The Role of Adenosine 5'-triphosphate in Mechanosensory Transduction in the Rat Colorectum

Gregory Robert Wynn

Autonomic Neuroscience Institute

Royal Free and University College London Medical School

M.D. Thesis

London 2005

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedicated to Kreon

Abstract

Adenosine 5'-triphoshate (ATP), along with other mediators, plays a role in peripheral sensory mechanisms and has been implicated in mechanosensory transduction in the urinary system. $P2X_2$ and $P2X_3$ receptors are expressed on small diameter sensory neurons in the dorsal root ganglia (DRG) and these are known to supply the rat colorectum. ATP appears to be particularly important during inflammation. This work aimed to investigate whether purinergic signalling contributes to mechanosensory transduction in the rat colorectum and whether these mechanisms play a more important role during colitis. The degree of interaction between ATP and other mediators that activate visceral afferents was also studied. Novel in-vitro rat colorectal preparations (either normal or colitic) were used to investigate whether ATP is released from the mucosa in response to distension and whether this contributes to sensory nerve discharge. Pelvic nerve recordings were made during application of ATP and other mediators to the preparations and computer analysis allowed calculation of single unit activity. Results indicated that $P2X_3$ receptors present on DRG neurons were up-regulated during colitis. Distension of the colorectum led to pressure-dependent increases in ATP which were substantially higher in the colitis preparations. Distension-evoked pelvic nerve excitation was mimicked by application of ATP and these discharges were potentiated by purinergic agonists and by ATPase inhibition and attenuated by various P2X antagonists. These effects were exaggerated in the inflammatory setting. Single fibre analysis showed that high threshold fibres were particularly affected by α,β-methylene ATP, suggesting correlation between purinergic activation and nociceptive stimuli. 5-hydroxytryptamine, capsaicin and protons when co-applied with ATP may act synergistically. It is concluded that ATP contributes to mechanosensory transduction in the rat colorectum, particularly during inflammation and this is probably associated with pain. The underlying mechanisms appear to involve distension-evoked release of ATP as well as an increase in the number of DRG neurons expressing $P2X_3$ receptors during colitis, especially those containing calcitonon gene-related peptide (CGRP). The pattern of neuronal activation to a variety of agents suggests that visceral afferents are polymodal but the receptor expression on their terminals can vary markedly.

Preface

The three experimental chapters contained in this thesis try to shed light on the role of ATP as an extracellular signalling molecule in the gut. This is an important topic because the purinergic field continues to expand very quickly and has been implicated in many areas of interest including development, regeneration, proliferation and cell death, in addition to more acute functions such as secretion, vasodilation and neurotransmission. ATP and related compounds have been shown to influence the neuronal response to visceral distortion in other models, but little similar work had been done in the gut. However, considering the clinical burden of functional bowel disorders and the evidence to suggest that the underlying mechanism to these disorders may be a hypersensitivity to normal intraluminal pressures, it seemed logical to investigate purinergic signalling in a gastrointestinal animal model.

In the general introduction, the theme concentrates on purinergic signalling, but attempts to keep this in perspective in a field that is overflowing with a huge array of other signalling systems. It is made clear that ATP is already established as an extracellular signalling molecule in living tissues, especially the nervous system, but special reference to the gastrointestinal tract is made in this section. Chapter three deals with the initial work that presented strong evidence that ATP is important in relaying information about distension in the rat colorectum and in the next chapter, similar experiments are carried out in an established model of colitis to assess the impact of chronic inflammation. Finally, in chapter five, assessment is made of how ATP relates to other common mediators that are known to be important in the gut. The general discussion selects some of the wider issues that are relevant to the work including potential clinical applications and ideas for future studies.

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CHAPTER ONE

INTRODUCTION

General sensory mechanisms

All living organisms require information about the environment in which they exist. This is important in order to seek nutrition, protect themselves from dangers in their immediate vicinity and communicate with other organisms. All vertebrates and most invertebrates use specialised signalling cells and a network of sensory neurons in order to achieve this combined goal for survival. The interaction of environmental energy (the stimulus) with a specialised receptor located on a sensory neuron gives rise to a response, which, when combined with many other responses and integrated in the central nervous system, gives rise to meaningful information which will influence behaviour. In vertebrates, sensory neurons gather information from smell, taste, sight, hearing and sensation from the face and throat via cranial nerves with their cell bodies within the brain. The tenth cranial nerve, the vagus, also receives sensory information from the thoracic and abdominal viscera. All other sensation arrives from the periphery into the spinal cord via neurons with their cell bodies in the dorsal root ganglia (DRG). These are arranged as pairs, one either side of each vertebra. DRG neurons receive sensory information from skin and musculoskeletal structures as well as from the viscera, the somatic sources out-numbering the visceral sources by about ten to one (Grundy and Scratcherd 1989; Sengupta and Gebhart 1994). This arrangement allows very high levels of perception from the external surface of the body, where large amounts of information are required in order to influence motor systems. The internal structures, whose stimuli rarely need to reach levels of conscious perception, require less central innervation. DRG neurons send their central processes into the dorsal horn of the spinal cord, where they terminate in distinct layers according to subpopulation.

One characteristic of cells of visceral origin is the tendency to terminate in areas above and below the point of entry into the spinal cord (Neuhuber et al. 1986; Gebhart 2000) and this is thought to be responsible for the poorly localised nature of visceral sensation compared with that from the skin. Also, because disparate neuronal pathways converge within the spinal cord, noxious signals arriving from the viscera can occasionally activate pathways of somatic origin giving rise to the phenomenon of referred pain. The dorsal horn, or second order neuron, crosses the spinal cord and ascends in the spinothalamic, spinoreticular, spinomesencephalic and dorsal column pathways to terminate in the thalamus, reticular formation or midbrain. Third order neurons then projects through the internal capsule and corona radiata, to the postcentral gyrus and cortex for somatic sensation and the anterior cingulate gyrus and sensory cortex for visceral sensation (Camilleri 1996). Somatosensory information is highly organised in its representation in the cortex, whereas that from the viscera is not. Pathways to the reticular formation are particularly important in visceral perception, leading to stimulation of autonomic centres and the amygdala, from which major pathways containing corticotrophin-releasing factor originate (Gray 1993). The amygdala also sends fibres to the parabrachial area, the region containing neurons that encode colonic distension in the noxious range (Bernard et al. 1994).

Visceral sensation from the gastrointestinal tract

Unlike somatic tissue, the gastrointestinal tract is innervated by two distinct groups of extrinsic primary afferent fibres (extrinsic as opposed to intrinsic or enteric nerves whose cell bodies reside within the wall of the gut). Neurons with their cell bodies in the nodose ganglia send axons via the vagus nerve to innervate all layers of the gut wall between the oesophagus and the transverse colon (Cervero 1994; Berthoud

and Neuhuber 2000). 80% of all vagal fibres are afferent in nature, leaving only 20% as preganglionic parasympathetic motor fibres. Vagal afferents project centrally to the nucleus tractus solitarius and the area postrema of the brainstem (Grundy and Scratcherd 1989; Sengupta and Gebhart 1994; Berthoud and Neuhuber 2000). Spinal afferent fibres that travel in the pelvic nerve via the lumbosacral DRG (sacral DRG only in humans) supply the remainder of the colon and rectum. The whole of the gastrointestinal tract is also supplied by another population of spinal afferents (DRG neurons) that travel with post-ganglionic sympathetic fibres in the splanchnic nerves and then on into the spinal cord as discussed above. Extrinsic afferent neurons, transmitting information to the central nervous system via vagal or spinal pathways, are involved in long loop reflexes that coordinate regions of the gut that may be physically meters apart and transmit or control noxious information to the brain (Grundy 2002).

In contrast to spinal afferent neurons that respond at both low and high thresholds, encoding physiological and noxious signalling (Sengupta and Gebhart 1994), vagal fibres have low thresholds of activation and reach maximal responses within physiological levels of stimulus (Sengupta et al. 1990). Those fibres that respond to distension play important roles in regulating normal gastrointestinal motor function and two specialised types of sensory endings have been identified. Intramuscular arrays are varicosed nerve endings lying in parallel to longitudinal muscle fibres that respond to wall tension (Wang and Powley 2000). Intraganglionic laminar endings are located within the myenteric plexus and are chemosensitive transducers of stretch (Kressel and Radespiel-Troger 1999; Zagorodnyuk et al. 2001). The vast majority of vagal afferent signalling fails to reach levels of conscious perception. Rather, they tend to activate regions of the brain (such as the limbic system) that are involved in the behavioural and emotional aspects of sensory processing (Sawchenko 1983). This is consistent with observations in patients with spinal injuries that have lost all sensation from the gut

(Lembo et al. 1994). However, some vagal afferents project to cervical regions in the spinal cord and have been implicated in the control of noxious signalling to the brain (Janig et al. 2000). Electrical stimulation of these cervical vagal afferents suppresses transmission of impulse activity in spinothalamic relay neurons with nociceptive function at all levels of the spinal cord and the central pathways that mediate this effect are neurons in the locus ceruleus, dorsolateral pontine tegmentum and the nucleus raphe magnus of the rostro-ventral medulla which project to the dorsal horn of the spinal cord (Fields and Basbaum 1999). Transmission of nociceptive impulses from both skin and colon in the dorsal horn could be enhanced by electrical stimulation of myelinated and depressed by unmyelinated vagal afferents in the rat (Gebhart and Randich 1992; Randich and Gebhart 1992), suggesting that both augmentation and inhibition can be achieved by this system, according to functional differences of the two fibre types.

The immune response to injury can also activate vagal pathways. Il-1β and TNF-α injected intraperitoneally can generate behavioural hyperalgesia that is abolished by vagotomy (Watkins et al. 1995) and similar endotoxin injection generates increased c-fos expression in the nucleus tractus solitarius and hypothalamic nuclei in addition to induction of Il-1β mRNA in the pituitary gland, hypothalamus and hippocampus (Ericsson et al. 1994; Wan et al. 1994; Laye et al. 1995). These changes are also abolished by subdiaphragmatically vagotomised animals. The results indicate that vagal afferent neurons are responsible for responding to products of the immune system in order to relay information to areas of the brain that are involved in the behavioural responses to injury and illness, such as immobility, anorexia and malaise.

About a quarter of spinal afferent neurons respond to a noxious stimulus, such as high-pressure colorectal distension (CRD), with a high threshold and the remaining three-quarters respond at lower thresholds but continue to increase activity into the noxious range (Sengupta and Gebhart 1994). Mechanosensitive spinal afferents have

their terminals in the mesentry, serosa, muscle layers and mucosa (Sengupta and Gebhart 1995; Gebhart 1996). In-vivo, The onset of reflex pressor and tachycardic responses known to be associated with pain correspond to the same degree of stimulus that activates high threshold neurons (Ness et al. 1991) and therefore these neurons, along with their low threshold counterparts that continue to increase their firing into this range, are thought to relay painful signals to the brain. Both vagal and spinal extrinsic afferents give off axon collaterals to both plexuses of the enteric nervous system. However, only small diameter mechanosensory spinal afferent neurons give off collaterals within the prevertebral ganglia (PVG) to form synapses with sympathetic ganglion cells (King and Szurszewski 1984; Szurszewski and Miller 1994). These neurons release the neuropeptides Substance P (SP) and calcitonin gene-related peptide (CGRP) and have high thresholds of activation (Parkman et al. 1993; Ma and Szurszewski 1996a). Interestingly, axon collateral release of neuropeptides within the PVG can be modulated from central peptidergic preganglionic neurons without altering the afferent signal referred centrally (Ma and Szurszewski 1996b).

The enteric nervous system

The number of extrinsic afferent neurons pale into insignificance when compared to the many millions of intrinsic primary afferent neurons (IPANs) located within the enteric nervous system. These neurons orchestrate the on-going function of the gastrointestinal tract, feeding information into complex networks of fibres to elicit reflex motor, secretory and vasoactive responses (Furness et al. 1999). Their cell bodies reside in the myenteric and submucous ganglia and they send multiple axons to other neurons and also to the muscular layers and mucosa (Wedel et al. 1999). Both submucosal and myenteric neurons respond to chemical and mechanical stimuli

(Furness et al. 1998), but IPANs within the myenteric plexus comprise the most important group of mechanosensory neurons. They form interconnecting networks that respond to longitudinal stretch in order to regulate peristaltic reflexes (Kunze et al. 1998 and 1999).

Another group of mechanosensitive neurons that regulate normal gastrointestinal function are those cells within the myenteric ganglia that send axons outside the wall of the gut, intestinofugal afferent neurons (IFANs). Their axons terminate in the PVG and respond to circular muscle stretch, as occurs during luminal filling (Crowcroft et al. 1971; Szurszewski and Miller 1994). The majority of IFANs terminating in the coeliac, superior mesenteric and inferior mesenteric ganglia arise from the colon and rectum (Messenger and Furness 1993; Timmermans et al. 1993), in contrast to the inhibitory efferent limb of these reflexes that is evenly distributed throughout the intestine (Trudrung and Furness 1994). This indicates that the colon and rectum reflexly modulate their own motor activity in response to filling, in addition to more proximal areas of the intestine and thereby not only maintain the colorectal wall in a relaxed state during filling but also ensures that the transit of intestinal contents into the colon is appropriate.

The visceral afferent terminal

The terminal endings of visceral afferent neurons are of particular interest in the study of gastrointestinal sensation. It is here that impulses are initially generated by activation of membrane-bound receptors and as a result, neuronal activity is potentially amenable to pharmacological manipulation. A wide variety of mediators are able to activate or interact with the visceral afferent terminal and in variable combinations they can relay specific physiological information via these neurons about the current state of

the tissues in which they lie. These substances can bind directly with receptors and open ion channels, sensitise the neuron without directly activating it or change the phenotype of the terminal. In addition to generating or modulating sensory signals, receptors may mediate initiation or promotion of the release of mediators in the afferent nerve endings, either locally or from axon collaterals (see Kirkup et al. 2001). These efferent functions are an important aspect of sensory nerves, allowing further modulation of function. Some of the main mediators and receptor types involved in activation of visceral afferents are briefly described below. One omission is the purinergic signalling system, which will be described in greater detail later.

The vanilloid receptor, VR1, is a non-selective cation channel with a high permeability for Ca^{2+}. It is a member of the transient receptor potential family of store-operated Ca^{2+} channels (Caterina et al. 1997) and acts as a polymodal detector of potentially harmful stimuli, including heat above 42°C, acidity and several lipoxygenase products (Caterina et al. 1997; Tominaga et al. 1998). The receptor is found on the majority of spinal afferents supplying the gut but rather less so on vagal fibres (Blackshaw et al. 2000). VR1 channel activity is enhanced by sensory neuron stimulants that activate protein kinase C, without being strongly active at the VR1 receptor itself, for example bradykinin (Premkumar and Ahern 2000). Genetic deletion of VR1 has revealed that sensory neuron responses to capsaicin, protons or noxious heat are suppressed and inflammatory hypersensitivity to heat is attenuated (Caterina et al. 2000; Davis et al. 2000).

5-Hydroxytryptamine (5-HT) is released from enterochromaffin cells in the mucosa of the gut and acts on 5-HT_3 receptors that are present on both spinal and vagal afferents (Holzer 2001). Those receptors located on vagal afferents are involved in the emetic reflex, while spinal neurons activated by 5-HT are involved in afferent signalling

of colorectal distension (Kozlowski et al. 2000a). Both 5-HT_3 and 5-HT_4 are found on intrinsic neurons (Bertrand et al. 2000).

