch fit this paradigm. Interestingly, in the one study in which the tissue was reflected, one of the receptive fields was associated with a blood vessel and the other was not. The fact that one of the receptive fields was not associated with a blood vessel was interesting but the physiological reason for having one associated with a blood vessel and the other not remains puzzling. However, there remains the possibility that that particular receptive field was indeed associated with a blood vessel, but one that was not visible under the dissecting microscope. This error is more likely to occur *in vitro* where there is often little blood associated with smaller vessels than it is *in vivo* with a full perfusion system in place.

6.5.3 Primary Afferent fibres or Viscerofugal Fibres?

An alternative to primary afferent fibres having multiple receptive fields is the possibility that viscerofugal fibres projecting from the gut to the spinal cord act as second order neurones with multiple intrinsic primary afferent fibres projecting onto them. There has been some discussion as to whether viscerofugal fibres can act as primary sensory neurones or whether their role is that of second order integrative neurones (Sharkey *et al.*, 1998). If they are the latter, then they would be able to project information from a number of different receptive fields at any one time. Viscerofugal neurones were not believed to project from the distal colon as far as the spinal cord, but they do in the rectum where they are called rectospinal neurones (Doerffler Melly & Neuhuber, 1988). However, it is also now known that

viscerofugal fibres from the distal colon can project beyond the IMG (Luckensmeyer & Keast, 1995) into the intermesenteric bundle. Thus there is the potential for recordings from afferent fibres central to the IMG (as in the current study) to be made from a small population of second order neurones. With the small amount of data presented here this theory was not tested adequately. However, as viscerofugal neurones have their cell body in the gut, it would be expected that hexamethonium would prevent the cell from transmitting the signals projecting onto it from intrinsic primary afferent fibres or collaterals of external primary afferent fibres. This clearly was not the case in Rat 176 where hexamethonium had no effect on the response of the fibre to NaCl 308mM at either receptive field. It would have been beneficial to confirm this result either with a nicotinic agonist or a stronger concentration of hexamethonium. It is possible that a second order viscerofugal fibre would not necessarily have identical intrinsic afferent fibres projecting onto it. In the data presented however, all receptive fields presented as serosal fibres - not one had the characteristics of a mucosal or muscular fibre. If the role of the second order viscerofugal fibre was an integrative one, it would be possible, indeed probable that some stimuli would cause an inhibition in discharge as well as the excitatory response usually expected in primary afferent fibres. Yet the response characteristics of the fibres - apart from having multiple receptive fields - were in no way distinguishable from other serosal fibres discussed in this thesis. These arguments combined give evidence (though none conclusive) for the afferent fibres described here to be primary afferent fibres with multiple receptive fields rather than second order viscerofugal fibres.

6.5.4 Limitations of Technique

Using the present technique it is possible that not all the receptive fields were documented for these multiple receptive field fibres. Fibres with multiple receptive fields have been documented to have up to 7 punctate receptive fields (Morrison, 1973), yet the greatest number seen in this study were 3 discrete receptive fields. Apart from the obvious fact that only a portion of the distal colon was removed and therefore other receptive fields could have been cut off, it has been discussed elsewhere in this thesis, that there is probably a loss of receptive fields due to the

tissue being opened off-centre to the antimesenteric border. The possibility then arises that there are other fibres that have been described elsewhere in this thesis as having single receptive fields that in actual fact had more than one receptive field but the others had been severed in the preparation of the tissue. Considering that there are no distinct differences between the characteristics of the fibres with multiple receptive fields described in this chapter and other serosal fibres described in this thesis, it may be inappropriate to classify these separately from others in the future.

6.5.5 Neurone Processes

In the dog (Floyd & Morrison, 1974) receptive fields from the same fibre were up to 100mm distant from each other and in the cat (Morrison, 1973) up to 40mm. The furthest distance documented in this study was 40mm, which was most of the full length of the preparation. Therefore distances documented in the rat in this study are relatively much larger than those documented for the cat and dog. This suggests that a single fibre can have extremely long processes, capable of sensing environment along the full length of a piece of gut.

6.5.6 Physiological Role

The serosal fibres are generally thought to be nociceptive (Gebhart, 1999) although this is a premise I have challenged elsewhere in this thesis. Whatever the case, one wonders at the physiological advantage to having a single fibre with such distant receptive fields. Floyd and Morrison (1974) suggest that these fibres may have a role in the diffuse sensations experienced in the viscera. If these fibres are primarily nociceptive then this study provides further basis for the observation that many painful sensations in the gut are difficult to localise.

It is unfortunate that so many of the multiple receptive field fibres encountered over the full course of this study were discarded and not studied. In the first instance, these fibres were discarded in disbelief, on the premise that this phenomenon was just a documentation of two fibres with remarkably similar wave characterisitics. As this phenomenon became more convincing, only those fibres for which I could

find no reason to doubt were studied and any others that could be doubted for any reason were discarded. Consequently, the fibres described here, though a small group are that much more convincing because no reasonable error was allowed.

Chapter 7. A Role for Endogenous Prostaglandins in Colonic Afferent fibre Chemosensitivity

7.1 Abstract

1. Elsewhere prostaglandins have been found to potentiate or mediate responses of primary afferent fibres to adequate stimuli. The aim of this study was to investigate the role of prostaglandins in colonic afferent fibre activation.

2. 13 fibres (6 serosal, 7 mucosal) were tested with PGE_2 100μM. 1 mucosal fibre responded. 1 serosal fibre was tested with dose response curves of PGE_2, PGD_2, PGI_2 and $PGF_{2\alpha}$ on mucosal and serosal surface without effect.

3. 25 fibres (8 time-controls) were used for investigation of effect of endogenous prostaglandins in chemosensitivity.

4. Hypertonic NaCl (308mM) was added to a small chamber placed on the mucosal surface surrounding the receptive field of the fibre for 2 min. The response to hypertonic saline was investigated with indomethacin (3μM) in the superfusates, and without after a washout period of 12 minutes. Indomethacin was then replaced and the protocol was repeated.

5. 9 serosal and 6 mucosal fibres responded to NaCl 308mM (4 serosal and 4 mucosal time controls responded). 2 fibres did not respond to NaCl 308mM and did not develop sensitivity after indomethacin was removed.

6. Basal discharge, latency, time to peak, magnitude of peak and washout effect of responsive fibres were not affected by the removal of indomethacin.

7. Mean increase in discharge rate was greater ($p<0.01$) with indomethacin absent (3.02 ± 0.81 spikes s^{-1}) from the bath than in the presence of indomethacin 3μM (1.89 ± 0.59 spikes s^{-1}), but not in time controls. There were no differences between responses of serosal or mucosal afferent fibres.

8. Endogenous prostaglandins may therefore contribute to augmentation of chemosensitivity of colonic afferent fibres. Mucosal PGE_2 does not directly activate stretch-insensitive colonic afferent fibres.

7.2 Introduction

The role of prostaglandins in the gut has received considerable attention over the last few decades with the realisation that the levels of prostaglandins played an important role in the damage caused by both aspirin in the gastric mucosa and the diarrhoea caused by cholera toxin (Hawkey & Rampton, 1985). My interest in prostaglandins in the colon had two main motivations. Prostaglandins are released into the venous blood flow and into the lumen of the gut by events such as mechanical stimulation (Beubler & Juan, 1978). Also, there is an increased rate of prostaglandin release in the colon soon after the colon is removed from the whole animal. For example the rate of *in vitro* prostaglandin release in the rabbit colon peaked during the first hour after explantation (Nygård & Berglindh, 1989). Interestingly this was decreased significantly in the presence of indomethacin 660nM and abolished in the presence of indomethacin 10μM. Thus, in a preparation such as this *in vitro* rat preparation, endogenous release of prostaglandins would be expected to reach unphysiological and maybe even noxious levels. In order to decrease the effect on prostaglandin by trauma, a cyclo-oxygenase inhibitor such as indomethacin can be added to the superfusate of the tissue.

7.2.1 Aims

The aim of the next series of experiments was to investigate the role of prostaglandins in the response to a consistent chemical adequate stimulus. This involved investigating the effects of endogenous prostaglandins on the chemosensitivity of both serosal and mucosal afferent fibres and investigating primarily the role of PGE_2 by exogenous application to the site of the receptive field of the fibre under investigation.

7.3 Methods

A full description of the tissue preparation used in this chapter can be found in Chapter 2.

Thirteen fibres were tested with exogenous prostaglandins and 17 fibres (and 8 additional time-controls) were tested for endogenous effects on chemosensitivity.

After characterisation of mechanical sensitivity, NaCl 308mM was applied as a stimulus in the presence of indomethacin 3μM. After a washout period of at least 3 minutes, new reservoirs of Krebs solution, taken from the stock solution but containing no indomethacin replaced the normal reservoirs. Superfusion rate was kept constant at 15mls min^{-1} and the tissue equilibrated for a minimum of 12 min. In most fibres, mechanical stimuli were again applied followed shortly by a second application of NaCl 308mM. This was usually followed by one or more chemical stimuli, before the original reservoirs were replaced and the tissue again equilibrated for a minimum of 12 minutes. Again, in most fibres mechanical stimuli were applied followed shortly by a final application of NaCl 308mM. Time controls followed an identical protocol without the introduction of indomethacin-free Krebs.

In 3 experiments, after the indomethacin-free part of the protocol but before the recovery period, PGE_2 100μM was applied into the ring over the mucosal site of the receptive field and allowed to equilibrate for 10 min. At this time, the ring was removed and mechanical stimuli were quickly applied before a new application of NaCl 308mM. If this sub-protocol was undertaken, another 12min were allowed to elapse before a third and final application of NaCl 308mM during the indomethacin free period before the recovery part of the main protocol was undertaken. The data from the sub-protocol was not included in this study.

The latency, peak discharge rate (1s bins), time to peak, control discharge rate, mean discharge rate (1 min after latency) and discharge rate after washout (1 min after onset of washout) were investigated (Fig 7-1).

One fibre was tested with dose response curves to PGE_2, PGD_2, PGI_2 and $PGF_{2\alpha}$. In the above order, each prostaglandin was applied into the ring at 1μM, 10μM, 100μM and 1mM consecutively and for a duration of 2min for each concentration. 20min recovery period was given between the dose response curve of each prostaglandin. The tissue was then reflected exposing the serosal

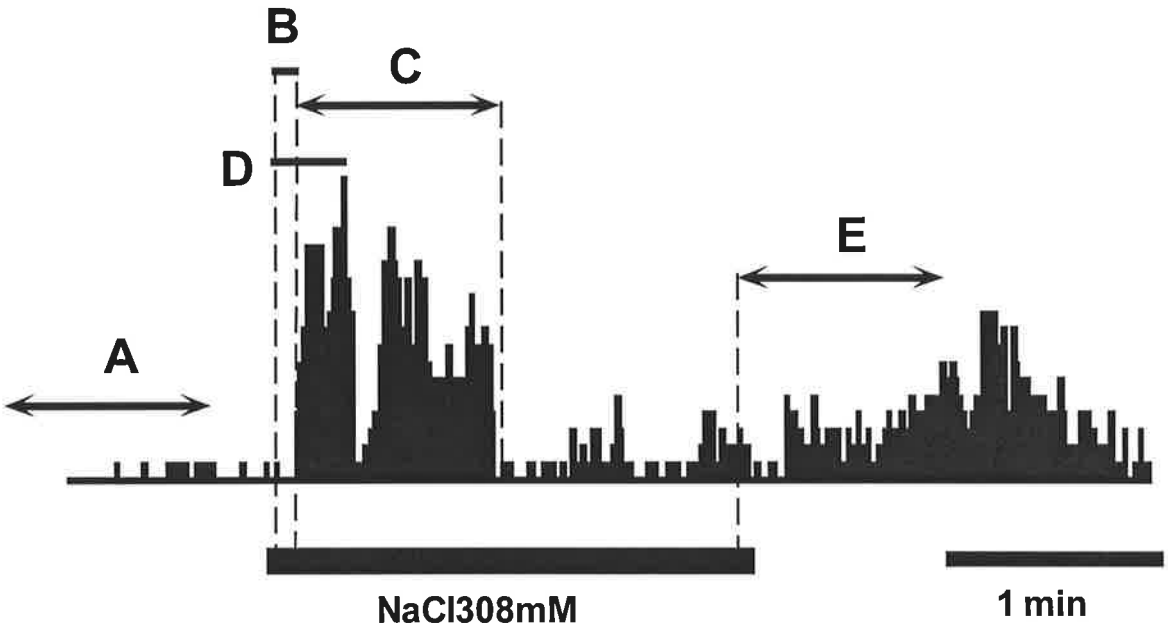

Figure 7-1. Group analysis for prostaglandin investigation. A. Control period was 1min prior to the application of NaCl 308mM. Mean discharge rate (spikes s^{-1}). **B.** Latency of the response (s). From application of NaCl 308mM to the point where mean discharge rate increases from control discharge rate by 50%. **C.** Mean discharge rate of response (spikes s^{-1}) was measured over a 1 min period after the point of latency determination. **D.** Peak discharge was the highest 1 s bin (#spikes), and time to peak (s) was the time from the application of NaCl 308mM to the peak. **E.** Washout response was the mean discharge rate (spikes s^{-1}) for 1min immediately after washout of NaCl 308mM.

receptive field. The ring was placed on the serosal surface and the protocol was repeated.