Acid-sensing ion channels (ASICs) are proton-gated Na^+ channels that are made up of different subunits, some of which are expressed exclusively on primary afferent neurons (Chen et al. 1998; Waldmann et al. 1999). This receptor may play a nociceptive role, given that acidosis is present in inflammatory tissues and a lowered pH is known to sensitise afferent neurons (Reeh and Steen 1996).

Two members of the family of voltage-gated Na^+ channels are specifically located on spinal and vagal afferents. They are the tetrodotoxin-resistant SNS/PN3 and SNS2/NaN (Bielefeldt 2000; Baker and Wood 2001). Many inflammatory mediators enhance currents generated by these receptors (England et al. 1996) and increase their expression on sensory neurons (Okuse et al. 1997).

Bradykinin (BK) can stimulate extrinsic afferents from the intestine and this is mediated by the metabotropic B_2 receptor (Maubach and Grundy 1999). Antagonists at this receptor can attenuate the inflammation-induced increase in abdominal constriction responses to colorectal distension and intra-peritoneal acetic acid (Julia et al. 1995).

Sensory neurons express prostaglandin EP_1, EP_3, EP_4 and IP receptors (Bley et al. 1998; Ek et al. 1998; Nakamura et al. 2000). Prostaglandin E_2 excites mesenteric afferent nerves supplying the rat intestine (Haupt et al. 2000) and sensitises visceral afferents to other algesic substances (Maubach and Grundy 1999). It can also modulate tetrodotoxin-resistant Na^+ channels located on rat colonic sensory neurons (Gold et al. 2002).

In contrast to somatic sensory nerves, the vast majority of visceral afferents release the neuropeptides SP, which acts at the neurokinin 1(NK_1) receptor and CGRP (Perry and Lawson 1998). These mediators can be released centrally in the spinal cord or peripherally via axon collaterals and tend to play a protective role during tissue

damage by effecting local vasodilator and secretory functions (Reinshagen et al. 1998; McVey and Vigna 2001). NK_1 knock-out mice fail to develop primary mechanical hyperalgesia in the colon after administration of capsaicin and acetic acid (Laird et al. 2000). The enteric nervous system also contains high levels of SP and CGRP (Holzer 1998).

Peripheral opioid receptors (μ, δ and κ subtypes) are present on visceral afferent terminals (Ji et al. 1995; Minami et al. 1995; Rusin and Moises 1998; Aicher et al. 2000) and stimulation of these receptors can inhibit visceral nociception (Reichert et al. 2001). Distension-evoked discharges in extrinsic afferent neurons are attenuated by κ-receptor agonists (particularly after inflammation) but not by μ- or δ-receptor agonists (Sengupta et al. 1999).

Cannabinoid CB_1 receptors are expressed on capsaicin-sensitive, non-peptidergic DRG neurons (Ahluwalia et al. 2000) and are thought to be involved in the modulation of excitatory nociceptive stimuli (Di Marzo 2000). The formation of endocannabinoids during tissue damage, such as anadamide, can prevent and reverse inflammation-associated hyperreflexia of the urinary bladder by activation of CB_1 (Jaggar et al. 1998).

Serine proteases (mast cell tryptase, trypsin and thrombin) can stimulate subpopulations of visceral afferents by binding to protease-activated receptors (PARs). Two of these subtypes, PAR-1 and PAR-2 are expressed on DRG neurons and agonists at these sites cause delayed hypersensitivity to colorectal distension (Vergnolle et al. 2001).

The ability of gastrointestinal afferents to detect stretch, contraction or other mechanical deformity of the gut wall is related to the expression of specific mechanoreceptors, one of which, a mechanosensitive K^+ channel, has been characterised in sensory neurons supplying the rat colon (Su et al. 2000).

Some mediators have their main actions at central terminals within the spinal cord. Glutamate is released by spinal and vagal afferents at their central terminals to act on *N*-methyl-D-aspartate (NMDA) receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainite receptors, in addition to group 1 metabotropic receptors. These receptors are involved in the excitatory spinal input evoked by noxious colorectal distension in rats (Zhai and Traub 1999; Kozlowski et al. 2001b). γ-amino-butyric acid (GABA) receptors, expressed by spinal and vagal afferents, are involved in the central inhibition of substance P release in the spinal cord via the $GABA_B$ subtype (Riley et al. 2001). Stimulation of presynaptic α_{2B} and α_{2C} receptors in DRG neurons can inhibit the release of substance P and glutamate from afferent terminals in the spinal cord (Birder and Perl 1999; Millan 1999) and reduce the sensation of discomfort associated with colorectal distension (Malcolm et al. 2000).

It is clear that in order to respond to the array of physiological and pathophysiological messengers that can be encountered within the gut wall, complex chemical signalling systems must interact with precision in order for the gastrointestinal tract to function properly in regard to secretion and absorption in addition to protecting itself from the constant threat of bacterial invasion. Many of the mediators outlined above activate or sensitise visceral afferents in order to initiate protective reflexes that involve behavioural changes and the immune system. These neurons are known to undergo almost continual remodelling and plasticity in response to tissue damage and the ongoing need for the mucosa to renew itself (Stead et al. 1991). If abnormal or prolonged sensitisation of receptors occurs due to an inflammatory visceral insult, then this mechanism might contribute to some of the long-term symptoms seen in functional bowel disorders such as constipation, diarrhoea, bloating and abdominal pain.

Abdominal pain arising from the gut as a clinical problem

There are really only two mechanisms that cause pain from the gastrointestinal tract: distension and inflammation. In the acute setting, these clinical problems are usually either self-limiting or require immediate medical intervention to treat the underlying cause. Examples of these situations are large bowel obstruction secondary to a tumour, which is acutely painful but will resolve after operative removal the cause of the obstruction; in the case of inflammation, an acute exacerbation of ulcerative colitis can be very painful but often resolves after medical treatment with steroids or immunosuppressants. A greater problem to the clinician is the management of chronic functional bowel disorders. These patients present with abdominal symptoms that have no organic cause demonstrable by conventional diagnostic tests. Because the underlying pathophysiology remains elusive, the diagnosis of syndromes such as functional dyspepsia and irritable bowel syndrome (IBS) has been based entirely on clinical criteria (Drossman and Thompson 1992).

Abnormal processing of sensory traffic from the gut will lead to altered and inappropriate perception as well as manifestations in the efferent loops of sensorimotor reflexes. This could happen at any point along the brain-gut axis and evidence suggests that there are peripheral as well as central components. As a population, patients with IBS demonstrate higher levels of past physical and sexual abuse (Drossman et al. 1990; Talley et al. 1994). It is possible that chronic stress may lead to long-lasting changes at the CNS level. In animal models, infant-maternal separation and electric shock stress create chronic colonic hypersensitivity and hypercontractility resulting in diarrhoea (Coutinho et al. 2001; Stam et al. 1996). The autonomic nervous system is known to modulate visceral sensitivity in humans and increased sympathetic tone leads to increases in the level of perception of gastrointestinal stimuli (Iovino et al. 1995). There

is also evidence that intestino-intestinal reflexes may be impaired in patients with functional bowel disorders (Coffin et al. 1994). However, a common theme in IBS is the demonstration of a visceral hypersensitivity to distension (Ritchie et al. 1973; Whitehead et al. 1990; Accarino et al. 1995; Mertz et al. 1995). Studies using mechanical stimuli or transmucosal nerve stimulation in patients with IBS have shown that mechanosensitive afferent pathways are selectively affected (Accarino et al. 1995). However, neither the cause nor the level of afferent dysfunction has been established.

Although the clinical features of IBS are clear and some of the associated factors have been well documented, the underlying mechanisms remain obscure. Evidence has now accumulated for inflammation within the gut wall as a cause of sensitisation of visceral afferents. In fact, a specific subset of IBS patients report that their symptoms commenced after a severe bout of gastroenteritis. This phenomenon is known as post-dysenteric IBS (PD-IBS) and is estimated to affect between 7% and 32% patients after acute bacterial gastroenteritis (McKendrick and Read 1994; Neal et al. 1997; Garcia-Rodriguez and Ruigomez 1999). Spinal afferent neurones supplying the gut are known to exhibit enhanced excitability following enteric inflammation (Olivar et al. 2000) and a prolonged or abnormal response to this normally protective mechanism is a possible candidate for causing persistent abdominal pain.

The first evidence of possible involvement of an inflammatory component in the intestinal wall of patients with IBS was reported in the early 1960's by Hiatt and Katz, who detected an increased number of mast cells in the muscularis externa of surgical specimens (Hiatt and Katz 1962). This has been followed up by studies that show an increased density of mast cells in the mucosa of both small intestine and colon of patients with IBS (Weston et al. 1993; O'Sullivan et al. 2000). Patients with IBS also have increased numbers of T-cells in the colonic mucosa and these powerful immunocytes lie in close proximity to axonal fibres of the enteric nervous system

(Barbara et al. 2000a). Activated T-cells are known to evoke changes in neuromuscular function in the gastrointestinal tract (Collins 1996).

A variety of mechanisms may contribute to the low-grade mucosal inflammation seen in IBS. PD-IBS patients may be unable to down-regulate intestinal inflammation after an episode of infection (Spiller et al. 2000). Undiagnosed food allergies may contribute (Smout et al. 2000), as might changes in intestinal microflora (Barbara et al. 2000b). Good evidence exists that genetic factors play a role. DNA analysis of patients with IBS has shown lower genotype frequencies of genes producing anti-inflammatory cytokines (Chan et al. 2000) and monozygotic twins (and dizygotic twins to a lesser extent) have a particularly high prevalence of IBS (Morris-Yates et al. 1998). What is clear from the evidence so far is that the complex relationship between the gastrointestinal immune system and the enteric nervous system is critical for the peripheral component of functional bowel disorders.

The purinergic signalling system

Descriptions of the physiological effects of adenine compounds on the heart were first described by Drury and Szent-Györgyi in 1929 and many years later, experiments were carried out showing that adenosine-5'-triphoshate (ATP) could be released from sensory nerves in sufficient quantities to cause vasodilatation in rabbit ear arteries (Holton 1959). The concept that purines could behave as extracellular signalling molecules was not given early recognition, despite the fact that extacellular enzymes responsible for their breakdown were known to be present. In 1972, ATP was proposed as one of the transmitters responsible for non-adrenergic non-cholinergic neurotransmission in the bladder and gut (Burnstock 1972) and four years later purinergic receptors were first defined (Burnstock 1976). Since then, a classification of

a whole family of purinoceptor subtypes has been established that is described below. ATP was one of the earliest biological molecules to appear and because of this, it is not surprising that it has widespread important intracellular (metabolic) and extracellular functions (Burnstock 1996). The molecule is made up of a nitrogenous base (adenine), attached to a five-carbon sugar group (ribose), to which three phosphate groups are joined. This allows stepwise removal of the phosphate groups, releasing energy at each stage, converting it to adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and eventually the nucleoside, adenosine. Although most adenine bases are salvaged in order to re-use them, degradation of purines is achieved by metabolism to the common base xanthine and then to uric acid, which is excreted in the urine.

The extracellular actions of ATP are mediated via membrane-bound receptors on a huge variety of cell types, resulting in actions on vasodilatation, platelet aggregation, immune responses, endocrine and exocrine secretion, inflammation and pain (see Burnstock 2001a). Receptors were initially classified according to their responses to adenosine (P1 receptors) and ATP or ADP (P2 receptors) (Burnstock 1978). Since then, four subtypes of G protein-coupled P1 receptor have been cloned: A_1, A_{2A}, A_{2B} and A_3 (Olah and Stiles 1995; Ralevic and Burnstock 1998; Baraldi et al. 2000; Fredholm et al. 2001). Like other G protein-coupled receptors, there are seven transmembrane domains and these areas are critical for their ligand binding characteristics. P2 receptors have been divided into P2X and P2Y subtypes (Burnstock and Kennedy 1985; Abbracchio and Burnstock 1994). Currently there are seven recognised P2X ligand-gated ion channels ($P2X_{1-7}$) and at least eight G protein-coupled P2Y receptor subtypes, including those that are sensitive to pyrimidines as well as purines (see Ralevic and Burnstock 1998; Burnstock 2003).

P2X receptors have two transmembrane domains with intracellular N and C termini that form binding sites for protein kinases. The extracellular loop contains 10

conserved cysteine residues that form a series of disulphide bridges, allowing formation of an ATP binding site and a hydrophobic region for possible channel modulation by cations. The stoichiometry of P2X receptors is thought to involve three subunits that form a stretched trimer (Khakh et al. 2001) and these can be expressed together in a variety of combinations, forming heteromultimers. This arrangement is clearly established for $P2X_{2/3}$ in nodose ganglia and DRG (Lewis et al. 1995; Radford et al. 1997; Dunn et al. 2001), $P2X_{4/6}$ in CNS neurons (Lê et al. 1998), $P2X_{1/5}$ in some blood vessels (Torres et al. 1998; Haines et al. 1999) and $P2X_{2/6}$ in the brainstem (King et al. 2000). Pharmacological profiles of recombinant homomeric receptors demonstrate that $P2X_1$ and $P2X_3$ show rapid densensitisation in response to ATP, whereas the other subtypes show slow desensitisation (Chizh and Illes 2001). Heteromultimers involving both fast and slow types (e.g. $P2X_{2/3}$) show a biphasic response.

Metabotropic P2Y receptors are characterised by a subunit topology of an extracellular N-terminus and intracellular C-terminus, the latter possessing consensus binding motifs for protein kinases. There are seven transmembrane-spanning regions, which help to form the ligand-docking pocket and the intracellular loops contain enough structural diversity to influence the degree of coupling with G proteins. Each receptor binds to a single heterotrimeric G protein, typically $G_{q/11}$. P2Y receptors have low sequency homology at the peptide level (19-55%) and consequently show significant differences in their pharmacological profiles (Burnstock 2003). Some are activated by both purine and pyrimidine nucleotides ($P2Y_{2,4,6}$) and others by purine nucleotides alone ($P2Y_{1,11,12}$). In response to nucleotide activation, recombinant P2Y receptors either activate phospholipase C and release intracellular calcium or affect adenylyl cyclase and alter cAMP levels (see Burnstock 2003). P2Y receptors are expressed on a wide variety of cells and have been implicated in vasodilatation, platelet function, insulin secretion and renal function. There are also examples of P2Y-mediated signalling in long-term

events such as development, regeneration and cellular proliferation. In relation to visceral pain, $P2Y_1$ and $P2Y_4$ receptors have been demonstrated on subpopulations of DRG neurons that also express $P2X_3$ receptors (Ruan and Burnstock 2003) and the $P2Y_2$ and $P2Y_4$ ligand UTP can activate a large proportion of capsaicin-sensitive DRG neurons (Stucky et al. 2003). The majority of these neurons also respond to α,β-methylene ATP (α,β-meATP), indicating that P2Y and P2X receptors are widely coexpressed.

Evidence for the role of ATP as an extracellular signalling molecule in sensory transduction and in particular, nociception, is growing (Burnstock 2000, 2001a). Attention has focused on $P2X_2$ and $P2X_3$ receptors because they coexist in sensory ganglia (see Dunn et al. 2001). The $P2X_3$ subunit is selectively expressed on small diameter sensory neurons in dorsal root ganglia (Chen et al. 1995; Bradbury et al. 1998) and sensory neurons in culture respond to P2X agonists with a pharmacological profile suggestive of $P2X_3$ involvement (Cook et al. 1997; Dunn et al. 2001). $P2X_3$ immunoreactivity is seen in the peripheral projections of sensory neurons in a variety of tissues including the tongue (Bo et al. 1999), the tooth pulp (Alavi et al. 2001), the bladder (Cockayne et al. 2000; Vlaskovska et al. 2001) and gut (Yiangou et al. 2001) and also on the presynaptic membranes in inner lamina II of the spinal dorsal horn (Llewellyn-Smith et al. 1998; Vulchanova et al. 1998). The $P2X_2$ receptor, also present in sensory ganglia, is pH sensitive (King et al. 1996) and along with $P2X_3$ subunits can form heteromultimers that yield ATP-activated currents similar to those found in sensory neurons (Lewis et al. 1995).

In-vitro studies have demonstrated the ability of P2X agonists and antagonists to change afferent nerve activity in other models of pain: knee joint (Dowd et al. 1998) and skin (Hamilton et al. 1999a). Injection of ATP and related agonists into the rat hindpaw results in dose-dependent nocifensive behaviour and localised thermal hyperalgesia

(Hamilton et al. 1999b). In the absence of selective $P2X_3$ receptor agonists and antagonists, $P2X_3$ knock-out mice have been invaluable. These animals have demonstrated bladder hyporeflexia and reduced inflammatory pain-related behaviour (Cockayne et al. 2000; Souslova et al. 2000). This observation that ATP may be a more important factor in the inflammatory setting has been consistently demonstrated. Behavioural studies in rats (Hamilton et al. 1999b; Jarvis et al. 2001;) and humans (Hamilton et al. 2000;) have demonstrated that the pain-inducing effects of ATP are enhanced in states of inflammation and nerve recordings show exaggerated responses to ATP from inflammatory tissues (Hamilton et al. 2001). SP and BK potentiate currents mediated by $P2X_3$ and $P2X_{2/3}$ receptors expressed by Xenopus oocytes (Paukert et al. 2001) and $P2X_3$ receptors are up-regulated in colitis specimens obtained from patients with inflammatory bowel disease (Yiangou et al. 2001) and in DRG neurons in models of chronic nerve injury (Novakovic et al. 1999).

Purinergic signalling in the gut

Neuromuscular transmission

It was in the guinea-pig taenia coli that ATP was first demonstrated to be a non-adrenergic, non-cholinergic (NANC) transmitter involved in the inhibitory neural control of gut smooth muscle (Burnstock et al. 1970). Since then, NANC inhibitory neuromuscular transmission has been shown to be dominant in many regions of the gut (see Hoyle and Burnstock 1989; Burnstock 2001c). While ATP, nitric oxide (NO) and vasoactive intestinal peptide (VIP) appear to be cotransmitters in many of these NANC nerves, there are wide variations between different species and regions of the gut. In general, NO seems to be more important in the foregut regions whereas ATP is more involved in the hindgut regions (see Burnstock 2001c). For example, NO is the most

dominant NANC transmitter at the lower oesophageal sphincter (Murray et al. 1991; Tottrup et al. 1993) but both ATP and NO mediate fast relaxation of the circular muscle of the guinea-pig colon (Maggi and Giuliani 1996).

In bullfrog stomach, ATP was shown to be released by electrical field stimulation of the vagus nerve, which was associated with smooth muscle relaxation (Burnstock et al. 1970; Satchell and Burnstock 1971). In addition, in the presence of α- and β-adrenergic blockers, ATP and related substances were shown to inhibit both spike activity and slow wave generation in isolated smooth muscle of the stomach, suggesting a direct action (Ohkawa and Watanabe 1976). Later it was shown that ATP and analogues could mimic the relaxation and hyperpolarisation produced by NANC nerve stimulation. The effect of ATP on the rat stomach is biphasic (Burnstock et al. 1970) and involves an early and brief relaxation followed by a sustained contraction. The latter effects may indicate the presence of NANC excitatory transmission (Rhee et al. 1996) or an indirect action via the production of prostaglandins (Burnstock et al. 1975). The receptor types involved in these responses are still debated. P2Y receptors are thought to be responsible for relaxation, whereas P2X receptors are involved in contraction (see Burnstock 2001c).

Evidence exists to support purinergic inhibitory neuromuscular transmission in the small intestine. One study in the human jejunum concluded that the fast inhibitory junction potential (IJP) was mediated in part by an ADPβS-sensitive P2Y receptor (Xue et al. 1999). ATP and α,β-meATP have both been shown to contract guinea-pig ileum and these contractions were inhibited by atropine, suggesting that P2 receptors mediated release of acetylcholine (ACh) from cholinergic nerves (Northway and Burks 1980; Moody and Burnstock 1982). ATP might also cause a fast contraction by stimulation of cholinergic neurones within the myenteric plexus (Sakai et al. 1979). Receptor types governing small intestinal motility are predominantly P2Y in nature, but P2X receptors

play a role in some areas of the gut, for example contraction of the rat duodenum (Johnson et al. 1996; Burnstock 2001c).

In the taenia coli, Burnstock et al. (1970) showed a block of responses to exogenously applied ATP and NANC inhibitory nerve stimulation by high concentrations of quinidine. A high concentration of phentolamine was also effective in blocking IJPs and the actions of ATP (Tomita and Watanabe 1973). Further evidence for purinergic neuromuscular transmission in the large bowel came from studies that showed that stimulation of NANC inhibitory nerves in taenia coli preincubated with [^{3}H]adenosine, which was taken up by the nerves and retained primarily as [^{3}H]ATP, led to release of tritium (Su et al. 1971). The P2 receptor antagonist, suramin, has been shown to block IJPs in guinea-pig taenia coli (Hoyle et al. 1990) and theophylline, a P1 antagonist, blocked the relaxations produced by adenosine without affecting the response to ATP (Brown and Burnstock 1981). More recent studies have shown that adenosine analogues have been shown to relax guinea-pig teniae coli via an A_{2B} receptor (Prentice and Hourani 1997). Both NO and ATP have been implicated in NANC inhibitory transmission in the circular muscle of the colon although there appear to be differences between species (Burnstock 2001c). There are believed to be three components of this transmission in the guinea-pig colon. Firstly, a fast relaxation in response to low frequency stimulation, probably involving ATP which mobilises intracellular calcium leading to the activation of apamin-sensitive K^+ channels. Secondly, a fast relaxation at higher frequencies of stimulation involving production and release of NO and thirdly, a slowly developing relaxation at higher frequencies of stimulation that is apamin-resistant probably utilising VIP (Maggi and Giuliani 1996). P2 receptors are involved in both contraction and relaxation in this preparation (Zagorodnyuk and Maggi 1998). ATP elicits relaxation of the rat colon longitudinal muscle via a P2Y receptor and also by a P1 (A_2) receptor following breakdown to

adenosine (Bailey and Hourani 1992). ATP (via P2Y receptors) and adenosine (via A_1 receptors) can contract the muscularis mucosa of the colon in the rat (Bailey and Hourani 1990), ATP possibly producing its effects via $P2X_1$ receptors (Burnstock 2001c).

NO is clearly established as an important transmitter in all the sphincters in the gastrointestinal tract (Wang et al. 1996). It appears to be dominant in the lower oesophageal and ileocolic sphincters, but there is good evidence for the involvement of ATP in the pyloric and internal anal sphincters (Soediono and Burnstock 1994; Rae and Muir 1996). Both adenosine and ATP are known to cause concentration-dependent relaxations of the internal anal sphincter in the rat (Nissan et al. 1984) and guinea-pig (Crema et al. 1983). In the latter preparation, IJPs could be blocked with suramin and inhibitors of nitric oxide synthase (NOS) indicating a role for both ATP and NO in NANC inhibitory transmission (Rae and Muir 1996).

Epithelial secretion in the gut

ATP has been implicated in the secretion of mucus from gastric goblet cells (Bertrand et al. 1999) and cAMP-mediated stimulation of active ion transport in small intestinal enterocytes (Korman et al. 1982). The mammalian colon is primarily concerned with the absorption of NaCl, however, rises in intracellular calcium and cAMP are important second messengers that activate NaCl secretion. Experiments in the rat distal colon have shown that ATP induces intracellular calcium in the basal crypt enterocytes and acts via a P2Y receptor to secrete NaCl (Leipziger et al. 1997). ATP also stimulates transepithelial secretion across rat cholangiocyte monolayers in culture (Roman and Fitz 1999).

Purinergic vascular control in the gut

ATP, along with noradrenaline (NA), is a cotransmitter in sympathetic nerves supplying the vasculature of the gut. It is responsible for the initial rapid response of the intestinal microcirculation to sympathetic nerve stimulation and this is mediated by P2X receptors (Taylor and Parsons 1989 and 1991). It is now known that excitatory junction potentials (EJPs) and constrictions of the submucosal arterioles are mediated solely through the activation of P2X receptors by ATP; the function of neurally released NA was to act at prejunctional α_2-adrenoceptors to depress transmitter release (Evans and Suprenant 1992). Paradoxically, if ATP is injected intra-arterially, the response of the vessel is to vasodilate, but this is likely to be due to activation of P2Y receptors on endothelial cells to release NO, a potent vasodilator or by breakdown to adenosine to act on P1 receptors (see Burnstock and Ralevic 1994).

Purinergic signalling in the enteric plexus

In experiments studying the effects of ATP and its analogues on myenteric neurons of the guinea-pig ileum, about 80% of AH neurons (sensory) and 90% of S neurones (motor) responded to the purinergic stimulus (Katayama and Morita 1989). The rank order of potency indicated that a P2X receptor was responsible for the rapid inward currents (Barajas-Lopez et al. 1996). These currents, found in both small and large intestine, were mostly slowly desensitising and were attributed to $P2X_1$ receptors, whereas a small minority of rapidly desensitising currents might be mediated by $P2X_2$ receptors (Zhou and Galligan 1996; LePard et al. 1997). Extracellular ATP caused rises in intracellular calcium and led to phospholipase C dependent signalling (Kimball et al. 1996). ATP regulates synaptic transmission by presynaptic and postsynaptic mechanisms and in doing so, augments nicotinic fast depolarisations produced by ACh, but inhibits muscarinic and SP-mediated depolarisations in both AH and S neurones

(Kamiji et al. 1994). Exogenous and endogenous ATP, released during increases in intraluminal pressure, inhibit intestinal peristalsis in guinea-pig via different apamin-sensitive purine receptor mechanisms. Exogenous ATP depresses peristalsis mostly via suramin- and pyridoxyl 5-phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS)-insensitive P2 receptors, whereas endogenous purines act via P2 receptors sensitive to both suramin and PPADS (Heinemann et al. 1999). Recent evidence suggests that ATP plays an important role in excitatory neuro-neuronal transmission in both ascending and descending reflex pathways to the longitudinal and circular muscles of the guinea-pig ileum and this is triggered by mucosal stimulation (Spencer et al. 2000). Adenosine suppresses nicotinic synaptic transmission in AH myenteric neurones by activating high affinity presynaptic A_1 receptors (Christofi et al. 1994a), although a small minority express the A_2 subtype and mediate excitatory responses in these neurones (Christofi et al. 1994b). The P1 agonist, 5'-*N*-ethylcarboxamidoadenosine (NECA) is a potent inhibitor of morphine withdrawal induced diarrhoea in rats acting by inhibiting secretion as well as intestinal peristalsis and is thought to exert its effects by A_1 receptors and A_{2B} receptors respectively. Stimulation of these receptors may be of clinical value in the treatment of diarrhoea (Tomaru et al. 1994; Hancock and Coupar 1995). Several enteric neurotransmitters have been claimed to modulate release of ATP from neurones in the myenteric plexus. Enkephalins have been shown to inhibit NANC IJPs recorded in human colon (Hoyle et al. 1990) and 5-HT can cause release of ATP from myenteric nerve varicosities (Al-Humayyd and White 1985). Evidence has been presented that gamma aminobutyric acid (GABA) receptors mediate relaxation of rat duodenum by activating intramural NANC neurones (Maggi et al. 1984).

Intracellular recordings from submucous neurons in the guinea-pig ileum showed that ATP induced fast transient depolarisations of the majority of AH-type neurons and fast transient depolarisation followed by slower onset longer lasting

depolarisation in S-type neurons (Barajas-Lopez et al. 1994). Recent immunohistochemical studies have shown that specific subtypes of enteric neurons express $P2X_2$ and $P2X_3$ receptors, including inhibitory motor neurons, non-cholinergic secretomotor neurons in the guinea-pig ileum and intrinsic primary afferent neurons in the guinea-pig colon (Castelucci et al. 2002; Van Nassauw et al. 2002; Poole et al. 2002).

Purinergic signalling in extrinsic enteric nerves

ATP can also activate extrinsic afferent neurons in the gut in a concentration-dependent way. Kirkup et al. (1999) showed that application of intra-arterial ATP and α,β-meATP gave rise to a biphasic increase in mesenteric afferent nerve activity in the rat mediated by P2X receptors, which was blocked by suramin and PPADS. Adenosine was also capable of increasing mesenteric afferent discharge via A_1 and A_{2B} receptors (Kirkup et al. 1998).

Experimental rationale

The $P2X_3$ receptor is expressed primarily on small diameter sensory neurons (Chen et al. 1995) and these receptors form heteromultimers with $P2X_2$ subunits in many DRG neurons (Dunn et al. 2001). Pelvic nerve fibres travelling via DRG are known to be important in the transmission of painful stimuli, in particular, colorectal distension in the rat (Sengupta and Gebhart 1994). ATP is known to be an algesic compound in humans (Bleehan and Keele 1977; Hamilton et al. 2000) and in the rat, especially in inflammatory conditions (Hamilton et al. 1999b). A working hypothesis of purine-mediated mechanosensory transduction has been proposed (Burnstock 1999 and 2001b). This states that ATP released during distension from epithelial cells lining tubes

(such as ureter or gut), and sacs (such as bladder), acts on $P2X_3$ and/or $P2X_{2/3}$ receptors on a subepithelial nerve plexus to initiate impulses that are relayed via the spinal cord to pain centres in the brain. It is with this background that we aim to test this hypothesis in relation to the rat colorectum.

CHAPTER TWO

METHODS

Animals

Experiments were performed using adult male and female Sprague-Dawley rats (240-320 g) that were allowed free access to food and water. Animals were humanely sacrificed by exposure to rising levels of carbon dioxide and cervical dislocation in accordance with U.K. Home Office regulations covering Schedule One procedures.

Induction of colitis

In those experiments where inflamed colorectum was compared to controls, colitis was induced by administration of an intrarectal enema (8cm from the anus) of 30% trinitrobenzenesulfonic acid (TNBS) in ethanol at a dose of 80mg/kg body weight (Morris et al. 1989). The enemas were given through 6-French medical-grade polyurethane enteral feeding tubes while the rats were under light halothane anaesthesia. Animals in the control group were given an equivilent enema of normal saline. A model of chronic inflammation was favoured because this most closely mimmicks human IBD. Previous work has suggested that in the TNBS model of colitis in rats, chronic inflammation is evident at day 2 and evolves over several weeks, the most severe period of inflammation starts at day 5 (Morris et al. 1989). Animals were therefore sacrificed between 5 and 7 days later for the in-vitro work and 10 days later for examination of the DRG. Assessment of colitis was based on both the macroscopic and microscopic features of the colorectum.