7.4 Results

13 fibres were tested with PGE_2 1μM. 6 of these were serosal, and 7 were mucosal (3 of these tested with PGE_2 with indomethacin absent). Only one fibre responded to the application of PGE_2 1μM and this was a mucosal fibre with indomethacin absent from the perfusate (Fig 7-2). In 3 unresponsive fibres (2 mucosal and 1 serosal) a reapplication of PGE_2 to the receptive site still yielded no response.

In two unresponsive serosal fibres (in the same study) application of PGE_2 1μM to the serosal surface also yielded no response. In this fibre, a dose response curve for PGE_2, PGD_2, PGF_2 and PGI_2 was carried out, both on the mucosal surface and by spritzing the serosal surface. Responses to these mediators were not observed.

15 of 17 fibres (and 8 of 8 time-controls) investigated with the indomethacin-free protocol responded to NaCl 308mM applied to the mucosal surface.

There was a great deal of variability in the magnitude and shape of responses to NaCl 308mM within and between fibres, but no difference either statistically or visually between grouped mucosal and serosal fibres responses.

The two fibres that did not respond to the initial application of NaCl 308mM developed no sensitivity to NaCl 308mM either in the presence or absence of indomethacin and have therefore been removed from the group data.

The response to NaCl 308mM of 15 fibres was investigated statistically. Six of these fibres were classed as mucosal and 9 fibres as serosal. Group response characteristics of serosal and mucosal fibres did not differ in any of the characteristics measured.

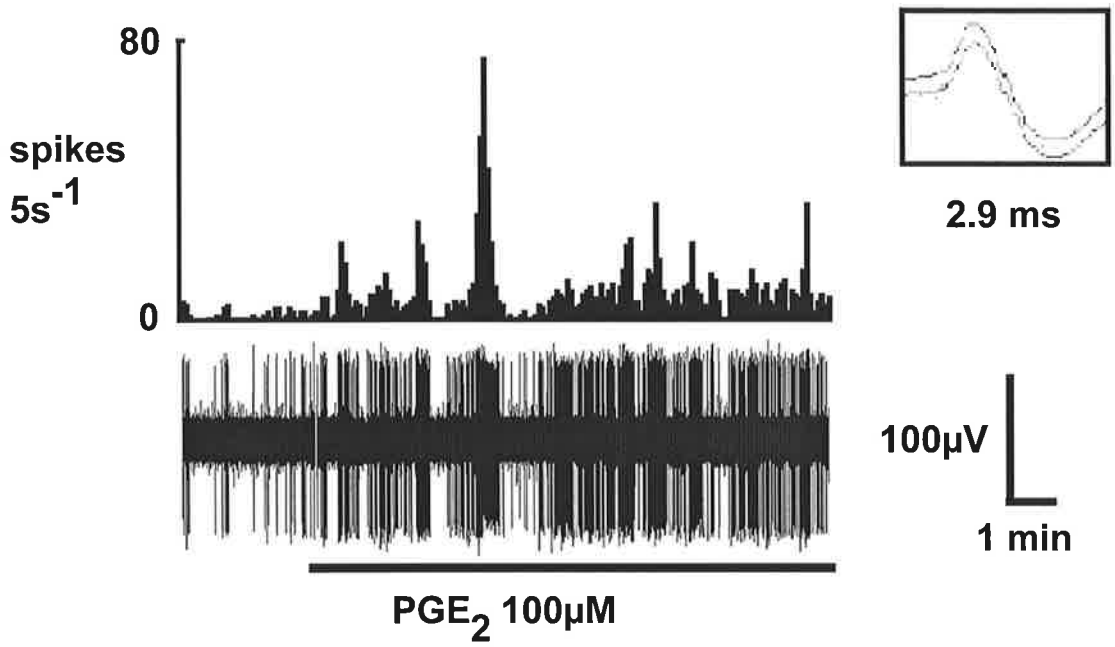

Figure 7-2. The response of a mucosal fibre to mucosal application of PGE_2 100µM. The discharge rate is shown above the raw tracing of the fibre. The fibre responded with periodic bursts of firing that was sustained for the full 10 minutes of application. It is not known how long the response persisted after the removal of the stimulus as mechanical stimulation followed by application of NaCl 308mM was immediately applied. This was the only response to PGE_2 observed in the fibres studied. The wave template for this fibre is shown in the top right corner.

The mean increase of firing in response to NaCl 308mM was greater ($p<0.01$) with indomethacin absent (3.02±0.81 spikes s^{-1}) from the bath than in the presence of indomethacin 3μM (1.89±0.59 spikes s^{-1}) (example in Fig 7-3). There was no recovery of the response (3.27±0.69 spikes s^{-1}) to control levels with the reintroduction of indomethacin into the bath. In time control fibres (1.56±0.49 spikes s^{-1}) there was no increase in the rate of firing (0.97±0.32 spikes s^{-1}) in response to NaCl 308mM. Nor was there any increase in the mean response on the third application of NaCl 308mM in time controls (0.52±0.16 spikes s^{-1}) (Fig 7-4).

There was no difference in the peak attained, the latency of response or the washout effect (observed in some fibres) in the presence or in the absence of indomethacin.

Sensitivity to Von Frey hairs in mucosal fibres was also investigated in the presence and absence of indomethacin. Quantification of these results proved impossible because of the variability of responses within fibres and difficulty in applying the stimuli to evoke consistent responses. Visual inspection of traces did not reveal any trends in mechanosensitivity in these fibres.

7.5 Discussion

7.5.1 Prostaglandins Sensitise Afferent fibres

This is the first time that prostaglandins have been shown to influence the response to luminal stimulation of afferent fibres supplying the gut. Nonetheless, the background for prostaglandins influencing the chemosensitivity of afferent fibres, is sound. PGE_2 enhances the response of testicular polymodal fibres to chemical applications of bradykinin and to heat- whilst causing no direct response in the fibre (Kumazawa *et al.*, 1995). PGE_1 also enhances the response of cutaneous afferent nerves to the application of bradykinin (Chahl & Iggo, 1977). Inflammation also increases the sensitivity of afferent fibres to mechanical and chemical stimuli (Handwerker & Reeh, 1991), and prostaglandins contribute to the

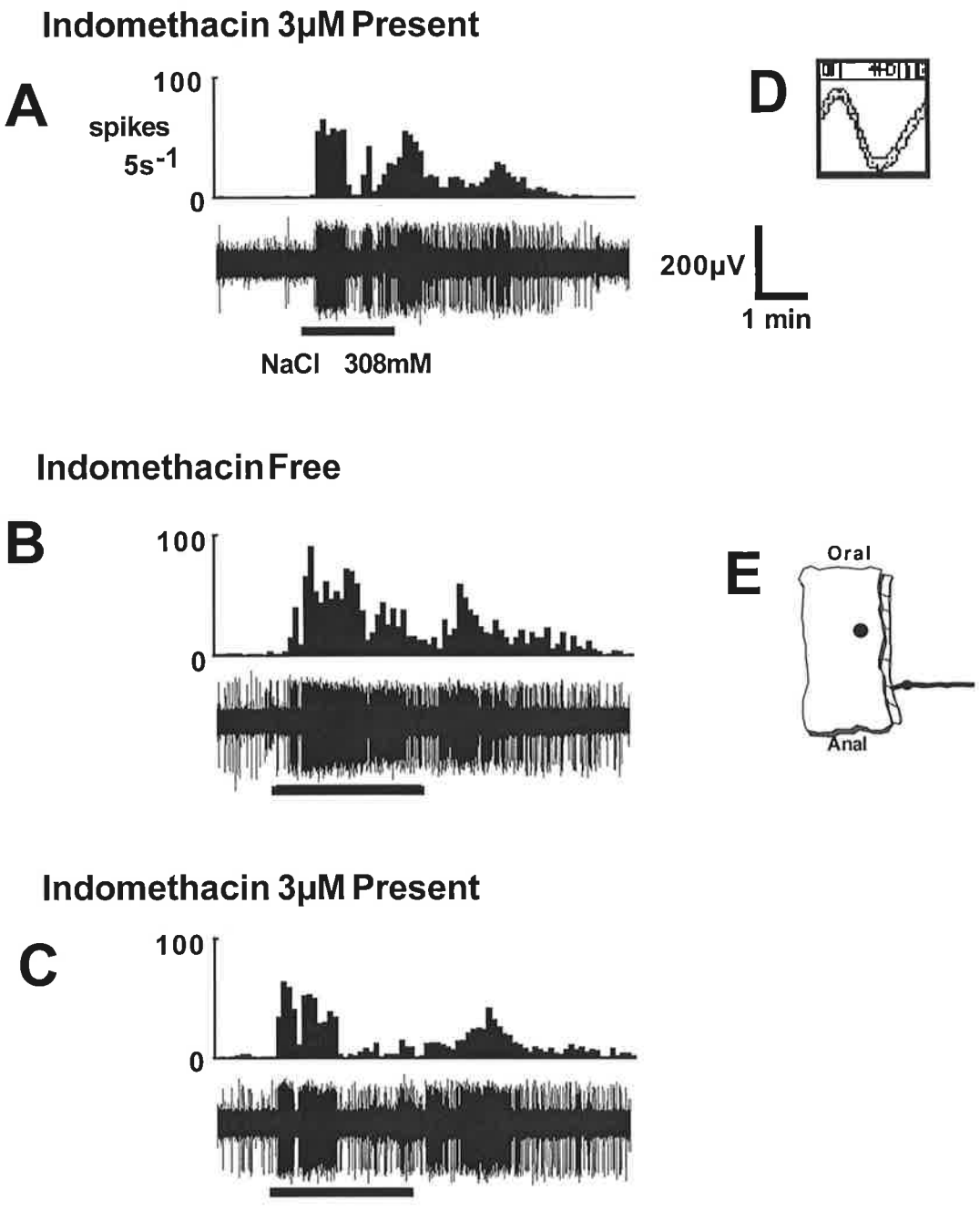

Figure 7-3. The effect of endogenous prostaglandins on the response of a mucosal fibre to NaCl 308mM. A. The discharge rate of the fibre is shown above the raw tracing. **B.** The response to NaCl 308mM was potentiated in the presence of endogenous prostaglandins. **C.** The response to NaCl 308 recovered with the reintroduction of indomethacin. This was not seen consistently. **D.** The wave template of the fibre investigated. **E.** A schematic of the precise location of the receptive field of the fibre.

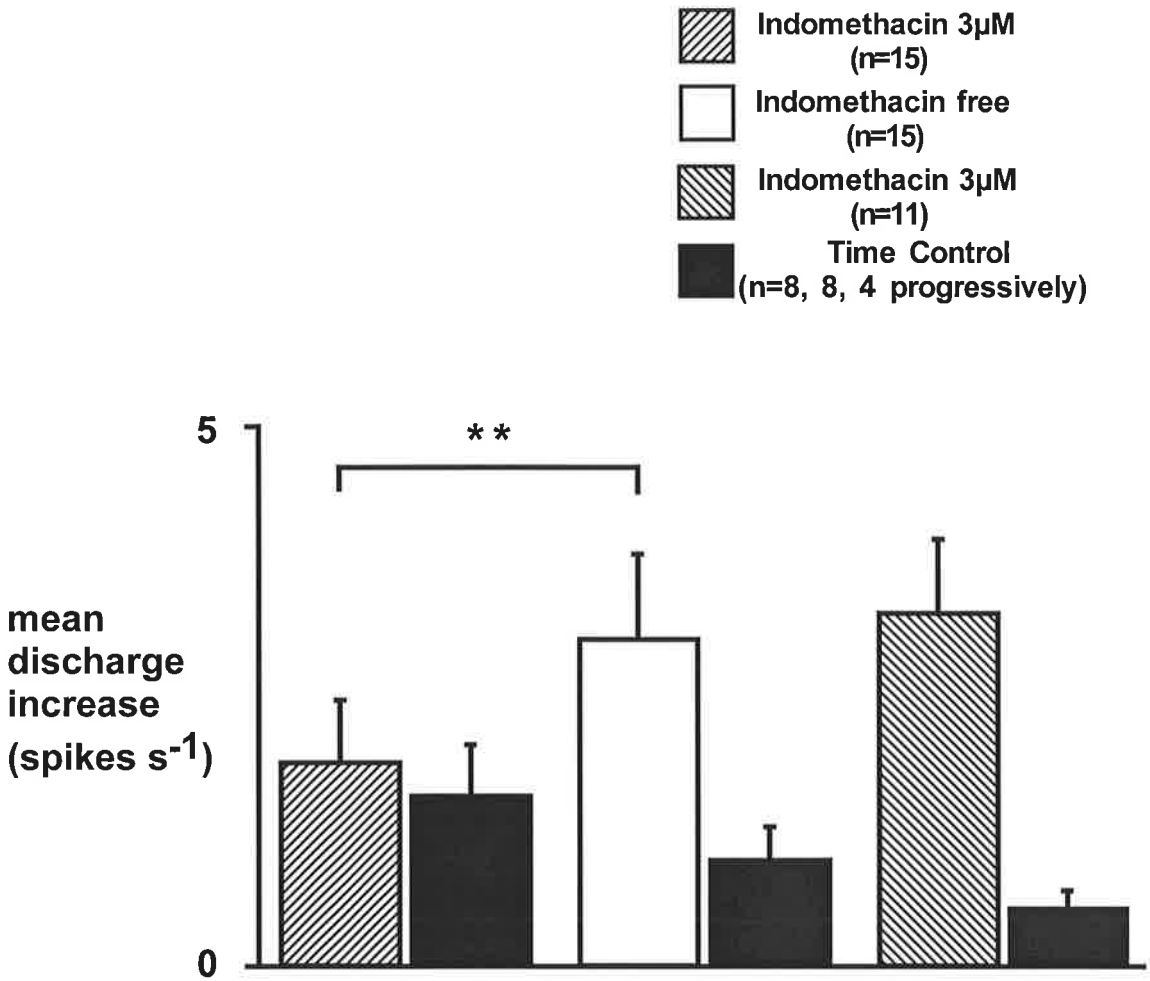

Figure 7-4. Group data for mean increase in discharge rate for the response to NaCl 308mM in the presence and absence of indomethacin 3µM in the superfusate. The increase of discharge rate from basal levels increases in the absence of indomethacin. This result is not recoverable with the reintroduction of indomethacin. Time controls show no change in the mean response to NaCl 308mM. Indomethacin 3µM (n=15), indomethacin free (n=15), reintroduction of indomethacin 3µM (n=11) and time control (n=8, 8, 4 progressively). Data presented as mean±SEM. Paired students t-test with no direction was used test between groups. ** $p<0.01$.

many agents involved in the inflammatory process (Heller *et al.*, 1993). Most applicable to the data at hand are the results of Maubach and Grundy (1999) who investigated the effect of prostaglandins on the sensitivity of serosal afferent fibres in the mesentery supplying the gut.

7.5.2 Specificity of Prostaglandin Action

The effect of endogenous prostaglandins on the response to mucosally applied NaCl 308mM was very specific. There was no change in basal discharge of the fibres, the latency of the response, but an effect on the magnitude of the response. This is consistent with observations in a study of the sensitising effect of prostaglandins on serosal fibres of the jejunum to bradykinin (Maubach & Grundy, 1999). The responses seen to NaCl 308mM were not dependent on the presence of endogenous prostaglandins, but permitting the endogenous production of prostaglandins by removal of indomethacin augmented the response. This suggests but does not confirm a sensitising effect of endogenous prostaglandins on luminal chemosensitivity in colonic afferent fibres. From studies in dorsal root ganglion cells it is becoming clear that prostaglandin sensitisation is mediated through the cAMP transduction cascade resulting in the modulation of a number of ion channels (Evans *et al.*, 1999; Lopshire & Nicol, 1998) thus increasing the excitability of the cell. The details of the combination of ion channels and how this is achieved remains to be elucidated. Complicating any interpretation of these studies with relevence to the current study is the fact that it is not known by what mechanism hypertonic saline exerts its effect on afferent fibres, be they mucosal or serosal or a mechanism common to both. However, given that the stimulus is eliciting the same responses in both groups of fibres, it is reasonable to assume that the prostaglandins are affecting a site or mechanism common to both. Both groups of fibres are likely to have unspecialised endings and have a homogenous sensitivity to hypertonic saline as discussed in Chapter 4.

7.5.3 Recovery of Control Responses

It was of interest that the response magnitude to NaCl 308mM was unrecoverable after the reintroduction of indomethacin but remained static, despite an extended

washout period after the treatment period. This was in contrast to time controls that demonstrated that the response to NaCl 308mM did not change significantly over the same timecourse. It is possible that prostaglandins synthesised in the absence of indomethacin were not washed out effectively due to the thickness of the whole organ preparation, or more likely, that there were permanent changes that occurred during the treatment period that were irreversible with the reintroduction of indomethacin into the bath. One possibility is that permanent damage had been done to the mucosa.

Prostaglandins also contribute to peristalsis in the colon (Bennett *et al.*, 1976). Therefore, indomethacin inhibits muscular activity in the colon. This part of the project used only mucosal and serosal fibres in the investigation, such that this influence on the muscular activity should not interfere with the results seen in afferent chemosensitivity. Indomethacin has fewer non-specific effects on the tissue than other cyclo-oxygenase inhibitors such as aspirin and can affect prostaglandin synthesis by penetration through the mucosa as well as by serosal application in the guinea pig colon *in vitro* (Bennett *et al.*, 1976).

Despite clear differences in the mechanosensitivity of mucosal and serosal fibres, there are no differences between the response seen to hypertonic saline seen in chemosensitive fibres. All the parameters mentioned in this chapter have been investigated to reveal any differences in the response to NaCl 308mM between serosal and mucosal fibres - even careful visual inspection of each response shows no difference in response patterns between the two groups. Therefore, despite the heterogeneity of mechanosensitivity between mucosal and serosal fibres, there appeared to be homogeneity with respect to this aspect of their chemosensitivity. This provided justification for the grouping together and classification only as stretch-insensitive fibres for the purpose of this investigation.

Hypertonic NaCl was the most consistent adequate stimulus for investigating chemosensitivity in both mucosal and serosal fibres. Therefore, it was the chemical stimulus of choice along with the appropriate mechanical stimuli whilst investigating the effects of indomethacin on the tissue. It was unfortunate, but not

unexpected that the variability within the mechanical stimuli, namely Von Frey hairs for the mucosal fibres, was so high that statistical analysis was impossible. Quantitative mechanical stimuli were also not identified for the serosal fibres and for this reason statistical analysis was also not a possibility.

7.5.4 Mechanism of Action of Sensitisation by Prostaglandins

It would be beneficial to determine whether endogenous prostaglandins also sensitise colonic afferent fibres to mechanical stimuli and other chemical stimuli as that would aid in understanding the mechanism by which endogenous prostaglandins exert their effect in the current preparation as has been determined in the cutaneous system. Not only do prostaglandins sensitise non-intestinal afferent fibres to chemical stimuli, but they also have a role in sensitising cutaneous nociceptors to mechanical stimuli (Heller *et al.*, 1993). But prostaglandins do not work alone in this effect. They interact with other substances such as histamine, 5-HT, bradykinin and substance P to cause sensitisation and ultimately hyperalgesia (Davies *et al.*, 1984). Indeed, these authors suggest that the main role of prostaglandins in nociceptive afferent fibres is to sensitise them to these other mediators which are in themselves sensitising agents. This can be translated across to the gut where prostaglandins sensitise serosal afferent fibres to bradykinin (Maubach & Grundy, 1999). It is also noted that mast cells can release mediators such as histamine and 5-HT in response to mechanical trauma (Davies *et al.*, 1984). Thus if hypertonic saline causes release of secondary mediators such as these, the main effect of prostaglandins may be to interact with them. Most prostaglandins are primarily released into the subepithelial space of the gut (Keenan & Rangachari, 1989), a prime position to affect mucosal pathways.

7.5.5 Interactions of Prostaglandins

Prostaglandins do not interact only with other algesic and inflammatory mediators, but also with each other. Indeed, the levels of eicosanoids within the gut are dependent in part upon each other (Keenan & Rangachari, 1989). In the distal colon, PGD_2 and $PGF_{2\alpha}$ account for a large portion of all eicosanoids, second only

to PGE_2 (Craven *et al.*, 1983). It is not yet known what role PGD_2 or $PGF_{2\alpha}$ have in sensitising afferent fibres, but it has been shown that PGE_2 and PGD_2 have contrasting effects on ion flux in the canine proximal colon (Keenan & Rangachari, 1991). Therefore, given that it is yet to be determined whether PGE_2 is the primary mediator for potentiating chemosensitivity in the current system, there may also be a role for PGD_2 or $PGF_{2\alpha}$, although these may not necessarily be identical to that of PGE_2.

7.5.6 Summary

It is clear that endogenous prostaglandins have a role in augmenting the effect of hypertonic saline on mucosal and serosal afferent fibres. The mechanism of action is not yet clear, but then the mechanism of action of hypertonic saline itself is also not yet clear. Evidence in cutaneous nerves and also in serosal afferent fibres of the jejunum would suggest that the effect would be direct on the nerve fibres. However, due to the lack of effects on spontaneous discharge of fibres and (to a lesser extent) of exogenous application of PGE_2, it is unreasonable to assume that in this system until further studies are undertaken. Of particular interest is the potential for a role for other prostaglandins such as PGD_2 and $PGF_{2\alpha}$ in afferent fibre sensitisation.

Chapter 8. *In Vitro* Mouse Preparation

8.1 Abstract

1. A novel *in vitro* preparation was developed for recording from colonic afferent fibres in the mouse.
2. 5/19 experiments yielded afferent fibre recordings. Experimental success increased with time highlighting the developmental process.
3. 9 afferent fibres were identified. 8/9 responded to probing. Receptive fields were small and difficult to quantify. 2/7 responded to a 10mg Von Frey hair and one other was identified as being mucosal due to extreme sensitivity to lightly brushing the surface with larger objects than a Von Frey hair. 7/8 afferent fibres were also chemosensitive.
4. Alternative classification criteria were investigated.
5. This is the first *in vitro* preparation allowing colonic afferent fibre recordings in the mouse.

8.2 Introduction

Transgenic and knockout mice are used increasingly in all aspects of physiological research. As they become more accessible, greater opportunities for their use are arising. Recently, publications have been documenting the use of genetically manipulated mice that are directly relevant to the study of visceral sensation. BDNF (brain derived neurotrophic factor) has been shown to be a survival factor for slowly adapting mechanoreceptors in the skin (Carroll *et al.*, 1998). This may also have relevance to the mechanosensitive 'stretch' fibres of the gut. Mice no longer responded to a noxious peritoneal stimulus when lacking the prostacyclin receptor (Murata *et al.*, 1997). Tetrodotoxin-resistant afferent fibre fibres have a role in sensation mediated by C-fibres. The sodium channel involved in this (sensory-neuron-specific (SNS) sodium channel) had been removed demonstrating a role for these channels in the mediation of sensation of noxious thermal, mechanical and inflammatory stimuli (Akopian *et al.*, 1999). No doubt there will be many more models to follow.

In vitro preparations of mouse gut have not been used in the investigation of visceral sensation using single unit recordings. We wanted to develop a similar preparation to that of the *in vitro* rat colon already described in this thesis using the mouse colon so that genetically manipulated animals could be utilised in preparations of this nature.

8.3 Methods

The preparation of the tissue was identical to the dissection of the rat colon preparation as much as was practicably possible, differing only in size of the tissue used. Mice were sedated with CO_2 and killed by cervical dislocation. After a midline laparotomy allowing exteriorisation of abdominal viscera, the animal was transferred into ice cold, carbogenated Krebs' bicarbonate buffer for dissection. Composition (mM) of the Krebs' solution used during dissection was as follows; NaCl 117.9, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.3, $MgSO_4(H_2O)_7$ 1.2, $CaCl_2$ 2.5 and glucose 11.1.* 1-2 cm of distal colon lying oral to the rim of the pelvis was removed, along with the lumbar colonic nerves and the neurovascular bundle containing the inferior mesenteric ganglion (IMG) the intermesenteric nerve and the lumbar splanchnic nerves (according to the classification of (Baron *et al.*, 1988) in the rat, in the absence of literature available for classification in the mouse). The pelvic and hypogastric nerves were not included in the preparation.

The distal colon was opened longitudinally off-centre to the antimesenteric border in order to orientate lumbar colonic nerve insertions to lie along the edge of the opened preparation. The faecal pellets were removed. After this dissection approximately 0.5cm (rat 1cm) of colon lay below the insertion point most closely related to the IMG and 1.5 cm (rat 4cm) lay above this point. The lumbar colonic nerves distal to the level of the IMG were severed from the colon until a length of 0.5 cm of neurovascular bundle could be pulled through to the adjacent chamber. This is in contrast to the rat preparation in which all of the lumbar colonic nerve insertions were left intact. Connective tissue was dissected away from the neurovascular bundle. The neurovascular bundle was cut and tied at the level of insertion of the artery into the abdominal aorta. Blood vessels were not dissected

* Due to the involvement of indomethacin in chemosensitivity in the rat, indomethacin was not used in the mouse superfusate.

away from the nerve, but were left adjacent to the nerve to add support to the preparation.

A response to chemicals was defined as an increase in mean rate of discharge greater than 50% of the basal mean rate of discharge.

8.4 Results

8.4.1 Organ Bath

The organ bath designed for the mouse distal colon had the following modifications in comparison to the bath for the rat distal colon (Fig 8-1).

The superfusion rate through both chambers was 6mls min^{-1}. Indomethacin was not used to supplement the modified Krebs solution. Stretch could not be applied to the tissue without damaging it, so thread was not attached to the edges of the tissue.