Immunohistochemistry

After death, the animals were perfused through the aorta with 60 ml of fixative (4% formaldehyde with 0.2% picric acid). The distal colon was removed and cut

transversely into 10 mm lengths in preparation for whole mount immunohistochemistry. After stretching the colon over a glass pipette, the outer layer of smooth muscle was carefully peeled off and the remaining colon was cut longitudinally (to provide a square of tissue) and placed in phosphate-buffered saline (PBS). For frozen sections, the colon was embedded in OCT compound (BDH/Merck, Leicester, UK) and frozen in isopentane, precooled in liquid nitrogen. Tissue was sectioned at 12μm using a Reichert Jung CM1800 cryostat. Preparations were firstly washed 3×5 min in 0.01 mol/L pH 7.2 phosphate buffer saline (PBS), then incubated in 1.0% H_2O_2 for 30 min to block the endogenous peroxidase. Pre-incubation in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min followed, then incubation overnight at 4°C with $P2X_2$ and $P2X_3$ antibodies, diluted 1:500 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS). Subsequently, tissues were incubated with biotinylated donkey-anti-rabbit IgG (Jackson ImmunoResearch, Luton, UK) diluted 1:500 in antibody dilution solution for 1 h at 37°C and then with streptavidin-HRP (Sigma, Poole, UK) diluted 1:1000 in PBS for 1 hour at 37°C. Finally, a nickel-intensified diaminobenzidine (DAB) reaction was used to visualize immunoreactivity. All the incubations and reactions were separated by 3×10 min washes in PBS.

For double staining among $P2X_2$, $P2X_3$ and calbindin D-28k, the preparations were washed 3×5 min in PBS, then pre-incubated in antibody dilution solution for 30 min. This was followed by incubation overnight at 4°C with $P2X_2$ and $P2X_3$ antibodies diluted 1:500 and calbindin (mouse-anti-rat, SWANT) diluted 1:5000 in antibody dilution solution. Subsequently, the preparations were incubated with Cy3 conjugated donkey-anti-rabbit IgG (Jackson) diluted 1:300 for P2X antibodies and FITC conjugated donkey-anti-mouse IgG (Jackson) diluted 1:200 for calbindin in antibody dilution solution for 1 h at room termperature. All the incubations and reactions were separated by 3×10 min washes in PBS. The preparations were mounted, dehydrated,

cleared, covered and observed under a Zeiss Axioplan microscope (Jena, Germany) at an excitation of 520 nm for immunofluorescent sections. Images were captured by digital camera (Leica, Germany).

Control experiments were carried out with $P2X_2$ and $P2X_3$ antibodies re-absorbed with $P2X_2$ and $P2X_3$ peptides. No staining was observed in those preparations incubated with the antibody solutions re-absorbed with $P2X_2$ and $P2X_3$ peptides.

ATP assay

This was an in-vitro preparation based on previous studies involving distension of the guinea pig ureter completed by Knight et al. (2002). The distal colon and rectum were dissected from the pelvis with attached pelvic nerve and placed in a bath superfused with oxygenated Krebs solution (contents in mM: NaCl 120; KCl 5.9; NaH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 15.4; $CaCl_2$ 2.5; Glucose 11.5). Both proximal and distal ends of the 30mm length of bowel were secured to 8.5 Fr. 3-way cannulae and the lumen perfused with Krebs solution. Ports on the cannulae were connected to a pressure transducer, large and small drainage tubing and infusion tubing which were connected in turn to a syringe driver (sp210iw; World Precision Instruments, Sarasota, FL). In all cases, the tissues were allowed to stabilise in the bath for 60 minutes before gathering data.

Either normal (or inflamed colon where necessary) was distended to pressures between 1 and 90mmHg at random. This was achieved by opening the infusion tubing to a reservoir of Krebs solution that was positioned at various heights in order to achieve a range of intraluminal pressures almost instantaneously. The pressure was held for 5 seconds before clamping the infusion tubing and allowing drainage. Fluid was drained through a short, small diameter tube with a calculated dead space of 50µl (this volume being discarded prior to collection). Samples were immediately frozen in liquid

nitrogen and collected for luminometry using the luciferin-luciferase assay (Taylor et al. 1998).

Pelvic nerve electrophysiology

The experimental apparatus was set up in a similar way to that described for the ATP release studies above. In addition, the attached pelvic nerve was carefully divided into small branches under the microscope and multifibre afferent activity was recorded using a suction glass electrode (tip diameter 50-100μm) connected to a Neurolog headstage (NL 100; Digitimer Ltd, UK) and an AC amplifier (NL 104). Signals were amplified (x10,000), filtered (NL 125, band pass 200-4000Hz) and captured by a computer via a power 1401 analogue-to-digital interface and Spike 2 software (version 4.03, Cambridge Electronic Design, UK). Those branches that did not yield a good response to distension were not used. Two types of distension were used. Graded distensions used the syringe driver (set at a constant rate) to slowly increase the intraluminal pressure against closed drainage, whereas flooding the lumen suddenly from a reservoir at a fixed height gave a phasic distension. Distensions were normally held at 50mmHg for 30 second periods. During set-up, control distensions to 50 mmHg with Krebs were repeated at 10-minute intervals until nerve responses were stable. This pressure is known to represent a noxious stimulus in in-vivo studies (Ness and Gebhart 2001). Applications of the various mediators were applied as a fast 10 ml bolus less than 10 mm away from the serosal surface of the colorectum and left to stagnate in the bath until activity had reduced to baseline levels. When dye was used in this manner, no discolouration was seen in the luminal fluid. Drug concentrations were decided upon by prior experimental dose-response data in our colorectal preparation.

During analysis of the multifibre recordings, single unit recordings were extracted by the Spike2 software. Advice from the manufacturers (Cambridge Electonic

Design, Cambridge, UK) was taken in trying to get the most sensitive discriminatory settings. We have also analysed the difference between small (< 12 units) and larger (> 12 units) recordings to find out if the percentage of units responding to a given agent were different in the two groups at the same discriminatory settings. This revealed that in our hands, the size of the pelvic nerve bundle did not alter the results.

DRG whole-cell voltage-clamp recordings

The ganglia were placed in Leibovitz L-15 medium (Life Technologies, Paisley, UK) and were desheathed, cut and incubated in 4 ml Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution with 10 mM Hepes buffer, pH 7.4 (HBSS; Life Technologies) containing 1.5 mg/ml collagenase (Class II, Worthington Biochemical Corporation, UK) and 6 mg/ml bovine serum albumin (Sigma, Poole, UK) at 37°C for 45 min. The ganglia were then incubated in 4 ml HBSS containing 1 mg/ml trypsin (Sigma) at 37°C for 15 min. The solution was replaced with 1 ml growth medium comprising of L-15 medium supplemented with 10 % bovine serum, 50 ng/ml nerve growth factor, 0.2% $NaHCO_3$, 5.5 mg/ml glucose, 200 i.u./ml penicillin and 2g/ml streptomycin. The ganglia were dissociated into single neurons by gentle trituration, then centrifuged at 160 g for 5 min. The resulting pellet was resuspended in 0.8 ml growth medium and plated onto 35 mm Petri dishes coated with 10 μg/ml laminin (Sigma). Cells were maintained at 37°C in a humidified atmosphere containing 5 % CO_2 and used within 30 hours. Whole-cell voltage-clamp recordings were carried out at room temperature using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) with membrane potential held at -60 mV. Data were aquired using pClamp software (Version 6.1. Axon Instruments). Signals were filtered at 2 kHz (-3 dB frequency, Bessel filter, 80 dB per decade), then digitized at 10-50 kHz (Digidata 1320A interface, Axon Instruments) and stored on the hard disk of a PC for viewing and analysis. Traces were acquired using

Clampfit (pCLAMP software) and plotted using Origin7 (Microcal, Northampton, MA, USA). External solution contained (mM): NaCl 154, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, Hepes 10 and glucose 5.6; the pH was adjusted to 7.4 using NaOH. Recording electrodes (resistance 2-4 M) were filled with internal solution which contained (mM): KCl 120, Hepes 10 and tripotassium citrate 10; the pH was adjusted to 7.2 using KOH. Solutions of ATP were prepared using deionized water and stored frozen, then diluted in extracellular bathing solution to the final concentration. They were applied rapidly through a manifold comprising 3 capillaries made of fused silica coated with polyimide with 250 μm internal diameter (SGE, Milton Kegnes, UK), connected to a single outlet made of the same tubing, which was placed about 200 μm from the cell. Solutions were delivered by gravity flow from independent reservoirs. One barrel was used to apply drug-free solution to enable rapid termination of drug application. Agonists were separately applied for 4 sec. at 2 min. intervals, which was sufficient for responses to be reproducible.

Chemicals

ARL-67156, ATP (disodium salt), α,β-methylene ATP (lithium salt), bradykinin (BK), capsaicin, 5-hydroxytryptamine (5-HT), 8-para-sulfophenyl-thoephylline (8pSPT), prostaglandin E_2 (PGE_2), pyridoxyl 5-phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS), substance P (SP), suramin (hexasodium salt) and trinitrobenzenesulfonic acid (TNBS) were all obtained from Sigma, Poole, UK. 2',3'-O-trinitrophenyl-ATP (TNP-ATP) was obtained from Molecular Probes, Leiden, Netherlands. All chemicals were diluted in Krebs solution to required concentrations before use. Titration of concentrated hydrochloric acid in Krebs solution was used to provide the correct pH in the relevant experiments.

Statistical analysis

The results for all experiments are presented as mean ±SEM. Data were compared by Student's t-test unless otherwise stated and differences considered statistically significant at $P< 0.05$.

CHAPTER THREE

PURINERGIC MECHANISMS CONTRIBUTE TO MECHANOSENSORY TRANSDUCTION IN THE RAT COLORECTUM

Introduction

There is now well-established evidence for the role of adenosine-5'-triphoshate (ATP) as an extracellular signalling molecule in sensory transduction and in particular, nociception (Burnstock 2000). Attention has focused on $P2X_2$ and $P2X_3$ receptors, two members of the larger P2X family of ligand-gated cation channels. $P2X_3$ is selectively expressed on small diameter sensory neurons in dorsal root ganglia (Chen et al. 1995; Bradbury et al. 1998) and sensory neurons in culture respond to P2X agonists with a pharmacological profile suggestive of $P2X_3$ involvement (Cook et al. 1997; Dunn et al. 2001). $P2X_3$ immunoreactivity is seen in the peripheral projections of sensory neurons in a variety of tissues including the tongue (Bo et al. 1999), the tooth pulp (Alavi et al. 2001), the bladder (Cockayne et al. 2000) and the gut (Yiangou et al. 2001) and also on the presynaptic membrane in inner lamina II of the spinal dorsal horn (Llewellyn-Smith et al. 1992; Vulchanova et al. 1998). The $P2X_2$ receptor, also present in sensory ganglia, is pH sensitive (King et al. 1996) and along with $P2X_3$ subunits can form heteromultimers that yield ATP-activated currents similar to those found in sensory neurons (Lewis et al. 1995).

In-vitro studies have demonstrated the ability of P2X agonists and antagonists to change afferent nerve activity in models of pain: knee joint (Dowd et al. 1998), skin (Hamilton et al. 1999a) and bladder (Vlaskovska et al. 2001). Injection of ATP and related agonists into the rat hindpaw results in dose-dependent nocifensive behaviour

and localised thermal hyperalgesia (Hamilton et al. 1999b). In the absence of selective $P2X_3$ receptor agonists and antagonists, $P2X_3$ knock-out mice have been invaluable. These animals have demonstrated bladder hyporeflexia and reduced inflammatory pain-related behaviour (Cockayne et al. 2000).

A working hypothesis of purine-mediated mechanosensory transduction has been proposed (Burnstock 1999 and 2001c). ATP released during distension from epithelial cells lining tubes (such as ureter or gut), and sacs (such as bladder), acts on $P2X_3$ and/or $P2X_{2/3}$ receptors on a subepithelial nerve plexus to initiate impulses that are relayed via the spinal cord to pain centres in the brain. Supporting evidence for this comes from demonstration of peripheral purinoceptors in sensory nerves and from studies that show ATP release after distension of the bladder (Ferguson et al. 1997; Vlaskovska et al. 2001) and ureter (Knight et al. 2002). Furthermore, recent studies show that pelvic afferent activity during bladder distension can be potentiated with P2X agonists and attenuated with P2X antagonists (Namasivayam et al. 1999; Vlaskovska et al. 2001; Rong et al. 2002).

The responses of afferent neurons to distension of the stomach and small intestine (Pan et al. 1996; Kunze et al. 1998; Thornton et al. 2002) and colon (Blumberg et al. 1983; Haupt et al. 1983; Kreulen et al. 1986; Bahns et al. 1987; Ness and Gebhart 1998; Janig and Koltzenburg 1991; Sengupta and Gebhart 1994; Lynn and Blackshaw 1999; Kalmari et al. 2001) have been described. A study exploring the effects of ATP on mesenteric afferents of the jejunum in the anaesthetised rat has shown excitatory effects (Kirkup et al. 1999) but there have been no experiments recording afferent nerve activity during distension in relation to purinergic signalling in the colon. In the present study, we aimed to test the hypothesis of purinergic mechanosensory transduction in the rat colorectum.

Results

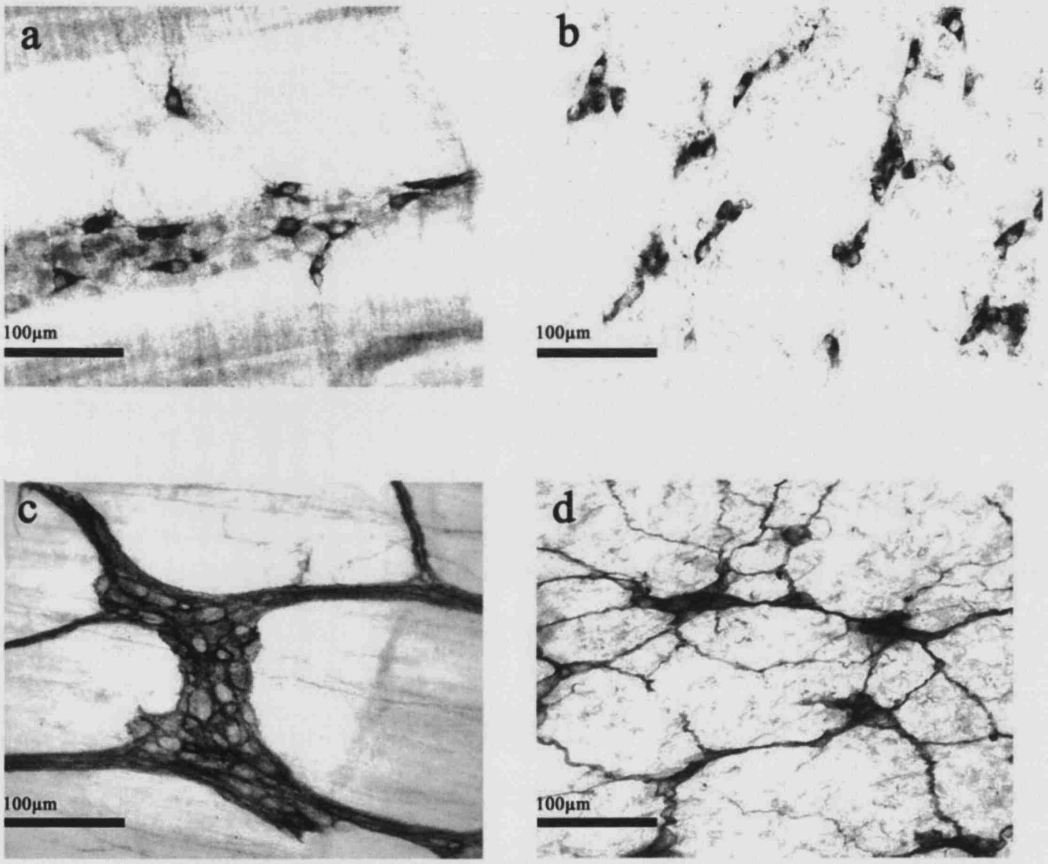
Immunohistochemistry

Many of the neurons in the L_1 and S_1 DRG in the rat show immunoreactivity for $P2X_3$ (fig. 3.2a and 3.2b). $P2X_2$ immunoreactivity was also demonstrated in a subpopulation of these DRG neurons and colocalisation of $P2X_2$ and $P2X_3$ occurred in approximately 20% of neurons. Immunostaining for the $P2X_3$ receptor subunit was also found in a subpopulation of cell bodies as well as their projections in the myenteric plexus (fig. 3.1a) and the submucous plexus (figs. 3.1b and 3.2c) of the rat colorectum. Similarly, immunostaining for the $P2X_2$ subunit in the myenteric plexus (fig. 3.1c) and submucous plexus (fig. 3.1d) show many heavily stained cell bodies and axons. Calbindin staining colocalises with both $P2X_3$ and $P2X_2$ immunostaining in a subpopulation of neurons in the submucous plexus (figs. 3.2e and 3.2f respectively) but colocalisation was not seen in the neurones in the myenteric plexus. Relatively strong nuclear staining for calbindin can be seen (fig. 3.2d) but other studies have confirmed that this is often a typical feature of calbindin immunoreactivity (German et al. 1997).