The mouse preparation was more susceptible to ambient interference than the rat preparation. Electrical interference was particularly problematic, including noise generated by the operation of the peristaltic pump, a problem not encountered with the rat preparation. Signal strength was very small being of the order of 50μV in comparison to 150μV in the rat. Signal to noise ratio was generally poor. Background noise was variable and unpredictable.*

8.4.2 Fibres Recruited

Of 19 mice used, afferent fibres were recorded in 5. Technical difficulties beset the project, but after these were resolved the last three studies yielded active fibres.

Generally the receptive fields of these fibres were very small, certainly smaller than 1mm^2 and the application of the blunt probe used in the rat preparation (with a tip diameter 2mm) easily covered more than one receptive field simultaneously.

* These observations were true phenomenom and were not a result of altered perceptions due to amplifier gain changes. Contributions to increased noise and electrical interference could include the qualities of the different electrodes used, smaller size in the bath and electrodes resulting in less physical stability, different flow characteristics of fluid in the bath compared with the rat preparation and different qualities of the tissue used.

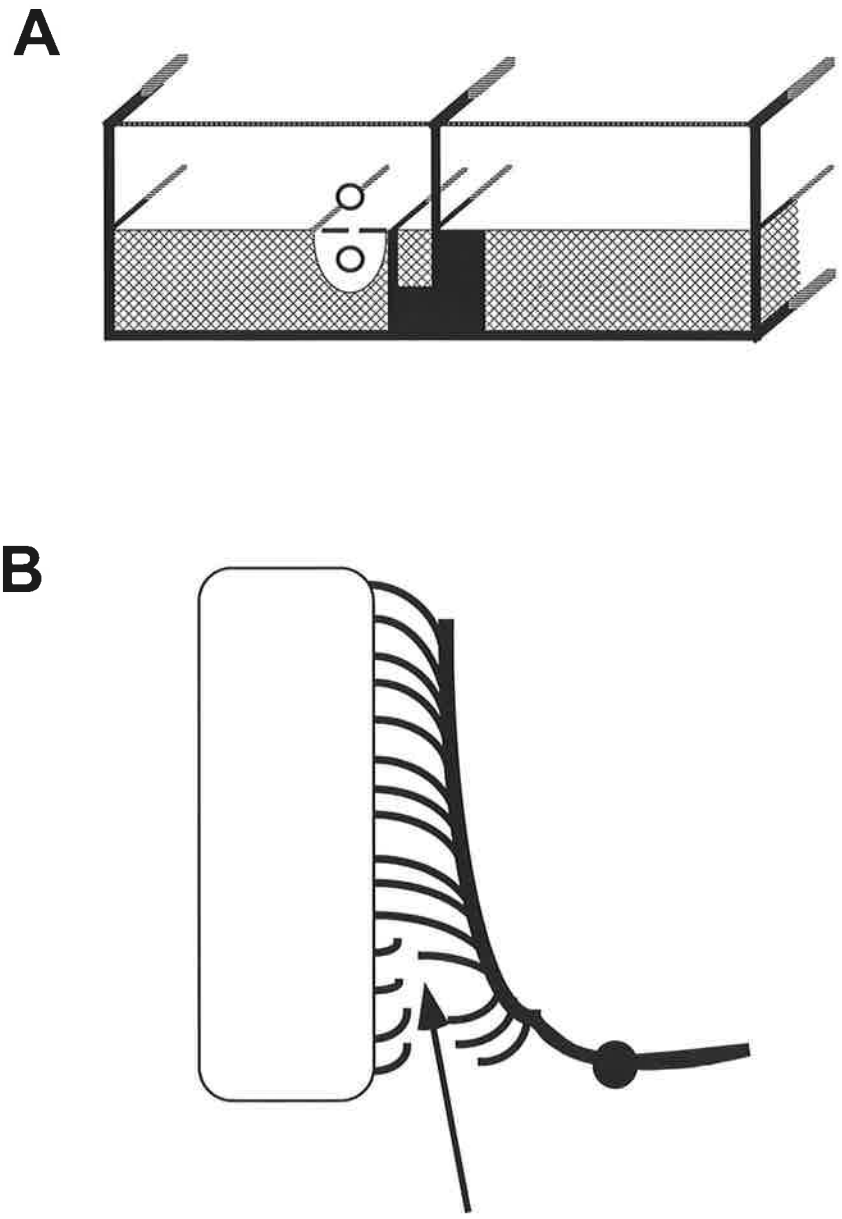

Figure 8-1. Organ bath and dissection used for the mouse. A. The organ bath was similar in most respects to the rat organ bath. The first major difference was the size. It was a third of the size of the rat bath. The second difference was the basement chamber. It was shallow and rounded to decrease deadspace and improve flow across the serosal surface. **B.** The lower lumbar colonic nerves were severed to create enough nerve tissue to pull through to the recording chamber. Thus, it was not possible to record from afferents originating distal to the major insertion point of the nerve bundle.

Group classifications were not possible due to small numbers. Table 8-1 summarises the chemosensitivity of these afferents. The observations of interest for each experiment will be individually documented in a descriptive manner.

Exp. 1

This fibre had very poor signal to noise ratio. It responded to probing (Fig 8-2).

Exp. 2

This strand of nerve responded to probing. The noise to signal ratio was poor. A single unit was unable to be identified off-line using Spike2 analysis. Visual and audio inspection determined that there was neural activity that was initiated in response to probing the mucosal surface, over the nerve trunk of the lumbar colonic nerves. This activity was characteristic of an undocumented observation in the rat studies. Some strands not yielding single units gave rise to spontaneous 'rumblings' that were increased when the nerve trunk itself was probed. The strand documented in this mouse study had no spontaneous activity but did respond with a characteristic rumble on probing down the length of the nerve strand (Fig 8-3). Activity was not elicited in response to the application of NaCl 308mM, PGE_2 100μM or capsaicin 100μM.

Exp. 7

A single unit was isolated in this study. The receptive field was located over the nerve bundle, oral to the insertion point of the neurovascular bundle. This fibre was silent and developed very little spontaneous activity over the course of the study. It responded reproducibly to Von Frey hairs (1000mg, 200mg, 50mg, 10mg) five times during the first hour of the study (Fig 8-4). Subsequently, the response was unrecoverable despite four more Von Frey hair applications. The amplitude of the action potential decreased over the course of the study.

Seven chemicals were applied at approximately 15 minute intervals in this order; NaCl 308mM, PGE_2 1mM, PGD_2 1mM, $PGF_{2\alpha}$ 1mM, bradykinin 1μM, undiluted ferret bile (gallbladder) and capsaicin 100μM (Fig 8-5). No stimulus was repeated.

	Probe	10mg Von Frey	NaCl 308mM	PGE_2 100µM	PGE_2 1µM	PGD_2 1µM	$PGF_{2\alpha}$ 1µM	Bradykinin 1µM	Bile	HCl 50mM	Capsaicin 100µM
Fibre 1	+										
Fibre 2	+		0	0							0
Fibre 7	+	+	+		+	+	+	+	+		+
Fibre 8	+	+	0		+						
Fibre 9-1	+	0	+		0	0	0	0	0	+	+
Fibre 9-2	+	0	0		0	0	0	0	0	+	+
Fibre 9-3	+	0	0		0	0	0	0	0	0	+
Fibre 9-4	0	0	+		0	0	+		+	+	0
Fibre 9-5	+	0	+		+	+	0	0	+	+	+

Table 8-1. Summary of responses of individual mouse colonic afferent fibres. A response to a stimulus or presence of spontaneous activity is indicated with **+**, no response or activity with **0**. A blank is left when a particular stimulus was not tested in a study. Mechanical sensitivity is shown as that present at the beginning of studies.

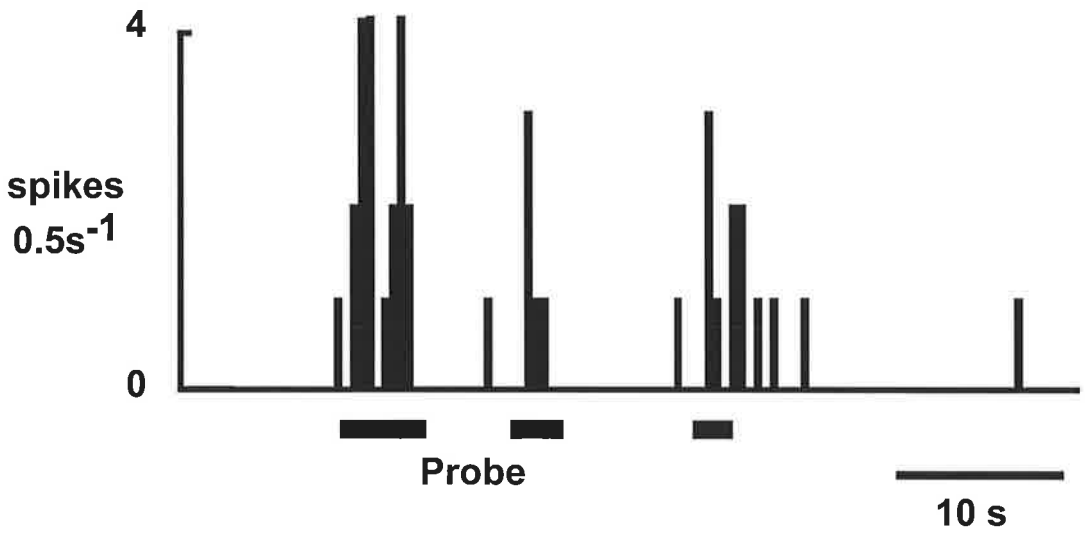

Figure 8-2. The response of the first mouse fibre to probing. Discharge rate of the fibre is shown. The fibre responded readily to probing.

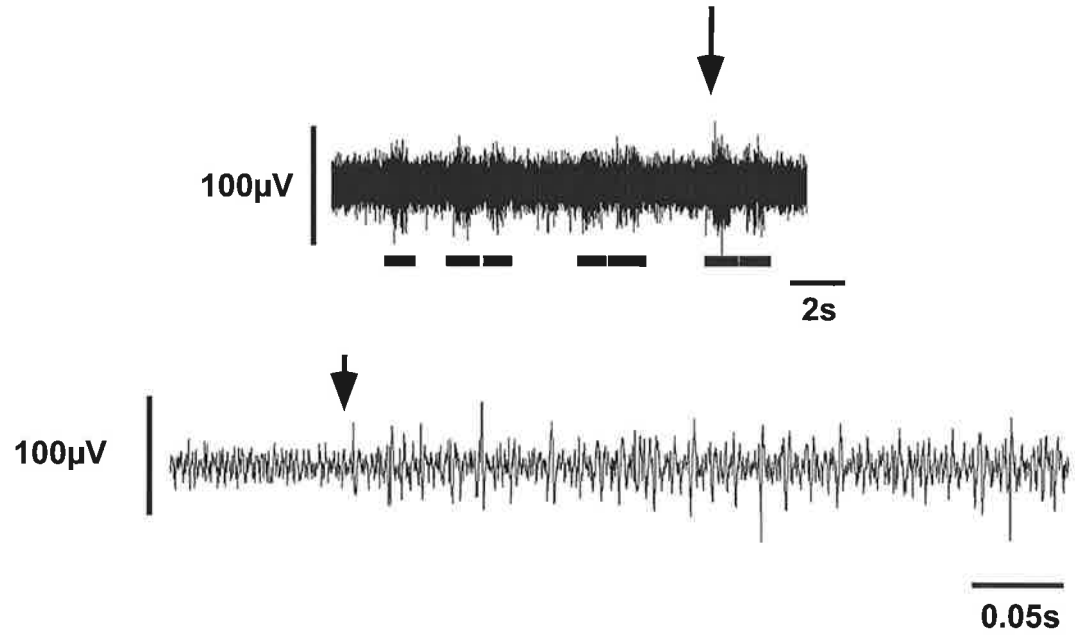

Figure 8-3. The response of the second mouse fibre to probing. The raw tracing of the response is shown in the top panel. The arrows indicate the same point in time, thus the lower panel demonstrates an expanded view of the response of this fibre to probing. The fibre responded readily to probing not with single unit activity but rather with a characterisitic 'rumble'.