ATP release

From 4 male and 4 female rats, 139 separate gut distensions ranging from 4 to 90 mmHg were performed. Fig. 3.3 shows the relationship between rising intraluminal pressure and concentration of ATP in the perfusate. Higher pressures were not used for fear of damaging the tissues. Control fluid was collected before each distension and the background level of ATP (mean 0.154 ±0.004 pmol/ml) measured from these samples remained stable regardless of intervening pressures. Pressure-release data were subjected to analysis of variance and were found to be highly statistically significant ($P \leq 0.001$). The distension-induced rise in ATP became significant at pressures over

Fig. 3.1 Immunoreactivity to $P2X_3$ is seen in a subpopulation of neurons in the rat colorectal myenteric plexus (a) and submucous plexus (b). A greater proportion of neurons stain for $P2X_2$ receptors in the myenteric plexus (c) and nerve fibres as well as cell bodies show positive immunoreactivity to $P2X_2$ in the submucous plexus (d).



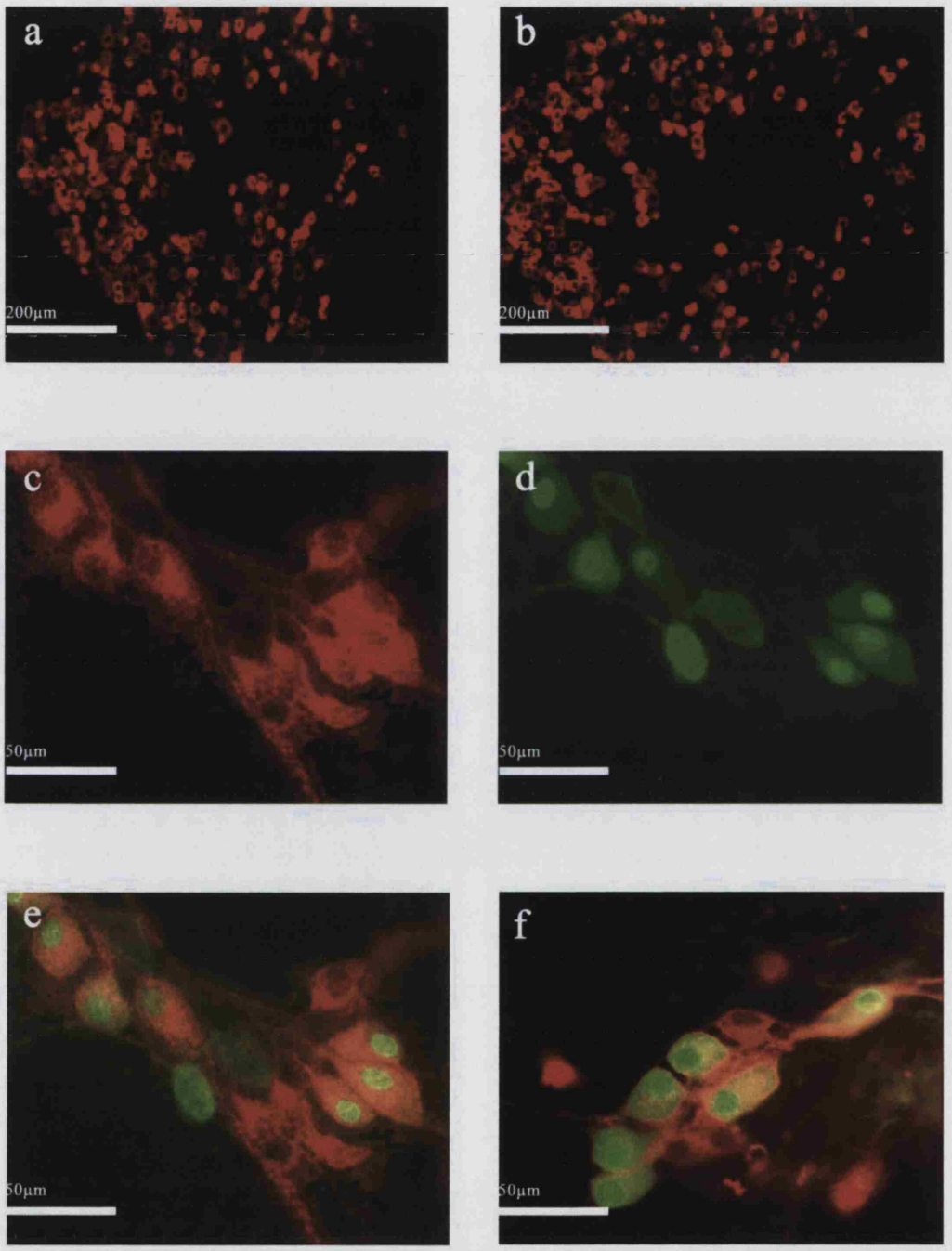


Fig. 3.2 DRG L_1 and S_1 are known to supply the distal colon and rectum and these ganglia show immunoreactivity with $P2X_3$ in many of their cells (a and b respectively). Neurons in the submucous plexus show immunolabelling of $P2X_3$ receptors (c) and calbindin (d) and colocalisation occurs in a subpopulation of these cells (e). Neurons in the submucous plexus are immunopositive for $P2X_2$ receptors and they also colocalise with calbindin in a subpopulation of cells (f).

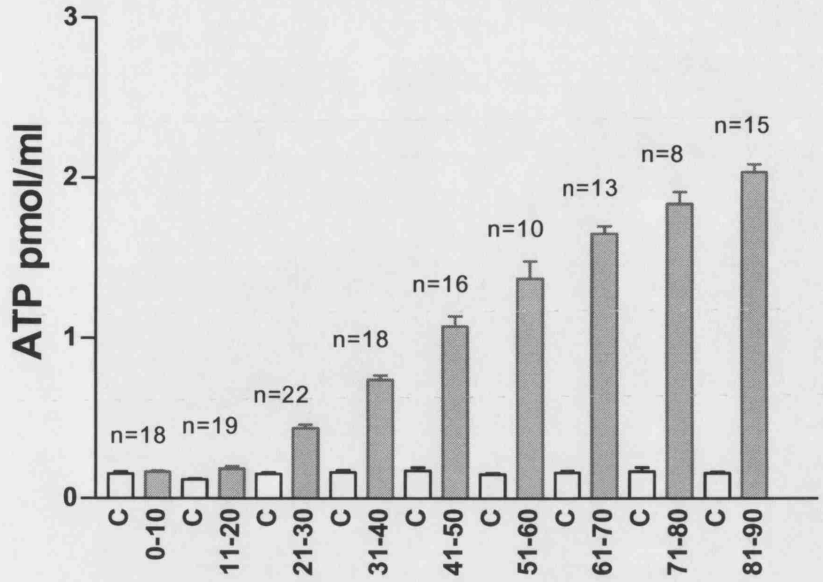


Fig. 3.3 ATP concentration of luminal fluid samples from the rat colorectum during distension. Each column shows the mean ATP release (pmol/ml) ±SEM for each of the pressure groups listed (in mmHg). Control samples were collected prior to each distension (C). The numbers above the columns refer to the number of distensions in each pressure group.

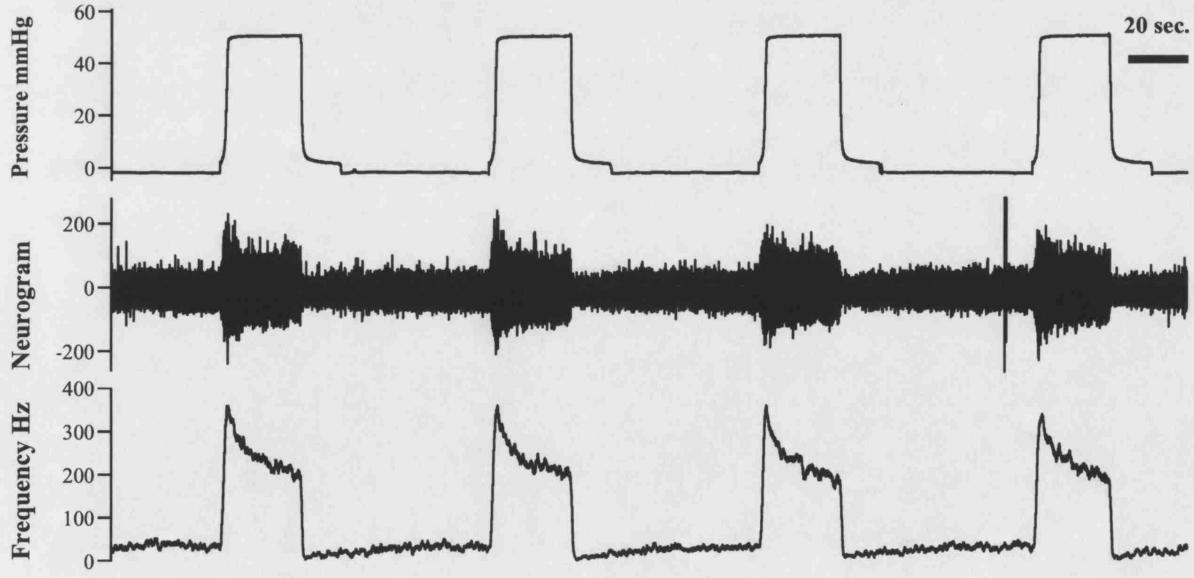


Fig. 3.4 Repeated phasic distensions to 50mmHg in the rat colorectum. Top: intraluminal pressure (mmHg), middle: pelvic afferent nerve activity (μV), bottom: frequency of spikes (Hz).

11 mmHg. Experiments were repeated after removal of the colorectal mucosa. Table 3.1 compares the percentage increase in ATP release during various distension pressures in the two groups. The graded relationship between intraluminal pressure and ATP release was abolished after removal of the mucosal layer. Removal of the mucosa was confirmed by routine histology.

Distension responses of colonic pelvic nerve afferents

Typical responses of multifibre recordings from the pelvic nerve in response to distension are shown in fig. 3.4. Phasic distensions typically produce a sudden burst of spikes that settle to a stable level after 30 to 60 seconds. Responses show good reproducibility even after short recovery periods.

Because the linear relationship between intraluminal pressure and ATP release was disrupted after removal of the mucosa, pelvic nerve recordings were performed to investigate the effect of mucosal ablation on the multifibre responses to distension. In 5 preparations with the mucosa stripped, the colorectum was subjected to 30-second phasic distensions at pressures of 10, 20, 30, 40, 50 and 60 mmHg. The responses were compared with 5 normal controls. There were reductions in mean nerve activity of 48.0 ±5.6% (10 mmHg), 26.9 ±2.6% (20 mmHg), 28.6 ±2.3% (30 mmHg), 26.5 ±2.2% (40 mmHg), 33.0 ±2.6% (50 mmHg) and 28.0 ±2.2% (60 mmHg). The overall mean reduction in afferent activity was 31.8 ±2.9% ($P = 0.007$; analysis of variance).

Pressure (mmHg)	Percentage increase in ATP release during distension	
	Normal colorectum	Mucosa removed
0-10	17 ±8	17 ±9
11-20	66 ±15	69 ±15
21-30	196 ±20	32 ±9
31-40	386 ±32	25 ±13
41-50	602 ±68	32 ±7
51-60	836 ±96	53 ±30
61-70	1023 ±103	10 ±12
71-80	1125 ±142	46 ±8
81-90	1216 ±62	80 ±19

Table 3.1 ATP release from the rat colorectum during distension: percentage increase from baseline levels from the intact gut (middle column) and from gut where the mucosa had been removed (right hand column).

Effect of ATP on colonic afferents

Application of ATP through the lumen of the colon did not produce consistent activation of pelvic nerve afferents. Those that responded (15 out of 23, 65%) were of long latency and variable character. In contrast, application of ATP to the serosal surface of the colon evoked consistent, rapid responses with a mean latency of 13.7 ±0.85 seconds. Fig. 3.5 shows that the percentage increase in peak firing rate from basal activity is dose-dependent. Serosal application of α,β-meATP, a stable ATP analogue that is active on the $P2X_3$ receptor, was able to evoke a response at a concentration that was sub-threshold for ATP (100 μM) and also produced a greater response than ATP at the same 1mM concentration (see fig. 3.6). The latency of evoked responses was similar for α,β-meATP and ATP. At a concentration of 100 μM, the P2X receptor antagonists, suramin and PPADS, were able to abolish the responses to both ATP and α,β-meATP.

Effects of P2X agonists and antagonists on the afferent response to distension

When the colon was distended in the presence of ATP, the peak response of the pelvic nerve was increased by 17.9 ±1.4% (1 mM; n=4), 22.6 ±4.2% (3 mM; n=8), 25.2 ±1.3% (5 mM; n=9). α,β-meATP increased the distension-induced response at lower concentrations: 21.4 ± 0.8% (100 μM; n=4), 24.8 ±3.1% (1 mM; n=5). Fig. 3.7 shows an example of distension-induced afferent discharge before and during serosal application of α,β-meATP.

Experiments on 7 preparations showed that the non-selective P2 receptor antagonists, PPADS 100 μM and suramin 300 μM, reduced peak firing in response to distension by 24.7 ±2.1% and 23.4 ±2.4% respectively. Fig. 3.8 shows the effect of the selective $P2X_1$, $P2X_3$ and $P2X_{2/3}$ heteromultimer antagonist TNP-ATP (60 μM). The

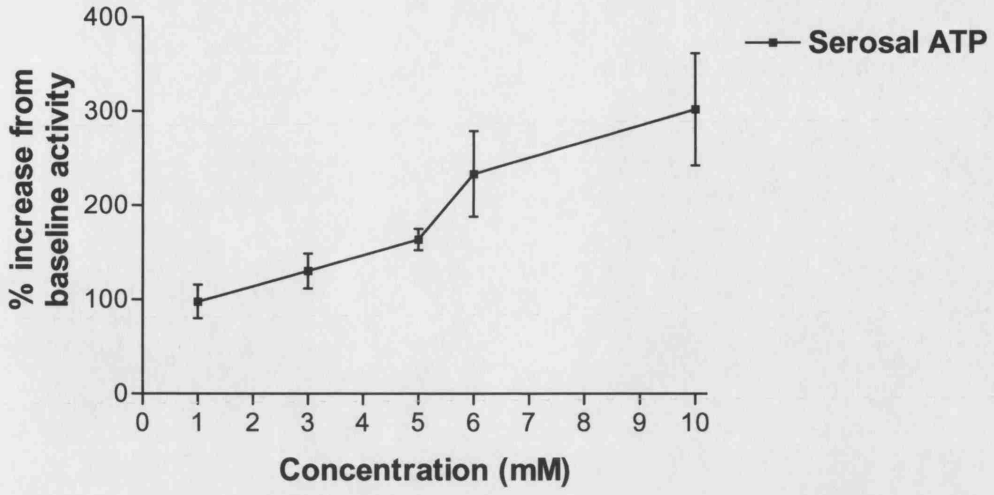


Fig. 3.5 Percentage increase from baseline activity of the pelvic nerve after administation of different concentrations of ATP to the colorectal serosa.