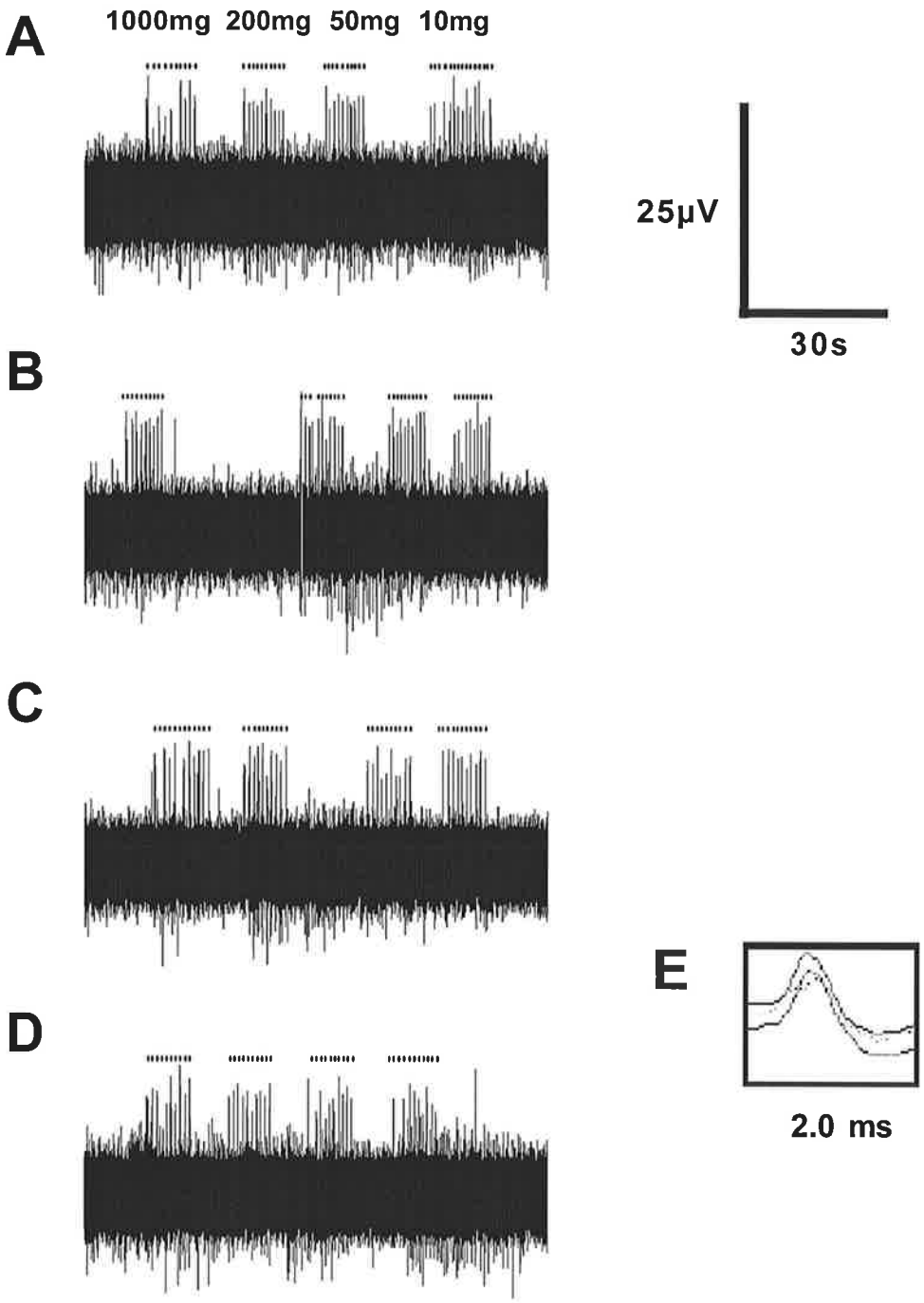

Figure 8-4. Response of a single unit to repeated application of Von Frey hairs. Each stroke with the Von Frey hair is indicated with a dot above the trace. Each hair was repeated at least ten times. The order of application is identical in every trace. **E.** Spike descrimination template. **A.** Not every stroke elicited a response from this fibre as can be seen clearly with 1000mg. This was usually due to human error in missing the small receptive field rather than true unresponsiveness. **B.** Von Frey hairs were applied 15min later after application of NaCl 308mM. **C.** Von Frey hairs were applied 13 min after B, and after an application of PGE_2 1mM. **D.** Von Frey hairs were applied 11 min after C and after an application of PGD_2 1mM. The response was beginning to deteriorate both in the consistency of being able to elicit a response with each stroke and amplitude of the fibre. Note particularly the variability in the visible amplitude of the fibre. Not shown; This fibre responded to 1000mg-10mg only once more, 60 min after the first Von Frey hair application and after the application of 5 different chemical stimuli. Despite 4 more attempts, the response to Von Frey hairs was not recoverable, even at 1000mg.

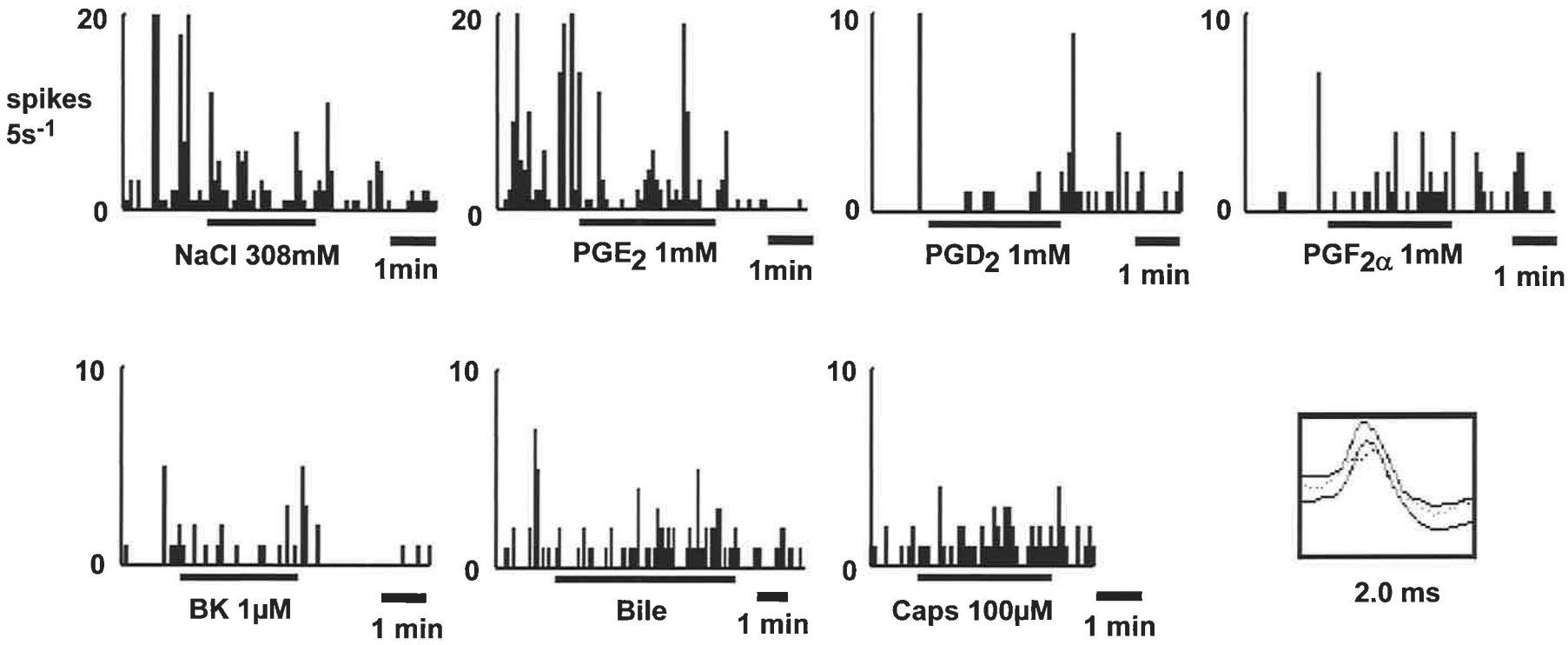

Figure 8-5. Response of a single unit to 7 chemical stimuli. Stimuli were applied consecutively with a break about 15 minutes between the application of each chemical. The wave template is shown. This is the same fibre that is shown with the repeated Von Frey hairs. In all but the Capsaicin trace there are distinct bursts of firing prior to the application of the stimulus caused by placing the ring over the receptive field. Note that this burst gets smaller as the experiment progresses. This is due to increasing expertise in placing the ring onto the tissue. This burst hides the fact that this was a silent fibre that developed very little spontaneous activity over the course of the study. This fibre responded to Von Frey hairs to a certain degree between each application of chemicals as defined in the other figure of this fibre. However, after the application of bile, this response to Von Frey hairs was lost completely, despite the fact that the fibre subsequently responded to capsaicin.

Inconsistent bursting responses were seen to all stimuli. In all but the capsaicin response there were distinct bursts of firing prior to the application of the stimulus caused by placing the ring over the receptive field. This burst became smaller as the experiment progressed, due to increasing expertise in placing the ring onto the tissue. This fibre responded to Von Frey hairs to a certain degree between each application of chemicals as defined in Fig 8-3. After the application of bile, the response to Von Frey hairs was lost and was not recoverable, despite the fact that the fibre subsequently responded to capsaicin.

Exp. 8

During the study it was thought that there were two identifiable fibres with discrete receptive fields. Off-line analysis however, revealed that although the fibre with the receptive field in the middle of the tissue was a single unit, the second fibre with the receptive field over the nerve trunk was in fact 'rumbling' in response to stimuli as described for expt 2. As a result of the variability within the response of this rumbling fibre, the single unit was unable to be discriminated confidently throughout the study period using Spike2. In consequence, results from this study should be treated with caution.

The 'rumbling' was elicited with a full range of Von Frey hairs, 1000mg-10mg. This was reproducible. A demonstration of this fibre is seen in Fig 8-6. Responses to the seven chemical applications cannot be quantified, but a qualitative description follows where appropriate.

The discrete fibre could be discriminated in the first part of the study. It responded to the repeated application of all Von Frey hairs, 1000mg-10mg. It did not respond to NaCl 308mM, PGD_2 1mM but did respond to PGE_2 1mM. Subsequent to this, discrimination was not clear. The fibre did not respond to $PGF_{2\alpha}$ 1mM or bradykinin 1µM. It appeared to respond strongly to HCl 50mM. The application of HCl 50mM bleached the mucosa. A small response was subsequently seen to capsaicin 100µM.

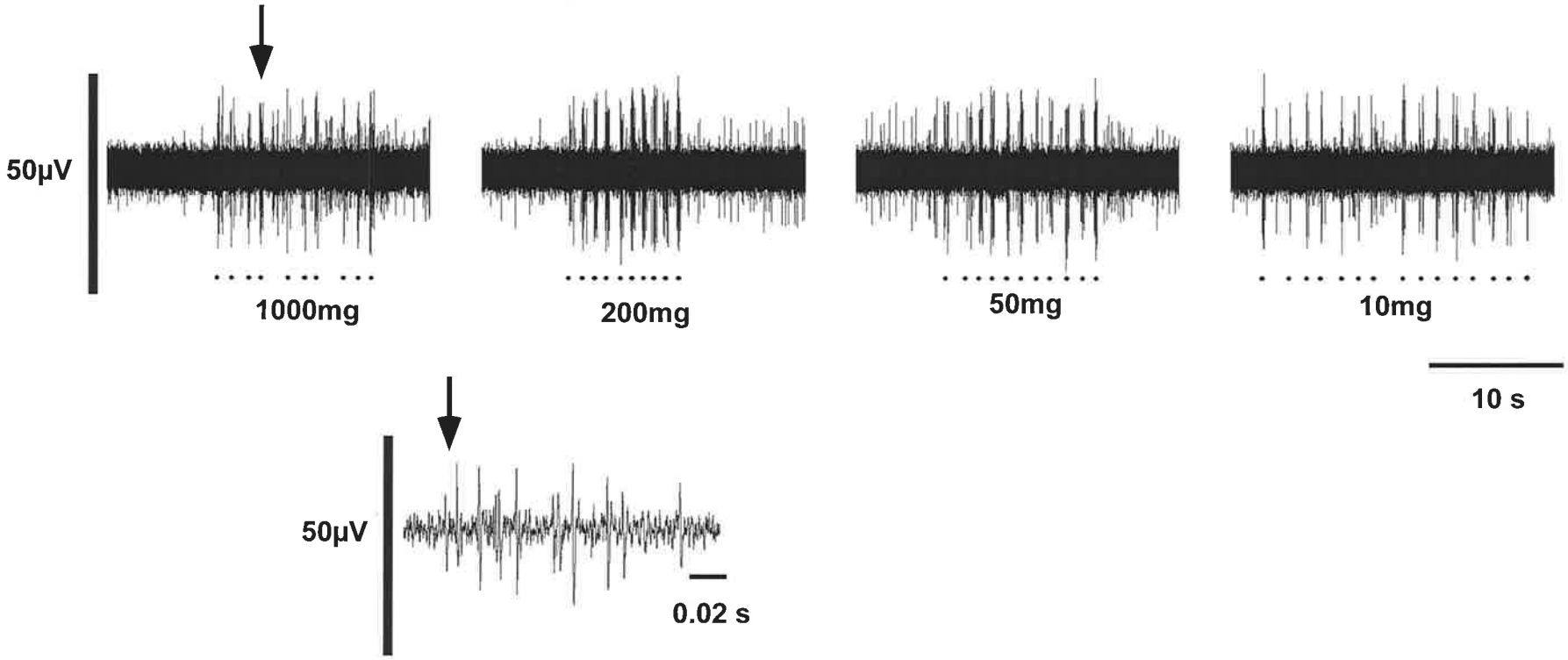

Figure 8-6. Application of Von Frey hairs to a discrete 'receptive field' situated over the nerve trunk. As can be seen from this trace, this recording was not from a single unit. A small portion of a burst in response to 1000mg has been expanded for accurate viewing. It can be appreciated from this expanded trace, it was not possible to discriminate any single units with accuracy using Spike 2, so responses to chemicals are not included. From previous experience with the rat, I make the assumption that I have one of those 'noisy' strands that seems to arise directly from the nerve trunk. Of particular interest in this preparation is the fact that this type of strand still responds at a very low threshold of 10mg. In the rat, this type of strand usually only responds to probing. This, along with other observations suggests to me that 10mg is a very heavy handed stimulus for this preparation and if possible we should try for at least a 1mg probe.