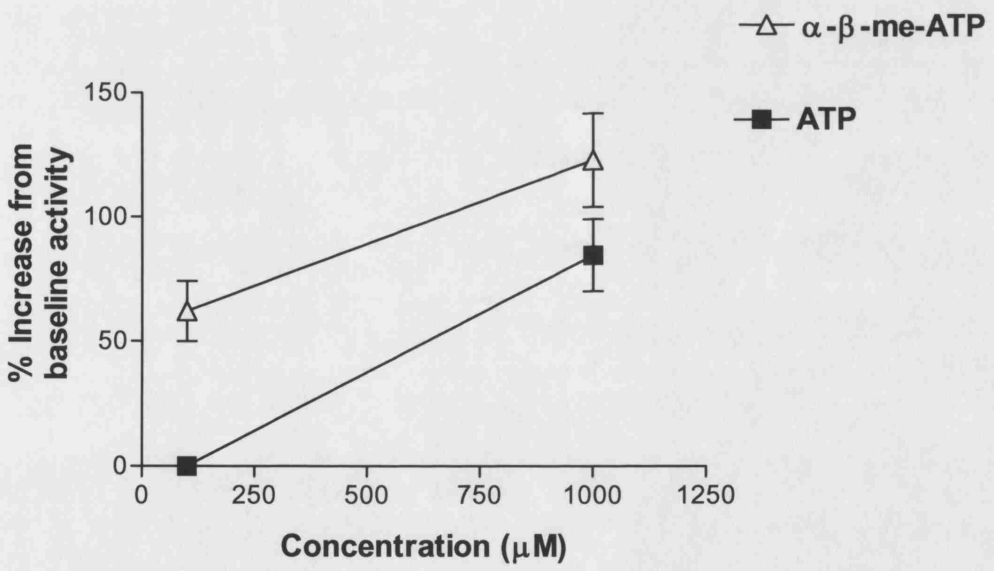


Fig. 3.6 Comparison between the magnitude of the responses of pelvic nerve afferents to ATP and α,β-meATP when applied to the colorectal serosa. For each concentration the percentage increase from baseline activity is shown and plotted as mean ±SEM. (P =0.0583 for the 1mM group).

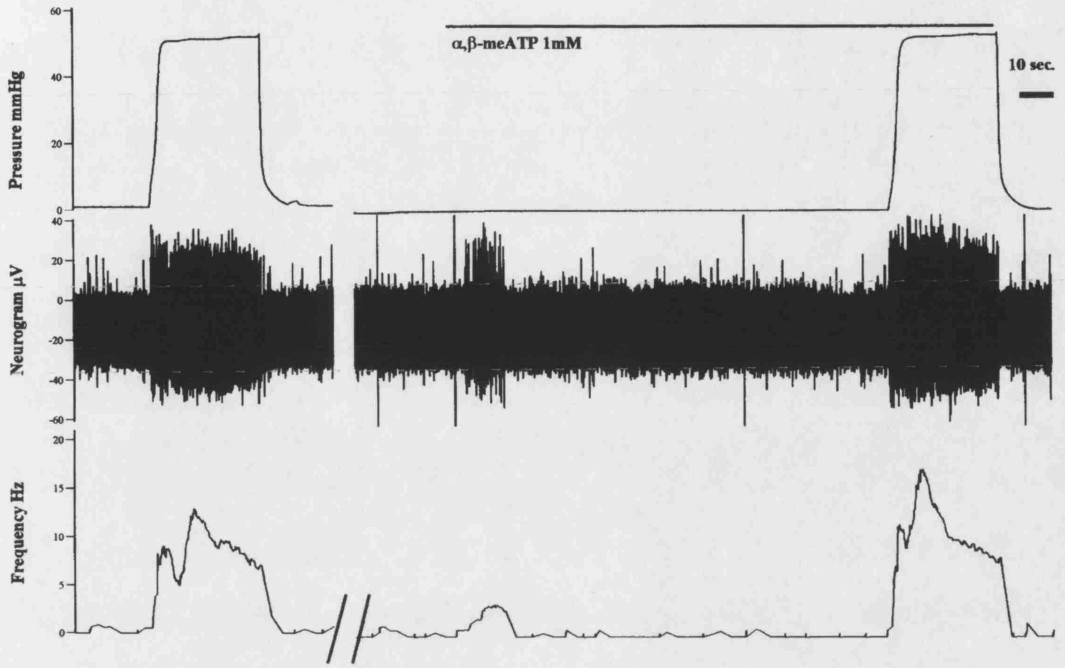


Fig. 3.7 Example of administration of α,β-meATP 1 mM to the rat colorectal serosa, producing a burst of activity and increasing the response to subsequent distension. Control distension is shown on the left.

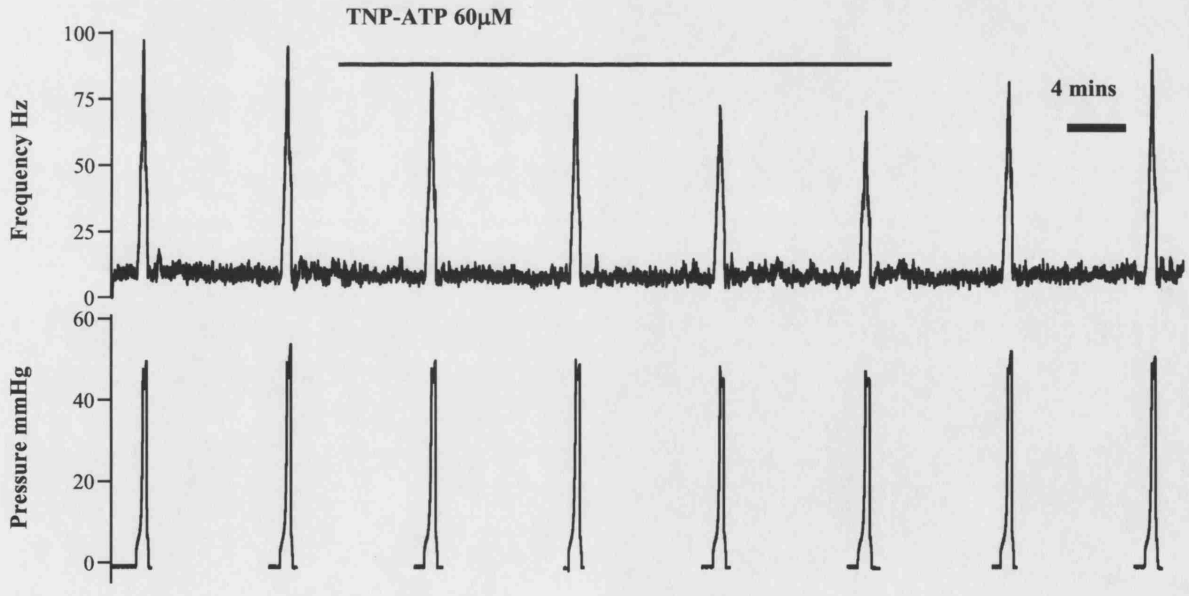


Fig. 3.8 Multiunit recording from the pelvic nerve in response to distension, showing inhibition of peak afferent activity during administration of the $P2X_1$, $P2X_3$ and $P2X_{2/3}$ antagonist TNP-ATP 60 μM. Recovery is seen with washout. Top: pressure (mmHg), middle: nerve activity (μV), bottom: frequency of spikes (Hz).

mean reduction in sensory nerve discharge in the presence of TNP-ATP was 26.2 ±3.3% (n=11). In order to exclude the possibility that the observed reduction in afferent activity was due to a non-specific effect of the antagonists, separate experiments were performed. 5-HT 1 mM was applied to the colorectal serosa before and after circulation of PPADS 100 μM. In the first 30 seconds after application, there was no significant difference ($P = 0.99$) in the mean nerve activity elicited by 5-HT either with or without PPADS (n=5). Similar results were obtained with TNP-ATP.

The effect of ATP metabolism on pelvic nerve excitation was studied. In 6 preparations, the colon was distended in the presence of the ATPase inhibitor ARL-67156. Mean activity was measured for every ten-second period during the distension and these were compared to controls. Activity was augmented during the early phase by up to 17.2 ±3.8% and reduced during the late phase of distension by as much as 12.9 ±5.2% during ATPase inhibition (see fig. 3.9). Analysis of variance of the two groups showed that they were significantly different ($P \leq 0.0001$).

In a small number of preliminary studies, adenosine was applied to the serosa and this resulted in afferent nerve excitation. To assess whether there was an adenosine component to the ATP-induced afferent activation or to the distension responses, the non-selective adenosine antagonist 8-para-sulfophenyltheophylline (8p-SPT) was used. In comparison to control responses to serosal ATP, there was a 27.5 ±5.0% (n=6) reduction in peak activity after 8p-SPT 100 μM was circulated for 20 minutes. No effect was seen on the nerve response to serosal α,β-meATP. For the distension response, sustaining intraluminal pressure at 50 mmHg for 2 minutes allowed assessment of afferent activity over 10-second intervals. Fig. 3.10 shows that although activity was reduced in all intervals (mean 25.6 ±0.78%), no period was especially affected.

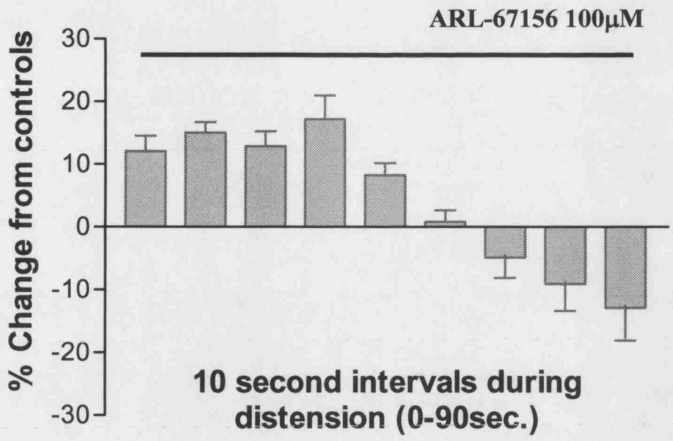


Fig. 3.9 Pelvic nerve activity during distension was compared before and after the application of the ATPase inhibitor, ARL-67156. The graph shows the percentage change from controls over ten second intervals.

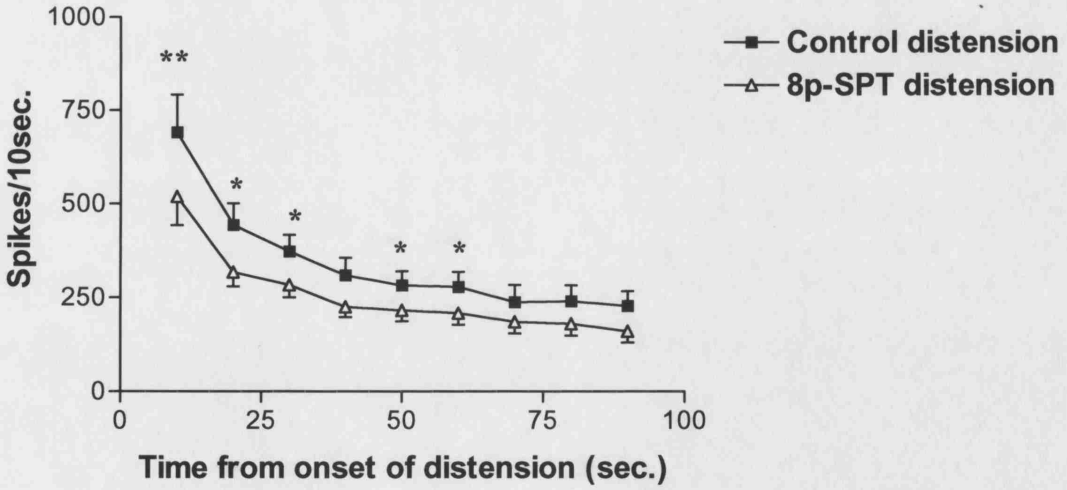


*Fig. 3.10 The effect of the general adenosine antagonist, 8-p-SPT 100 μM on pelvic nerve activity in response to distension. Each data point represents the average activity for the preceding 10 seconds. Statistical significance was assessed by paired Student's t-test; *P<0.05; ** P<0.01.*

Single unit analysis

Fig. 3.11 demonstrates a single unit that was activated by both ATP and distension. The response was dose-dependent. Computer analysis of 15 suitable multiunit recordings of distension responses revealed a total of 137 individual units. 106 of these (77%) were low threshold fibres with a mean threshold of activation of 6.83 ±0.29 mmHg and 31 units (23%) were high threshold fibres with a mean threshold of activation of 23.46 ±2.03 mmHg. Of those units that responded to a distension pressure of 50 mmHg, 78% responded to α,β-meATP. Three units were initially silent in response to distension but could be sensitised by α,β-meATP; one responded to distension at a low threshold and two at a high threshold following treatment with α,β-meATP. The majority of both low and high threshold fibres could be activated by α,β-meATP (77% and 82% respectively). Of those high threshold fibres that were activated by α,β-meATP, 86% of them contributed to the increased responses to distension, whereas the same could only be said of 46% of low threshold fibres. In the presence of PPADS, all of the high threshold fibres reduced their frequency of firing whereas only 63% of low threshold fibres were inhibited. Fig. 3.12 demonstrates that of the low threshold fibres that responded to α,β-meATP, the mean threshold of activation was similar before and after application of the agonist (7.60 ±0.42 mmHg vs. 6.62 ±0.49 mmHg). In contrast, the mean onset of activation of high threshold fibres was significantly reduced by α,β-meATP from 28.07 ±3.36 mmHg to 15.14 ±3.10 mmHg ($P = 0.0013$). PPADS was able to significantly increase the threshold of activation in both low (6.56 ± 0.42 mmHg to 10.42 ± 1.19 mmHg; $P = 0.0006$) and high threshold fibres (19.51 ± 2.08 mmHg to 28.98 ±4.16 mmHg; $P = 0.0092$).

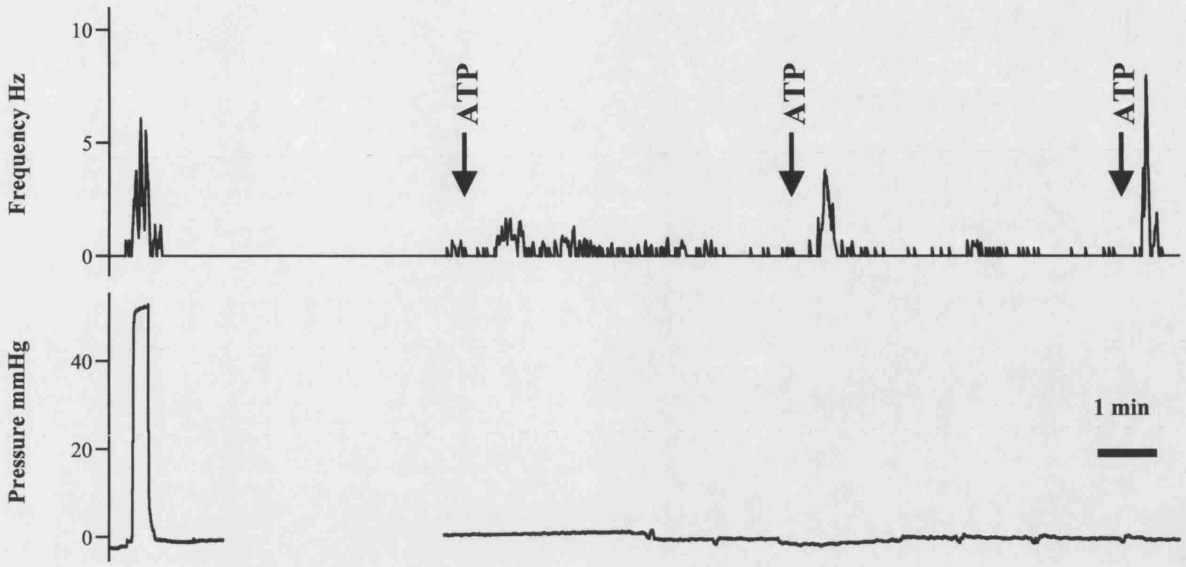


Fig. 3.11 Single unit analysis demonstrates that fibres responding to distension are also activated by ATP in a dose-dependent way. Top: frequency of single unit firing (Hz), bottom: pressure (mmHg).

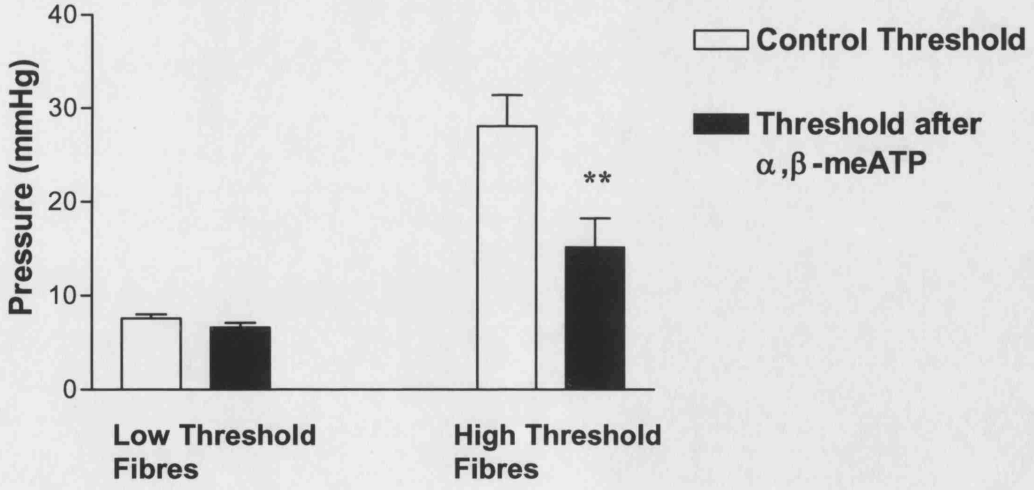


*Fig. 3.12 The effect of α,β-meATP on the threshold of activation of single units responding to colorectal distension: comparison between low and high threshold fibres. Statistical significance was assessed by paired Student's t-test; ** P<0.01.*

Discussion

A hypothesis of purinergic mechanosenory transduction in visceral organs has been proposed (Burnstock 1999 and 2001c). This hypothesis states that endogenous ATP is released from epithelial cells in response to stretch and acts upon $P2X_3$ or $P2X_{2/3}$ receptors to excite extrinsic afferent nerve fibres. This mechanism has already been shown to occur in the bladder (Cockayne et al. 2000; Vlaskovska et al. 2001; Namasivayam et al. 1999; Rong et al. 2002) and ureter (Knight et al. 2002 and unpublished data) where P2X antagonists reduced distension-induced sensory nerve discharge in both organs by about 40%, indicating that other signaling systems also contribute. This study presents data for the first time that suggests a similar mechanism may operate in the colorectum. We have demonstrated that ATP is released from the colorectal mucosa and that this release is proportional to the level of intraluminal pressure. Further, we have clearly shown that exogenous ATP activates pelvic nerve afferents and these same fibres are also responsive to noxious colorectal distension. In the presence of P2X antagonists, the pelvic nerve response to distension is reduced by about 25%. P2X agonists can also sensitise high threshold mechanosensitive units. These data give firm evidence that endogenous ATP released during noxious colorectal distension can activate and sensitise P2X receptors in the wall of the rat colorectum i.e. ATP acts as both a signalling molecule and a neuromodulator in this setting. This provides supporting evidence for the hypothesis of purinergic mechanosensory transduction in the colorectum. However, the results indicate that the purinergic component only contributes to this mechanism in part and other signalling systems must be present.

Both intrinsic and extrinsic nerves in the colorectum play a role in sensory mechanisms. In general, intrinsic afferents are concerned with local physiological reflexes such as peristalsis, which can occur if the gut is denervated of extrinsic nerves.

Extrinsic afferents are important for long loop reflexes when different parts of the gut or other body systems need to be coordinated. Clearly, extrinsic nerves form the pathways for transmission of discomfort and pain to the central nervous system. These two levels of gut control do not work in isolation; rather they must work in concert, providing overall control of gut mechanisms in a wide variety of physiological and pathophysiological scenarios. In the present study, it was therefore considered necessary to investigate the presence of $P2X_3$ and $P2X_2$ receptors in both the intrinsic and extrinsic nervous systems, although the immunohistochemical findings in the intrinsic nervous system are clearly of limited value in this discussion.

Other groups have studied P2X receptors in the myenteric and submucous plexuses. $P2X_3$ could not be identified on intrinsic sensory neurons in the guinea-pig ileum (Nassauw et al. 2002). However, another study comparing staining in the guinea-pig ileum and distal colon observed that colocalisation of $P2X_3$ and NeuN occurred in about 25% of submucous plexus neurons in the colon (Poole et al. 2002). It was suggested that $P2X_3$ receptors were expressed in intrinsic primary afferent neurons (IPANs) in this part of the gastrointestinal tract. Both studies implicate $P2X_2$ receptors with IPANs. $P2X_3$ staining has also been described on intrinsic nerves in human colon (Yiangou et al. 2001). In the present study, we have demonstrated colocalisation of $P2X_3$ and $P2X_2$ immunoreactivity with staining for calbindin in rat colorectal submucous neurons. This suggests that in this region of the gut, purinoceptors are likely to play a role in sensory mechanisms. Although we have not been able to demonstrate $P2X_3$ or $P2X_2$ receptors specifically on the terminals of extrinsic primary afferents, we have shown that $P2X_3$ and $P2X_2$ receptors are selectively expressed on small diameter L_1 and S_1 DRG neurons, which are known to supply the distal colon and rectum in the rat (Hicks et al. 2002). Further, experiments carried out to investigate the effects of spinal nerve ligation have shown $P2X_3$ receptor subunits accumulate just proximal to

the site of ligation, indicating that these receptors are transported to the periphery (Vulchanova et al. 1998). In any case, the fact that extrinsic afferents can be activated by α,β-meATP applied to the colorectal wall gives pharmacological evidence that P2X receptors exist on the peripheral projections of these neurons.

ATP is released from endothelial cells subjected to shear stress (Bodin et al. 1991) and there is good evidence that the mechanism of release is by vesicular exocytosis (Bodin and Burnstock 2001). ATP is also released from urothelial cells during bladder distension (Ferguson et al. 1997; Vlaskovska et al. 2001) and experiments have shown that release from the distended ureter is abolished after removal of the urothelial cells that line the lumen (Knight et al. 2002). In the rat colorectum, like in the urinary system, there is also a pressure-dependent ATP release and this is disturbed after mucosal ablation. ATP release was significantly elevated at intraluminal pressures of over 11mmHg. These data suggest that ATP is released in response to normal physiological distension and continues to be released proportionately into the noxious range, estimated to be about 30mmHg in the rat (Ness et al. 1991). The linear relationship between ATP release and intraluminal pressure is lost after removal of the mucosa and ATP release contributes to mechanosensory transduction, so we would expect a change in afferent nerve activity in this experimental condition. This study has shown a 32% reduction in pelvic nerve activity during distension after mucosal ablation. Other mechanisms of mechanosensory transduction must also be present in the rat colorectum. Sensory innervation of the colon includes nerve endings in the serosa and muscle layers and these may be directly activated by stretch; it is also possible that some of the basal layers of the mucosa remained after ablation giving rise to residual release of epithelial factors.

Mucosal application of ATP has been shown to activate sensory neurons in the myenteric plexus of the guinea pig ileum (Bertrand and Borstein 2002). In this study,

ATP was initially applied intraluminally but this did not elicit consistent results. It was unlikely that this was due to rapid breakdown by enterocyte ectonucleotidases, because α,β-meATP also gave unpredictable responses. Normally, the colonic lumen contains about 10 billion organisms per gram of stool and one major function of the colorectal mucosa is to provide a protective epithelial barrier. Passive permeation of hydrophilic molecules and ions across this epithelial barrier is conducted in the most part by tight junctions that allow selective absorption. The colon has a transepithelial electrical resistance much higher (10^6 ohms/cm^2) than the small intestine (10^2 ohms/cm^2) and hydrophilic molecules with a Stokes radius greater than about 11.5 Å are excluded (Madara et al. 1989). This may explain why luminal application of ATP did not always result in afferent excitation.

In contrast to intraluminal ATP perfusion, serosal application gave predictable, dose-dependent excitation of the same fibres that responded to distension, indicating that ATP activates mechanosensitive extrinsic afferents. The more stable α,β-meATP can mimic these results and its greater potency is in keeping with many other studies, where α,β-meATP has been reported to be more potent than ATP (Kirkup et al. 1999). When the colon was superfused with ATP, the multifibre afferent activity increased by between 100 to 300 percent above baseline. Of those fibres that were activated by distension pressures of 50 mmHg, 78% responded to α,β-meATP, showing a good general correlation between purinergic activation and nociceptive stimuli. Inhibition and occasional abolition of excitation by ATP or α,β-meATP could be achieved by prior application of P2X receptor antagonists.

It is likely that part of the afferent nerve excitation in response to ATP is due to adenosine. Previous studies have shown the ability of adenosine to activate extrinsic enteric nerves (Kirkup et al. 1998). In this study, the general P1 (adenosine) receptor antagonist 8p-SPT, reduced the sensory nerve discharge to ATP by 27.5%. Similarly,

the distension-evoked afferent excitation was reduced by about a third, indicating that endogenous adenosine is contributing to this response also. Adenosine is likely to appear as the result of rapid breakdown of ATP by ectonucleotidases. By preventing ATP breakdown, the weak ATPase inhibitor ARL-67156, enhanced the response to distension early on but reduced it in the later stages supporting the idea that adenosine contributes to the longer-lasting distension-evoked sensory discharge.

The pelvic nerve is important in colonic nociception in rats (Ness and Gebhart 1988). About 16% of the estimated 1,600 pelvic nerve afferents in the rat are responsive to colorectal distension (Sengupta and Gebhart 1994). At low intraluminal pressures, reflexes involving both the enteric nervous system and extrinsic pathways to lower brain centres maintain physiological mechanisms. As pressure rises, low threshold fibres increase their activity and high threshold fibres are recruited. Colorectal distension over 30 mmHg is noxious in the rat (Ness et al. 1991) and pseudoaffective pressor, tachycardic and visceromotor reflexes that precede this occur at 20-25 mmHg (Ness and Gebhart 1988). Interestingly, in the present study, the mean threshold of activation of high threshold units is similar to this value (23.46 mmHg).

Pelvic nerve afferents are activated by noxious colorectal distension, but in the presence of ATP or α,β-meATP, this activation can be potentiated. A smaller response to distension is achieved by blocking P2X receptors with PPADS or TNP-ATP, suggesting that a proportion of the afferent outflow involves purinergic signalling. Other mediators are likely to act alongside ATP in this process by directly opening ion channels at the nerve terminal (endogenous VR1 ligands, protons, 5-HT), or by sensitising the terminal to other stimuli (PGE2, bradykinin, substance P, histamine) or through alterations in receptor expression or their ligand-binding characteristics (Bueno et al. 1997 and 2000; Gebhart 2000; Kirkup et al. 2001; Holzer 2001). In this study, ATP and α,β-meATP were shown to alter the threshold of activation of low and high

threshold fibres and some fibres, having no background activity and being unresponsive to distension, were activated by α,β-meATP and subsequently responded to distension, providing evidence that colorectal afferents can be sensitised by a purinergic mechanism. Although ATP plays only a contributing role in visceral mechanosensory transduction in the normal colorectum, changes in the relative importance of different signalling molecules may occur in the transition between normal and pathological conditions. In fact, there is good evidence to indicate an enhanced role for ATP in inflammation and states of hyperalgesia (Hamilton et al. 1999b, 2000 and 2001; Cockayne et al. 2000; Souslova et al. 2000; Tsuda et al. 2000; Jarvis et al. 2001; Paukert et al. 2001; Yiangou et al. 2001). ATP would be a good candidate for signalling cellular damage in this context, being present intracellularly at millimolar concentrations. Work in this laboratory is currently being undertaken on purinergic signalling in a model of colitis in order to understand these processes further.

The role of ATP at the visceral afferent terminal and the physiology of gastrointestinal pain in general are only just beginning to be understood, but it is important that they are unravelled, not only to further our quest for selective analgesics, but because receptor mechanisms may well play a significant role in the peripheral component of functional bowel disorders.

CHAPTER FOUR

THE PURINERGIC COMPONENT OF MECHANOSENSORY TRANSDUCTION IS INCREASED IN A RAT MODEL OF COLITIS

Introduction

Genetic, environmental, microbial and immunological advances have all increased our understanding of the complex pathophysiological processes involved in inflammatory bowel disease (IBD), (for review see Ardizzone and Porro 2002). To the 4 million sufferers worldwide, these advances have brought about important clinical improvements. The aetiology, however, is still unknown.

One area of interest is the relationship between the enteric nervous system and the immune system. In the past, pelvic denervation or vagotomy has been used to treat refractory IBD (Dennis et al. 1946; Shafiroff and Hinton 1950; Thorek 1951). Inflammation in one area of the gut may profoundly affect the function of distant areas (Manousos and Salem 1965; Rao et al. 1987; Jacobson et al. 1995) and one episode of inflammation may give rise to future structural and functional abnormalities of enteric nerves (Stead et al. 1991; Collins 1996). Indeed, there is now good evidence that inflammation plays a role in the pathogenesis of irritable bowel syndrome (IBS) (Barbara et al. 2002).

Tissue concentrations of various gastrointestinal neurotransmitters are altered after inflammation (Goldin et al. 1989; Koch et al. 1987 and 1990; Swain et al. 1991 and 1992; Jacobson et al. 1997) and the intriguing relationship between patients with Ulcerative Colitis (UC) and non-smokers or ex-smokers (Lindberg et al. 1988) has been followed up with studies suggesting that nicotine, itself a parasympathetic agonist in the gut, can be used to induce clinical improvement (Pullan et al. 1994; Cohen and Hanauer

1996). Clonidine, an α_2 agonist has shown therapeutic promise (Lechin et al. 1985; Ardizzone et al. 1999) and sympathectomy in rats reduces the severity of experimental colitis (McCafferty et al. 1997). Local anaesthetic agents applied topically in UC patients have been able to induce remission (Bjorck et al. 1992; Arlander et al. 1996).