Exp. 9

Five fibres were successfully discriminated from this study. The times of these chemical stimuli from the beginning of the study are as follows; NaCl 31min, PGE_2 41min, PGD_2 49min, $PGF_{2\alpha}$ 58min, BK 69min, bile 88min, HCl 100min, 117min.

Fibre#5 responded to probing but not to Von Frey hairs. Responses were seen to the following chemical applications: NaCl 308mM, PGE_2 1mM, PGD_2 1mM, 50% bile, HCl 50mM and capsaicin 100µM (Fig 8-7). Of particular interest, above and beyond the responses to chemicals is the increasing basal discharge throughout the study from being spontaneously silent. This increase is a true increase of basal discharge not associated with any change in the shape or amplitude of the fibre.

Fibre #1 responded to probing. The receptive field was adjacent to fibre#5, the fibre of most interest. Spontaneous discharge did not change over the duration of the study (Fig 8-8). Responses were seen to HCl 50mM and capsaicin 100µM.

Fibre #2 responded to probing and stroking but not to Von Frey hairs. It also had an adjacent receptive field to fibre #5. This fibre responded to the application of NaCl with inhibition, a previously unseen response (Fig 8-9). Responses to chemicals are also seen to HCl 50mM and capsaicin 100µM.

Fibre #3 (of small amplitude) responds to probing but not Von Frey hairs or stroking. It also responds to the application of capsaicin 100µM (Fig 8-10).

The receptive field of fibre #4 was unidentified. However, the characteristic burst of firing before chemical stimuli suggests that the rim of the chemical chamber is stimulating the receptive field (Fig 8-11). This fibre responded to NaCl 308mM, $PGF_{2\alpha}$ 1mM, 50% bile and HCl 50mM.

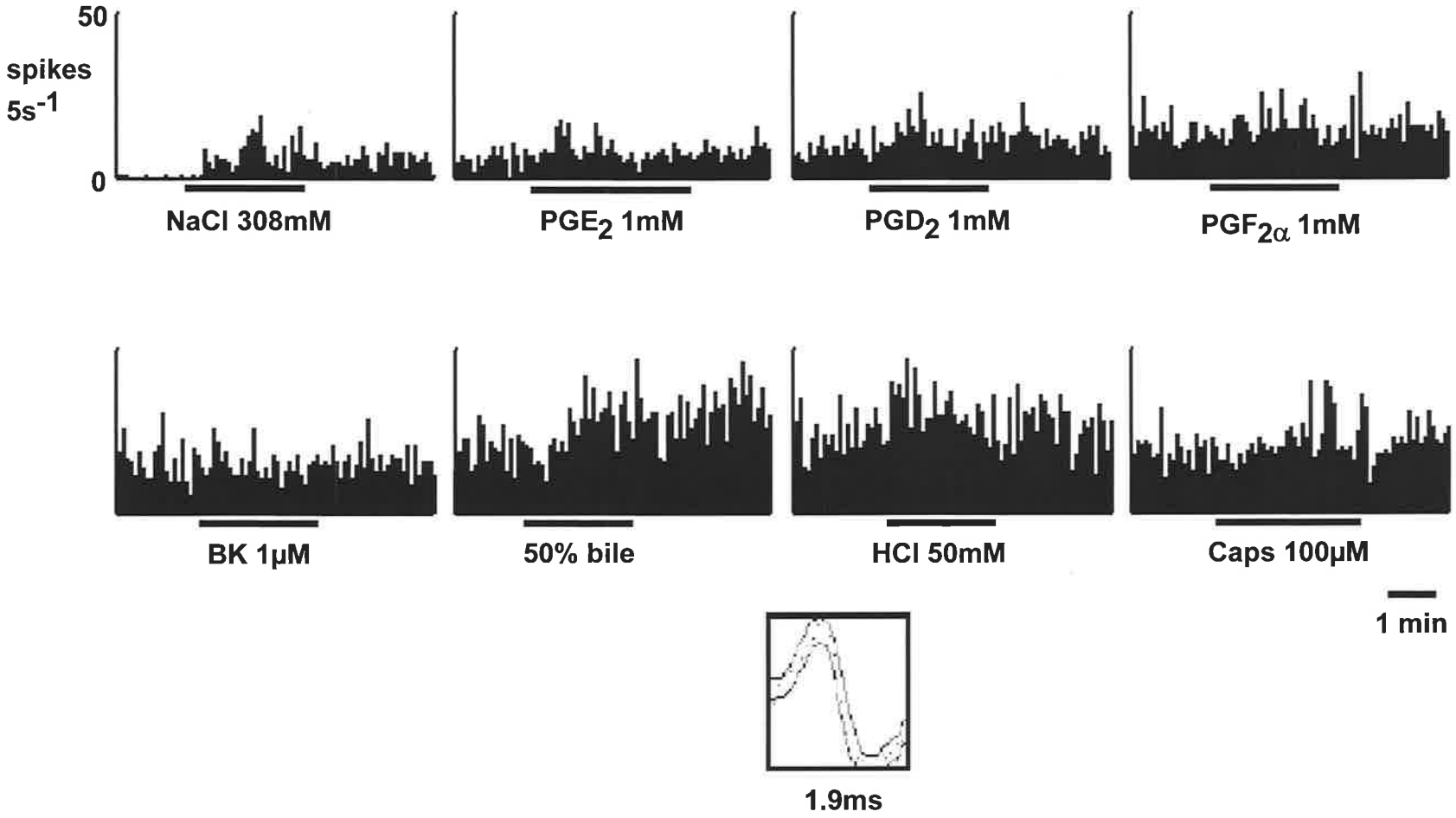

Figure 8-7. Response of fibre 5 that was discriminated from 5 fibres. It responded to probing but not to Von Frey hairs. Of particular interest, above and beyond the responses to chemicals is the increasing basal discharge throughout the study from being spontaneously silent. This increase is a true increase not associated with any change in the shape or amplitude of the fibre. The times of these chemical stimuli from the beginning of the study are as follows; NaCl 31min, PGE_2 41min, PGD_2 49min, $PGF_{2\alpha}$ 58min, BK 69min, bile 88min, HCl100min, Caps117min. The fibre responded to all chemical stimuli applied with the exception of $PGF_{2\alpha}$ 1mM and BK 1μM.

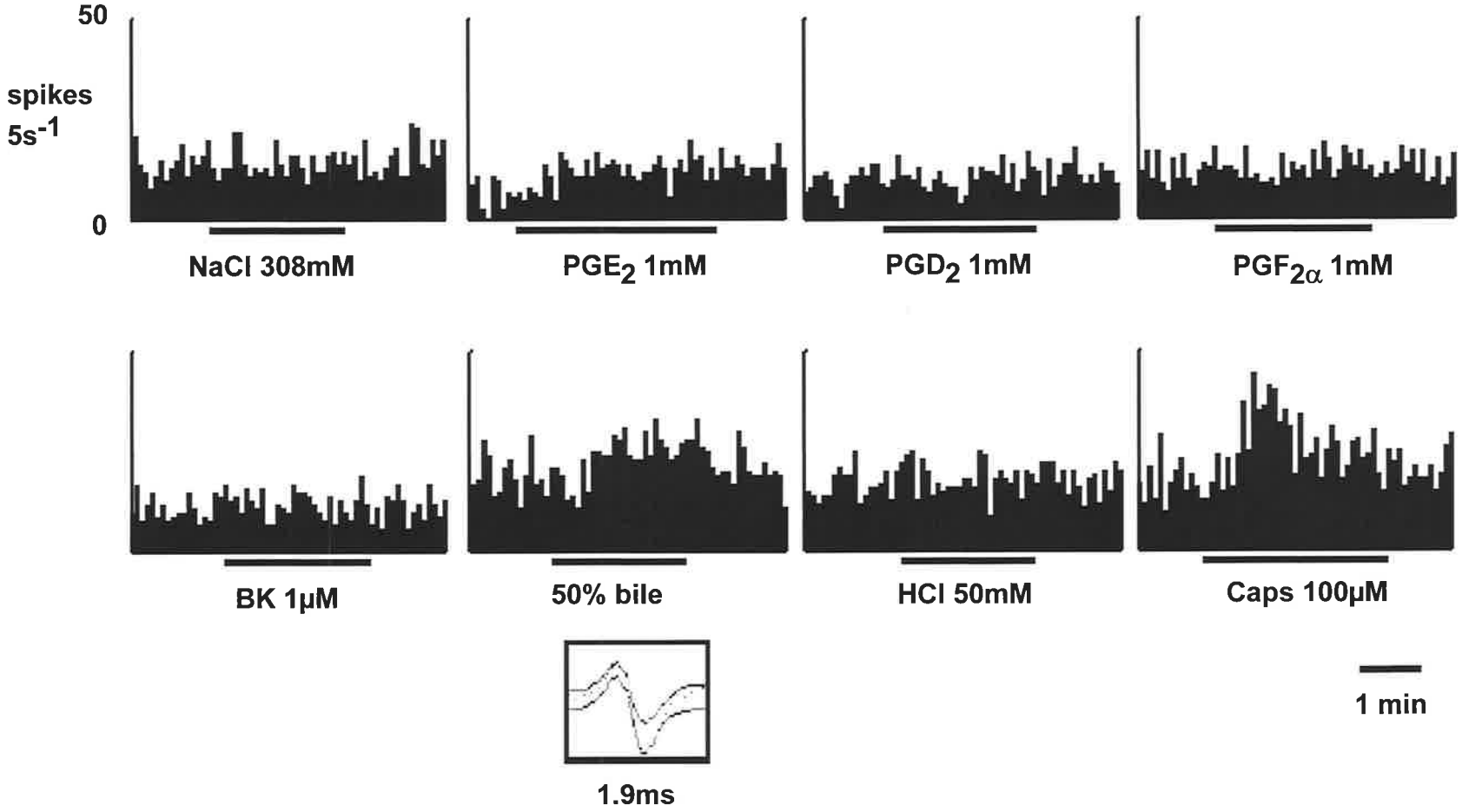

Figure 8-8. Response of fibre 1 discriminated from 5 fibres. This fibre responded to probing and had an adjacent receptive field to the fibre shown in Fig 7. This fibre responded to only two chemical applications, 50% bile and capsaicin 100μM.

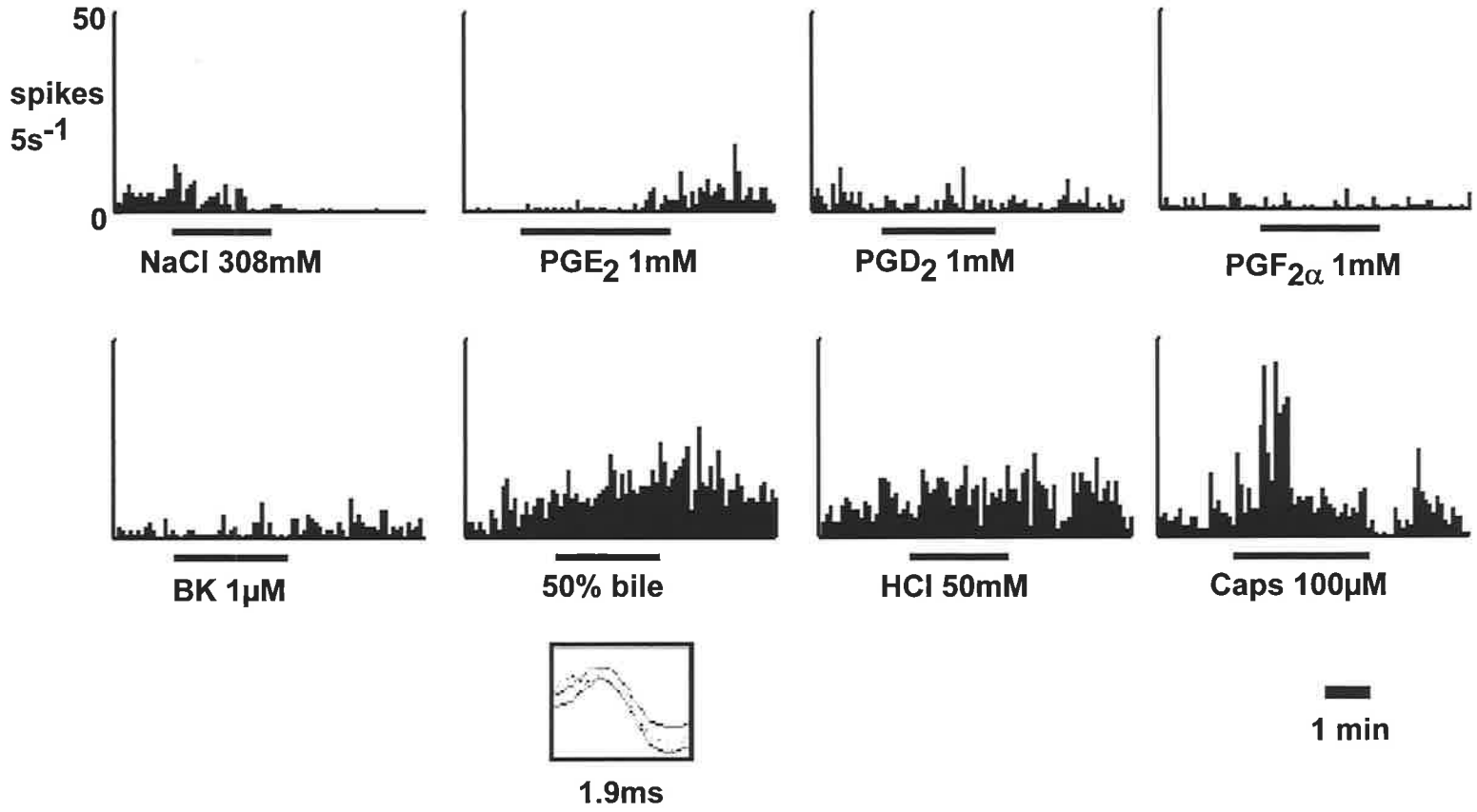

Figure 8-9. Response of fibre 2 discriminated from 5 fibres. This fibre responded to stroking and probing and had an adjacent receptive field to the fibre shown in Fig 7 - over the nerve bundle. The fibre responded to 50% bile and capsaicin 100μM. Inhibition to a chemical stiimulus had not been observed before this fibres response to NaCl 308mM.

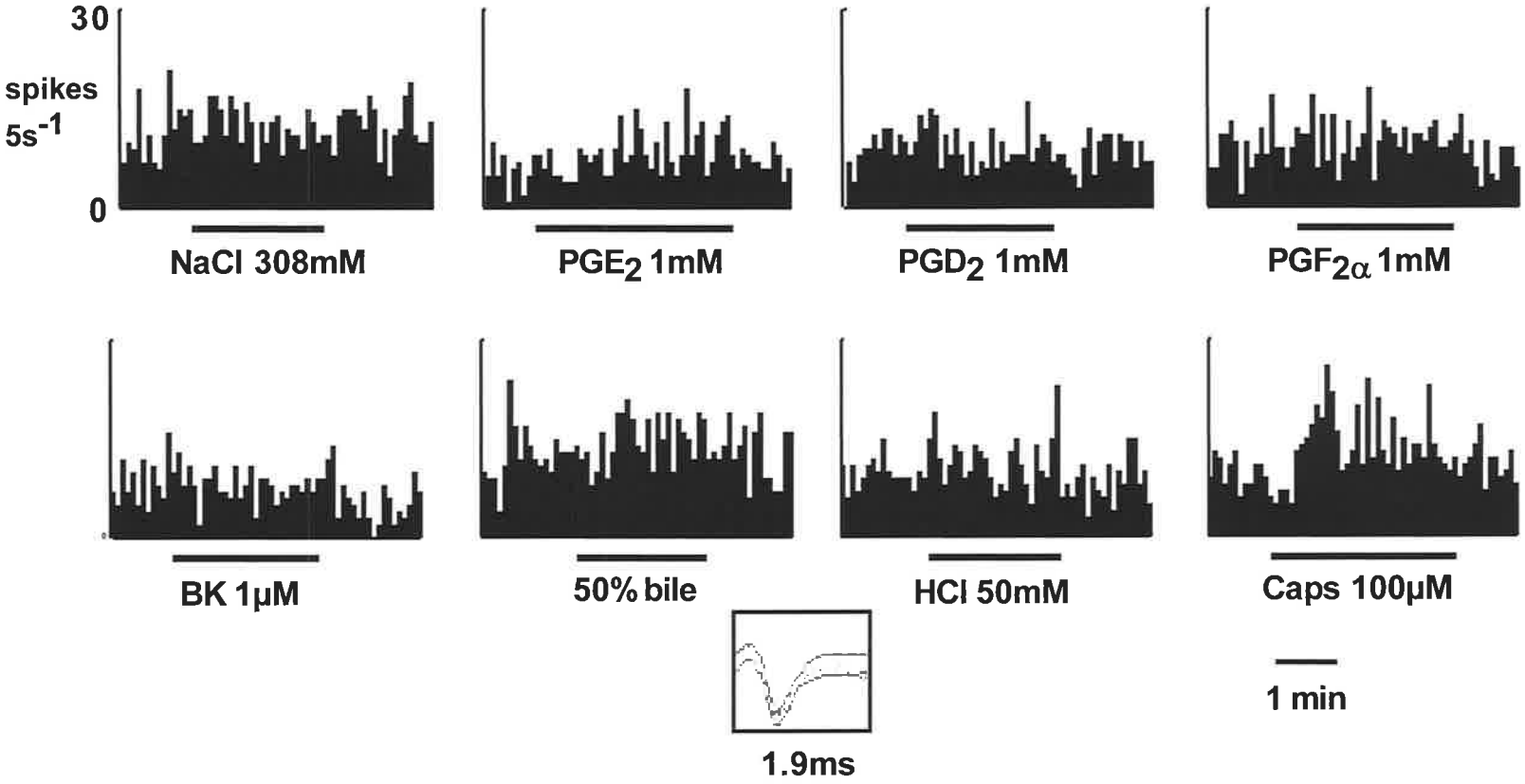

Figure 8-10. The response of fibre 3 discriminated from 5 fibres. This fibre responded only to probing. It had a high basal discharge and and was only chemosensitive to capsaicin 100μM.

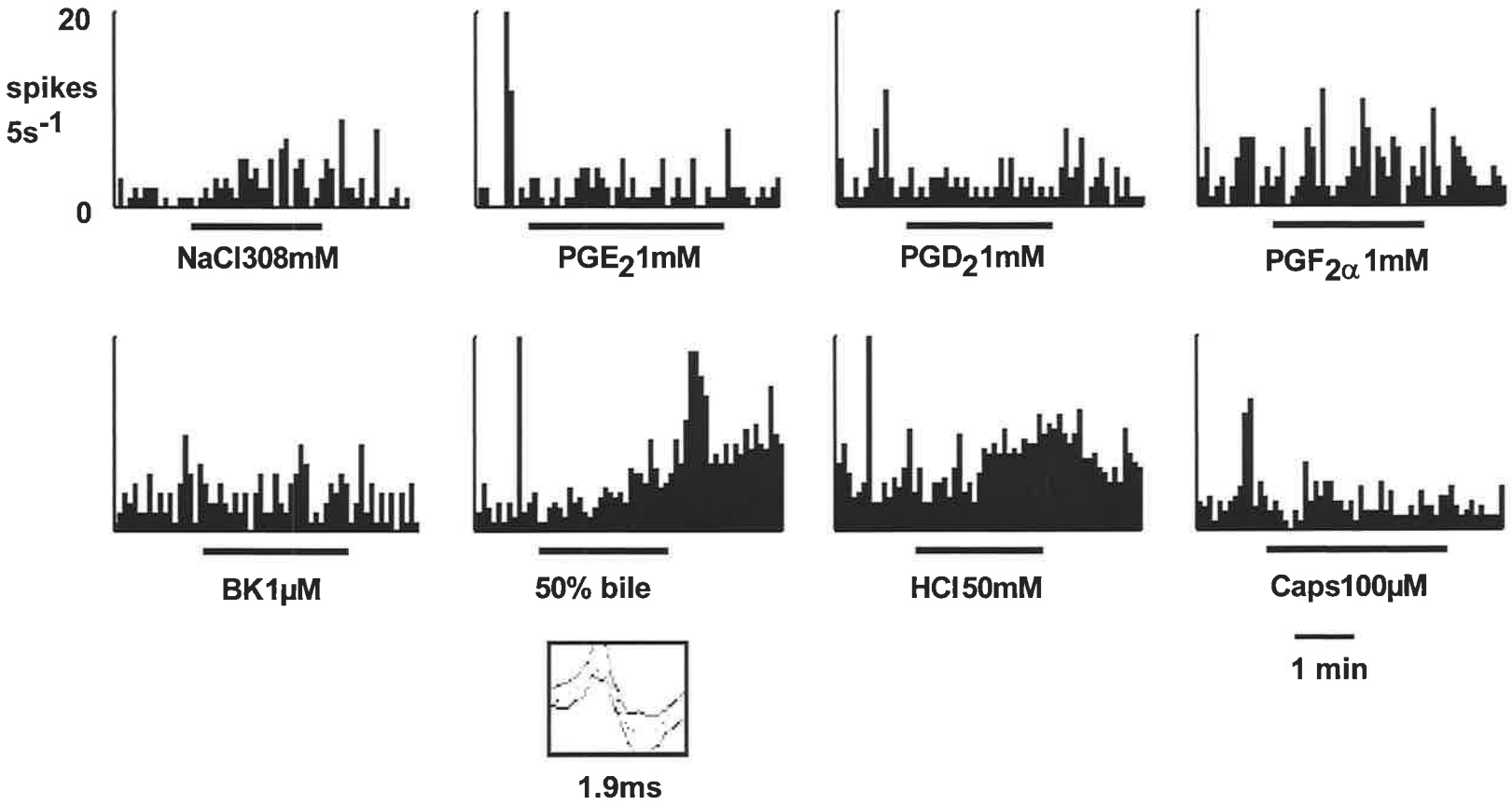

Figure 8-11. Response of fibre 4 discriminated from 5 fibres. This fibre did not respond to probing or von Frey hairs but there was a characteristic burst of firing before the chemical stimuli as the ring was placed over the receptive field sometimes seen in sensitive mucosal fibres in the rat The receptive field may not have been identified due to difficulty of applying von Frey hairs to the small tissue. This fibre responded to NaCl 308mM, $PGF_{2\alpha}$ 1mM, 50% bile and HCl 50mM.

8.5 Discussion

8.5.1 Success of the Technique

Using this technique it is possible to record from single afferent fibres in the intermesenteric nerve of the mouse, innervating the distal colon *in vitro*. However, it has yet to meet the original expectations of improving preparation time and efficiency in experimental protocol, as well as improving the rate of successful studies.

The final design of the organ bath was adequate, and enabled relative ease in dissection, layout of tissue and application of stimuli. In view of this experience, there are considerable improvements to be made to the design to increase efficiency and reproducibility. The mouse colon, as might be expected, was much more fragile than the rat colon and therefore more difficult to dissect, manipulate, prepare and apply stimuli to. This was not adequately taken into account in the design of the technique and greater measures can be taken in future designs to minimise the possibility of damage. Probing and Von Frey hairs could be applied to the mucosal surface without damaging the tissue but stretch could not. Thus, it is unlikely that any quantitative study of the effect of tension or length would be possible in this preparation. The use of probing and particularly Von Frey hairs was more unreliable than when used in the rat preparation. This was primarily because of the extremely small size of the receptive fields of these fibres. Reproducibility of responses to Von Frey hairs was poor because it was easy to miss the site of the small receptive fields. In addition, in preparations with more than one active fibre (for instance exp. 9), the receptive fields of clearly different fibres were so close that it was impossible to differentiate between them using the current mechanical stimuli. Chemical stimuli could be easily applied, but were more likely to visibly damage the mucosa at comparable concentrations to those used in the rat. This was particularly true of HCl 50mM and 100% bile. Most encouraging, was the 100% success rate in recording activity after some technical problems had been rectified. Considering the small size of the nerve and the small yield of strands possible, this preparation is unlikely to be of use in purely single unit studies. However, there is scope for a relatively high yield of data if it is used

for multiunit recordings, as stimuli - particularly chemical stimuli - can be very easily applied to the whole tissue.

8.5.2 Interpretation of Results

Nine distinct fibres (including two 'rumbles') were identified in five experiments. Of these, two showed characteristics of having mucosal endings by responding to 10mg Von Frey hairs, following the definition used for the rat mucosal afferent fibres. However, it was not possible to include a response to stretch as a defining parameter into afferent fibre definition in this preparation. A 10mg Von Frey hair has been shown to be adequate to identify mucosal fibres in the ferret oesophagus *in vitro* (Page & Blackshaw, 1998) and in the rat distal colon *in vitro*. However, in the mouse a 10mg Von Frey hair does not have a sensitivity great enough to differentiate a mucosal afferent fibre from other classes of afferent fibre. As the nerve trunk lies underneath the tissue, a mechanical stimulus must be strong enough to penetrate the thickness of the tissue to elicit a 'rumbling' response. Therefore, a stimulus that achieves this is clearly too strong to differentiate between a mucosal afferent fibre and a serosal afferent fibre. In Exp. 8, reproducible 'rumbling' responses to Von Frey hairs down to the level of 10mg were elicited over the site of the nerve trunk. This suggests that in this fragile mouse preparation, Von Frey hairs lower than 10mg must be employed to differentiate between serosal and mucosal afferent fibres. Therefore, it can be concluded using the definitions of the rat colon *in vitro*, only that these two afferent fibres showing characteristics of being 'mucosal' afferent fibres are mechanosensitive afferent fibres.

This mouse preparation yielded 'rumbling' activity with a seemingly discrete receptive field. In the rat, this activity was normally elicited down the length of the nerve trunk and was believed to be associated with stimulation of the nerve trunk. This activity could be elicited only with firm probing, but in the mouse it was elicited with a 10mg Von Frey hair. With the difficulty of stimulating very small receptive fields, and the overlapping of stimuli onto adjacent receptive fields of other fibres,

the ease with which this nerve trunk activity can be elicited will be a problem as it confounds the data being generated.

Responses to chemical stimuli were elicited in all studies that investigated chemosensitivity. Again, caution must be exercised in the interpretation of this data as the stimuli applied may have been of too high concentration, were not repeated and were not tested with respect to their vehicles. Concern that chemical concentrations may have been too high arose from the observation that no stimuli caused visible damage in the rat preparation, yet HCl (50mM) caused considerable visible damage to the mouse colon. This suggests that the mucosa of the mouse colon is more susceptible to damage than the mucosal of the rat colon. Whether this is due to the relative fragility of the tissue due to size, or whether the mouse colonic mucosa is more susceptible to damage than the rat colon *in vitro* because of physiological differences is not clear.

Some interesting observations have come about through the use of the relatively high concentrations of chemical stimuli. At these high concentrations, it cannot be argued that responsiveness had been unduly missed. All of the fibres (excluding rumblers) responded to some, but not all of the chemical stimuli. This suggests that there is true heterogeneity amongst the fibres. Compounding this conclusion is the distinctly different pattern and combination of responses in the five different fibres exposed to identical stimuli and environmental influences in Exp. 9. These observations support the heterogeneity of chemosensitivity that has been documented in the rat.

Of particular interest in Exp. 9, was the fibre that developed spontaneous activity over the course of the study. This was in contrast to the other four fibres that did not. Despite being unable to identify this fibre as a mucosal fibre, developing spontaneous activity over the course of the study duration is a characteristic of mucosal fibres in the rat preparation. The cause of this phenomenom is unknown at this stage, but speculation may include the increasing deterioration of the mucosa etc as discussed in Chapter 2. This may have relevance to the observation that of these five fibres three had activity affected by the visible

damage to the mucosa by bile. After the application of this stimulus, basal activity was increased and did not recover. Conversely, in the other two fibres there was no observable change in the basal activity. Of the three that did respond to damage to the mucosa, all had some characteristics of mucosal fibres. Fibre 5 developed spontaneous activity over the course of the study. Fibre 4 had large bursts as the ring was gently placed over the mucosa, a characteristic that has been used to define a mucosal fibre in these mouse preparations but not in the rat preparation. In addition, Fibre 2 responded to stroking of the mucosal surface. Thus, it can be speculated, but not concluded, that these three fibres had endings within the mucosa. The evidence from this study suggests that not all afferent fibres are designed to respond to damage to the mucosa.

8.5.3 Limitations of Technique

This preparation has not achieved the original aims of the project. In real terms this technique must be seen as a transitional preparation. It had been hoped that the setup and preparation time for this technique would be reduced in comparison to the rat technique. The primary dissection from animal to organ bath is straightforward. The positioning in the bath is awkward and the design of the bath could be improved to aid this. In particular, the walls of the bath are unecessarily high, making it difficult to access the tissue. With care this can be remedied. It would be advantageous having the outer walls angled to give even greater access to the tissue. Space within the bath was not utilised efficiently. Consequently, drug administration via the superfusate would include wasted volume. The bath size could be improved and dead space inside the bath reduced without compromising any other aspect of the technique. In addition, making the bath readily transportable, enabling the orientation to be changed substantially improves comfortable access.

The inability to use stretch of the tissue as a stimulus, the close proximity of adjacent receptive fields and the difficulty in quantifying mechanical stimuli compromises the type of information originally hoped for from this technique. If the technique is to be used for a high yield of data then it is not possible to rely on data

recorded from single units. However, a compromise on single units to include recording from multiunits would enable that yield to be realised. In view of the fact that it is not possible to characterise fibres properly on the basis of their response to mechanical stimuli, it is probably worth looking at multiunits to maximise the usefulness of the preparation.

8.5.4 Future Directions

There is probably little future for the preparation as it currently stands. It is cumbersome, unreliable and has a very low yield for the time expended. Nonetheless, with some judicious improvements and a shift in the aims of what can be achieved with it, I believe that it will prove to be a very useful tool.

In the advent of a reliable list of screening stimuli, whether they be mechanical or chemical, this preparation will be useful for comparision between transgenic and wild-type mice. In addition, this preparation would be easily adapted to compare the different effects of applying chemical stimuli to the mucosa in comparison to the serosal surface. This is becoming increasingly important as the use of mucosally applied chemical stimuli is being used in *in vitro* preparations instead of the more common serosally, intra-arterially and intravenous applications in the *in vivo* preparations. Certainly this was a question that arose as a result of the rat preparation but was not resolved.

General Discussion

The Spinal Mucosal Afferent Fibre

The importance of the documentation of colonic mucosal afferent fibres in the spinal innervation should not be underestimated. The observation that spinal afferents can be so exquisitely sensitive to luminal mechanical stimuli forces departure from what has been a longstanding but currently changing view that the spinal innervation is predominately a pain mediating pathway. When discussing pain it is important not to discount the central projections of these afferents. It is possible that spinal afferents with this degree of sensitivity can still mediate pain by virtue of their pattern of recruitment, their projection to the dorsal horn and the integration of the information that they mediate. However, it is reasonable to assume that this pathway which is likely to project information about the consistency, movement and chemical makeup of luminal contents is utilised in a regulatory pathway rather than exclusively pain generation. This aspect is compounded by the observation in this study that serosal fibres can be chemosensitive to luminal stimuli, thus negating the current view that serosal fibres are the only visceral fibres that are candidate for a purely nociceptive role.

With reference to disease, sensory disorders of the colon are primarily targeted by investigation of sensations arising from the stimulation of predominantly muscular fibres by balloon distension. Thresholds of discomfort and pain in response to balloon distension of the colon are lowered in patients suffering from irritable bowel syndrome (IBS) compared to normals (Lembo *et al.*, 1994; Munakata *et al.*, 1997). Interestingly, the nature of the hypersensitivity seen to gastrointestinal events in IBS has led to two studies to speculate a role for mucosal fibres contributing to the hypersensitivity in the pelvic innervation of the rectum (Plourde *et al.*, 1993) – where mucosal fibres have been documented – and normal sensation in the splanchnic innervation of the colon (Lembo *et al.*, 1994) – where they have not. Nonetheless, there has been little emphasis on pursuing the contribution of mucosal afferents and stimuli of those afferents in the study of IBS. This is probably important given the richness and complexity of afferent information that would be arising from the mucosal afferents in spinal pathways. The documentation of mucosal afferents from the colon also provides a new avenue for

therapeutic targets. This study failed to show any particular differences in the qualities of mucosal and serosal fibres in their chemo-sensitivity and differences in mechanosensitivity exist by virtue of their position in the layers of the gut wall. This may indicate that systemic application of therapeutic mediators for afferent modulation may not differentiate between the two groups but luminal application of therapeutic mediators unable to penetrate the wall of the gut effectively may target mucosal afferents. As yet evidence does not exist for a contribution to altered sensation by mucosal afferent fibres arising from the colon, but circumstantial evidence suggests that it is not unreasonable to speculate such a contribution. For example mucosal fibres in the urinary bladder become activated and sensitised during inflammation (Koltzenburg & McMahon, 1995) and mucosal fibres are in a prime position to be targeted by luminal disturbances, and inflammatory states in the mucosa. This will not be resolved until the relevent studies have been carried out, elucidating the potential role for mucosal fibres in altered sensation in the colon. The results presented in this thesis give justification for pursuing such studies.

Novel In Vitro Preparations

The techniques developed here, both the rat and mouse preparation, provide an opportunity to continue to investigate the physiological role of spinal afferent fibres innervating the colon in a manner that has not been possible before. *In vitro* preparations such as these enable much more discrete investigation of specific mechano- and chemosensitivity of afferent fibres than *in vivo* preparations, a situation that has also been described in the gastro-oesophageal region of the ferret (Page & Blackshaw, 1998). Indeed, in this study and in the current study, new classes of afferent fibres were described – a direct result of the control these preparations allow the investigator to have over the application of mechanical stimuli to the tissue. Results from preparations such as these contribute to clearer interpretation of results from *in vivo* studies. One such example resulting from the current study is a potential explanation of the physiological role of all or a sub-population of what have been termed silent nociceptors in *in vivo* investigations that have similar properties to those of the newly described colonic mucosal

afferent fibres. There is still scope for broadening the basic investigation of chemosensitivity of spinal afferent fibres innervating the colon as the information in the literature and the data gathered here does not equal the depth of information available about other areas of the gut.

Pharmacological intervention in whole organ *in vitro* preparations is considerably easier than in *in vivo* preparations and is in addition, often easier to interpret. In addition to the work on prostaglandins presented here, gastrointestinal preparations are increasingly being used as a tool for pharmacological manipulation (Brunsden & Grundy, 1999; Maubach & Grundy, 1999; Page & Blackshaw, 1999; Page *et al.*, 2000). It is interesting to note that all four studies (including the current study) all investigate aspects of sensitisation of afferents.

Future Investigations

Sensitisation of afferents and interaction of different mediators modulating afferent excitability are both areas that need to be explored in greater detail in the colon and in the rest of the gut. The effects of inflammation, sensitisation, pain generation and persistence of chronic pain is still not understood - particularly in the viscera. An understanding of the peripheral contribution to these phenomena will contribute greatly to the understanding of the aetiology of disease states – be they functional, neuropathic or physiological basis. Sensitisation of the gastrointestinal afferent and interactions between different mediators is currently being pursued from a number of different perspectives. The approach that has been pursued in the study of skin sensitisation and pain generation has recently been transferred to the study of colonic sensation through the pelvic nerves. This has included investigating the roles of intraluminally applied inflammatory soups (mixture of bradykinin, PGE_2, 5-hydroxytryptamine and histamine), pH and bile salts in sensitising responses of afferents to mechanosensitivity (Su & Gebhart, 1998). An alternative perspective investigating sensitising mechanisms involving PGE_2 and bradykinin in mesenteric afferents independently of the gut wall has also been investigated (Brunsden & Grundy, 1999; Maubach & Grundy, 1999). A novel approach investigating the opposite of sensitisation of mechanosensitivity –

inhibition – has demonstrated the ability of $GABA_B$ agonist baclofen to inhibit the responses of vagal mucosal and muscular afferents in the gastro-oesophageal region to mechanical stimuli (Page & Blackshaw, 1999).

One aspect of chemosensitivity in the gut that has not been pursued in detail is the interaction of different mediators. In this thesis, sensitivity to single mediators was pursued in a similar manner to that pursued in the majority of gastrointestinal afferent fibre studies of its kind. But it is unlikely that polymodal afferents respond to chemical mediators in isolation, but rather respond to a complex interaction of different mediators. Extensive work on this has been carried out in the skin, testis – spermatic nerve preparation (Kumazawa *et al.*, 1991; Mizumura *et al.*, 1992) and dorsal root ganglion dissociated cells (Kirschstein *et al.*, 1999) and it is known that there are interactive effects between most mediators investigated including bradykinin, heat, pH, prostaglandin, capsaicin and histamine sensitivity. Investigations into intracellular pathways of mechano- and chemosensitivity and sensitisation of these responses in dissociated dorsal root ganglion cells (Gold *et al.*, 1998; Gold *et al.*, 1996), skin–saphenous nerve (Kress & Guenther, 1999) and testis–spermatic nerve preparations (Mizamura *et al.*, 1997) also reveal an interaction of multiple mediators on the excitability of the afferent.

A study and comparison of the effects of short-term and chronic inflammation and recovery are pertinent to inflammatory bowel disease and Crohns disease but also to irritable bowel syndrome (IBS). Whilst inflammation is not a direct animal model for IBS, it has been proposed that for many patients, low-grade inflammation of the mucosa may be involved in the aetiology of the disease (Collins, 1992). It is not known what effect an inflammatory state has on colonic afferent fibres. Of particular interest to me is not the effect of single mediators on the mechano- or chemosensitivity of colonic afferents, but rather the lasting effects inflammation has on the mechano- and chemosensitivity of colonic afferent fibres. The following questions arise. Is the sensitivity of mucosal and serosal afferents modulated during and after inflammation? Is mucosal afferent mechano- and chemosensitivity more susceptible to mucosal disturbances than serosal afferent sensitivity, and if so, what are the determining factors? Do mucosal fibres in particular have a role in

disordered sensation as a result of sensitisation, and are they candidate for a role in sensation of gastrointestinal events that do not normally reach consciousness? In the colon answers for the aetiology of disordered sensation have primarily been sought in the study of muscular, distension-sensitive afferents and complete answers have not been found. The data presented in this thesis give precedent for investigation of the role of the mucosal and serosal afferent fibres in disordered sensation.

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