Sensory enteric nerves are important in transduction and transmission of painful stimuli and also in local and central reflexes that modulate gut function (Grundy 2002; Wynn et al. 2003). Neuropeptides such as substance P (SP), vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) are released from stimulated primary afferents via axon reflexes to influence local cellular function (Holzer and Sametz 1986; Mayer et al. 1990; Wallace et al. 1992; Roza and Reeh 2001). In particular, these neuropeptides are released in response to noxious stimuli such as vanilloid receptor type 1 (VR1) activation, acidosis or distension (Reinshagen et al. 1998; McVey and Vigna 2001). SP immunoreactivity is increased in afferent neuronal pathways during intestinal inflammation in the rat (De Giorgio et al. 2001) and VR1-null mice lose their ability to develop inflammatory thermal hyperalgesia (Davis et al. 2000). In accordance with this, loss of extrinsic sensory nerves in rats by neonatal capsaicin treatment worsens experimental inflammation in the gut (Evangelista and Meli 1989; McCafferty et al. 1997; Mazelin et al. 1998; Gay et al. 2000), as does the application of CGRP antagonists (Reinshagen et al. 1998). These data suggest that sensory innervation of the gut is essential for the normal inflammatory processes that lead to immunoprotection and healing. It is also clear that inflammatory mediators can influence afferent enteric neurons (for review see Sharkey and Kroese 2001) indicating a complex reciprocal relationship between sensory neurons and the inflammatory tissue in which they lie.

A wide variety of signalling molecules are involved in initiating and maintaining the inflammatory response including cations, amines, kinins, prostanoids, purines,

cytokines and growth factors. By lowering the threshold of activation and exaggerating the response to noxious stimuli, many of these inflammatory mediators are known to sensitise primary afferent terminals to produce pain (Dray 1995; Bueno and Fioramonti 2002). One of the molecules present during tissue injury, adenosine-5'-triphoshate (ATP), is a good candidate for signalling cellular damage, being present intracellularly in millimolar concentrations. There is now good evidence that ATP plays a role in nociception (for review see Burnstock 2000) and in particular, inflammatory pain. $P2X_2$ and $P2X_3$ receptors, two members of the larger P2X family of ligand-gated cation channels, are important in this process because $P2X_3$ is selectively expressed on small diameter sensory neurons in DRG that are known to supply, amongst other areas, the pelvic viscera (Chen et al. 1995; Bradbury et al. 1998). $P2X_2$, also present in these DRG, is pH sensitive (King et al. 1996) and along with $P2X_3$ subunits can form heteromultimers that yield ATP-activated currents similar to those found in sensory neurons (Lewis et al. 1995). Metabotropic $P2Y_1$ and $P2Y_4$ receptors are also present on a subpopulation of DRG neurons that also express $P2X_3$ receptors (Ruan and Burnstock 2003). Behavioural studies in rats (Hamilton et al. 1999; Jarvis et al. 2001) and humans (Hamilton et al. 2000) have demonstrated that the pain-inducing effects of ATP are enhanced in states of inflammation. Nerve recordings show exaggerated responses to ATP from inflammatory tissues (Hamilton et al. 2001) and $P2X_3$-null mice show reduced formalin-induced pain behaviour (Cockayne et al. 2000; Souslova et al. 2000). SP and bradykinin (BK) potentiate currents mediated by $P2X_3$ and $P2X_{2/3}$ receptors expressed by Xenopus oocytes (Paukert et al. 2001) and $P2X_3$ receptors are up-regulated in colitis specimens obtained from patients with IBD (Yiangou et al. 2001) and in DRG neurons in models of chronic nerve injury (Novakovic et al. 1999).

A working hypothesis of purine-mediated mechanosensory transduction has been proposed (Burnstock 1999 and 2001). ATP released during distension from

epithelial cells lining tubes (such as ureter or gut), and sacs (such as bladder), acts on $P2X_3$ and/or $P2X_{2/3}$ receptors on a subepithelial nerve plexus to initiate impulses that are relayed via the spinal cord to pain centres in the brain. Recent work in our laboratory has suggested that this mechanism contributes to afferent signalling during bladder distension in the mouse (Rong et al. 2002; Vlaskovska et al. 2001) and colorectal distension in the rat (Wynn et al. 2003). In the present study, a rat model of colitis was used to examine the effect of ATP on pelvic nerve recordings during noxious colorectal distension (CRD). The results were compared to controls to elucidate whether the purinergic component to mechanosensory transduction in the colorectum plays an enhanced role during inflammation. We also examined neurons of the DRG that supply the rat colorectum (Hicks et al. 2002) before and after the induction of colitis for possible changes in $P2X_3$ and $P2X_{2/3}$ expression and their electrophysiological responses to exogenous ATP.

Results

Assessment of TNBS-induced colitis

We quantified the effect of the TNBS enema in each rat by measuring body weight before the induction of colitis and on the day of sacrifice. In 22 controls, the mean body weight was 269 ±4.57 g and this was not significantly different from 22 pre-enema animals in the colitis group (272 ±4.89 g; $P = 0.622$). After induction of colitis however, mean body weight dropped significantly to 239 ±3.33 g ($P < 0.0001$), representing a 12.2% reduction. In addition, colorectal specimens were assessed for macroscopic damage such as adhesions, erosions and petechial haemorrhage. All colitis preparations had at least two of these features, whereas none were present in the controls. In randomly selected experiments (n = 7), inflamed colorectal specimens were

prepared for routine histology (haematoxylin and eosin staining) and inspected under the light microscope. In conjunction with a senior histopathologist, features of chronic inflammation such as lymphocytic infiltrates were consistently described. Although mucosal ulceration was a common feature, it was estimated that this accounted for less than 10% of the surface area of the lumen.

All pelvic nerve experiments were carried out on day 5 except for 3 experiments on day 6 (serosal ATP, α,β-methylene ATP and PPADS application) and 2 experiments on day 7 (ATP release). All DRG experiments were carried out on day 10. Analysis of the specimens showed no differences in the severity of inflammation between days 5 and 10.

Pelvic nerve afferent activity from the normal and inflamed colorectum

With intraluminal pressure at zero in 14 suitable colitis preparations, background activity in the pelvic nerve was compared with activity in 14 normal controls over a 100-second period. Using Spike 2 software, single unit analysis allowed calculation of the average firing rate of individual units. In the normal colorectum the mean firing rate per unit was 0.236 ±0.046 impulses per second. In the model of colitis this value increased to 0.457 ±0.074 impulses per second ($P = 0.018$). In recordings from both the normal and inflamed colorectum, phasic distensions in the rat typically produce a sudden burst of spikes that settle to a stable level after 30 to 60 seconds and responses show good reproducibility even after short recovery periods. Single unit analysis of pelvic nerve recordings from the colitis preparations, during 30-second phasic distensions with Krebs solution to 50 mmHg, revealed a mean firing rate per unit of 3.39 ±0.267 impulses per second. In the normal colorectum, distensions to the same pressure evoked a mean firing rate of only 1.95 ±0.113 impulses per second ($P < 0.0001$). Fig. 4.1 compares background and distension-evoked spike frequency in

recordings from a normal and colitis preparation. These examples were selected because single unit analysis demonstrated that each preparation contained the same number of fibres and therefore a meaningful comparison could be made of the multifibre activity in each.

Effect of ATP and α,β-meATP on pelvic nerve afferents in a model of colitis

In control colorectal preparations, intraluminal application of ATP or the $P2X_1$ and $P2X_3$ receptor synthetic agonist, α,β-meATP, did not cause consistent activation of pelvic nerve afferents. In the inflamed preparations, similar results were obtained. In contrast, application of ATP or α,β-meATP to the serosal surface of either the colitis model or the normal colon evoked consistent, rapid responses with a mean latency that was not significantly different in the controls (13.7 ±0.85 seconds) than in the colitis preparations (14.6 ±1.21 seconds). Table 4.1 compares the multifibre responses elicited by a bolus application of ATP or α,β-meATP. In both the normal colorectum and the colitis model, the mean percentage increase from baseline firing in response to a purinergic stimulus was dose-dependent. α,β-meATP was more potent than ATP in both experimental preparations. The colitis model, however, showed substantially greater magnitude responses for equivalent concentrations of agonist.

Application of the P2X receptor antagonist PPADS (100μM) to normal colorectal preparations resulted in the mean background firing rate being reduced by 14.8 ±2.57% (n=9). A greater reduction of 45.8 ±9.08% was seen in the colitis preparations (P =0.004; n=9).

The purinergic contribution to the afferent response to distension

In at least 8 normal animals and 8 colitis preparations, the effect of applying serosal ATP during distension to an intraluminal pressure of 50 mmHg was

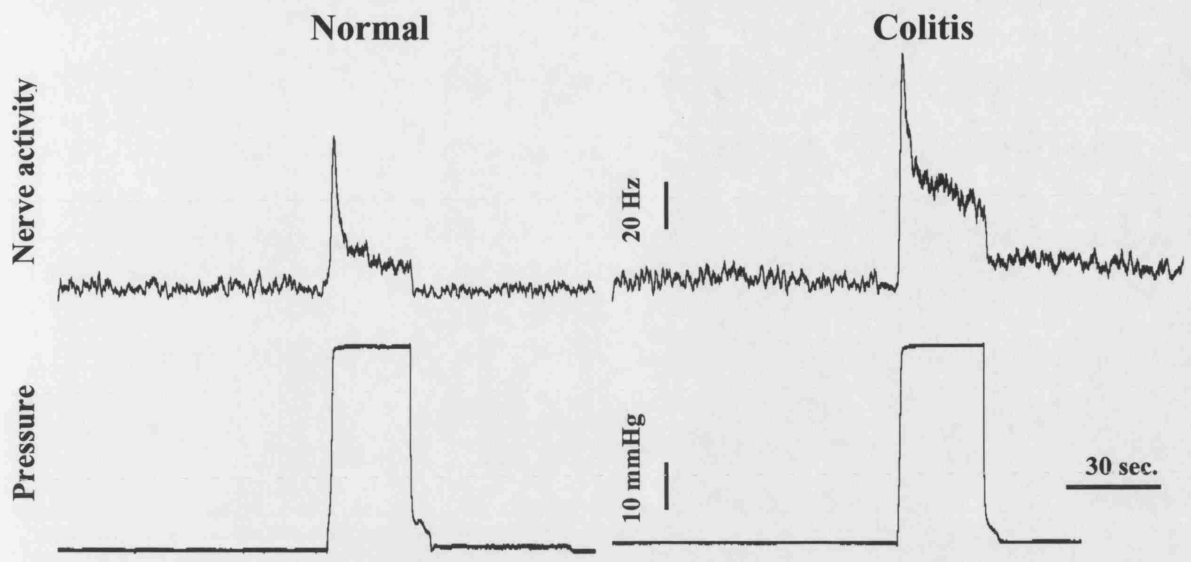


Fig. 4.1 Sample recordings from the pelvic nerve in a normal colorectal preparation (left) and a colitis model (right). Single unit analysis has confirmed that both preparations have the same number of active nerve fibres. Background activity and the response to 50 mmHg distension are both increased in the colitis model, demonstrating a greater firing rate per unit.

		Normal	Colitis	P value
ATP	**1mM**	97.5 ±12.0% (n=14),	149 ±17.4% (n=16)	0.0239
	3mM	114 ±16.5% (n=16)	240 ±27.0% (n=16)	0.0004
	5mM	164 ±11.3% (n=15).	261 ±34.3% (n=10)	0.0046
α,β-meATP	**100μM**	75.5 ±18.3% (n=6)	301 ±66.9% (n=6)	0.0086
	1mM	162 ±41.8% (n=5)	602 ±113% (n=4)	0.0051

Table 4.1 Comparison of the magnitude of responses of the pelvic nerve to bolus doses of ATP and α,β-meATP in the normal and inflamed colorectum. The figures represent the mean percentage increase in frequency of spikes from baseline activity.

investigated. Fig. 4.2 shows that in the normal colorectum (lower line), the presence of ATP increased peak distension-induced activity in the pelvic nerve compared to control distensions with Krebs solution and this potentiation was dose dependent. In the colitis preparations, the presence of ATP increased the afferent response to distension to an even greater extent (upper line). Distension of the colitic colorectum in the presence of α,β-meATP also increased the afferent response at 100μM (28.1 ±1.58%; n=4) and 1mM (37.6 ±3.84%; n=4) and this was to a greater extent than in the normal colorectum at the same concentrations: 19.3 ±1.81% (n=4) and 24.8 ±3.14% (n=5) respectively. As demonstrated before, α,β-meATP was more potent than ATP. Distension of the normal colorectum in the presence of the non-specific P2X receptor antagonist PPADS 100μM resulted in inhibition of the peak afferent response by 23.4 ±1.88% (n=9; range: 13.2% - 27.4%). A significantly greater reduction (P =0.026) was seen at equivalent doses in the colitis preparations: 37.2 ±5.34% (n=9; range: 18.9% - 74.8%). The $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptor antagonist TNP-ATP 60μM produced 26.2 ±3.3% reduction in the normal preparations (n=11; range: 8.2% - 36.7%) and a 34.5 ±2.9% reduction in the distension response of the colitis preparations (n=6; range: 27.2% - 45.6%). Again, the difference was statistically significant (P =0.03). Pelvic nerve recordings from normal and colitis preparations during application of TNP-ATP and PPADS can be compared in figs. 4.3 and 4.4 respectively.

The metabolism of ATP to adenosine and their effect on the pelvic nerve response to distension at 50 mmHg was studied using the ATPase inhibitor, ARL-67156 and the general P1 (adenosine) antagonist 8p-SPT. In 6 normal preparations, the presence of the ATPase inhibitor gave a mean increase in nerve activity of 12.0 ±2.45% over the first 10 seconds of colorectal distension. However, this figure increased to 26.1

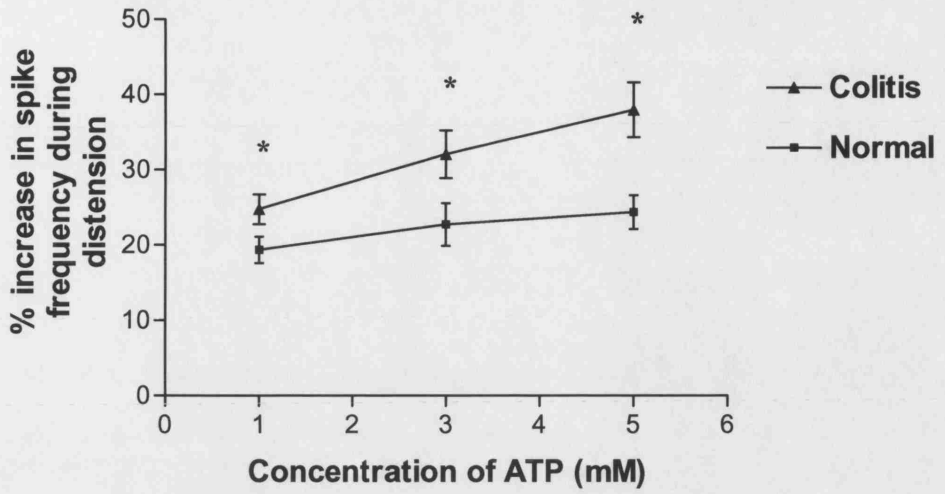


*Fig. 4.2 The augmentation of the pelvic nerve response to colorectal distension (50 mmHg) in the presence of ATP is dose-dependent (lower line). In the colitis models (upper line), this potentiation is increased to a greater extent. *P ≤0.05.*

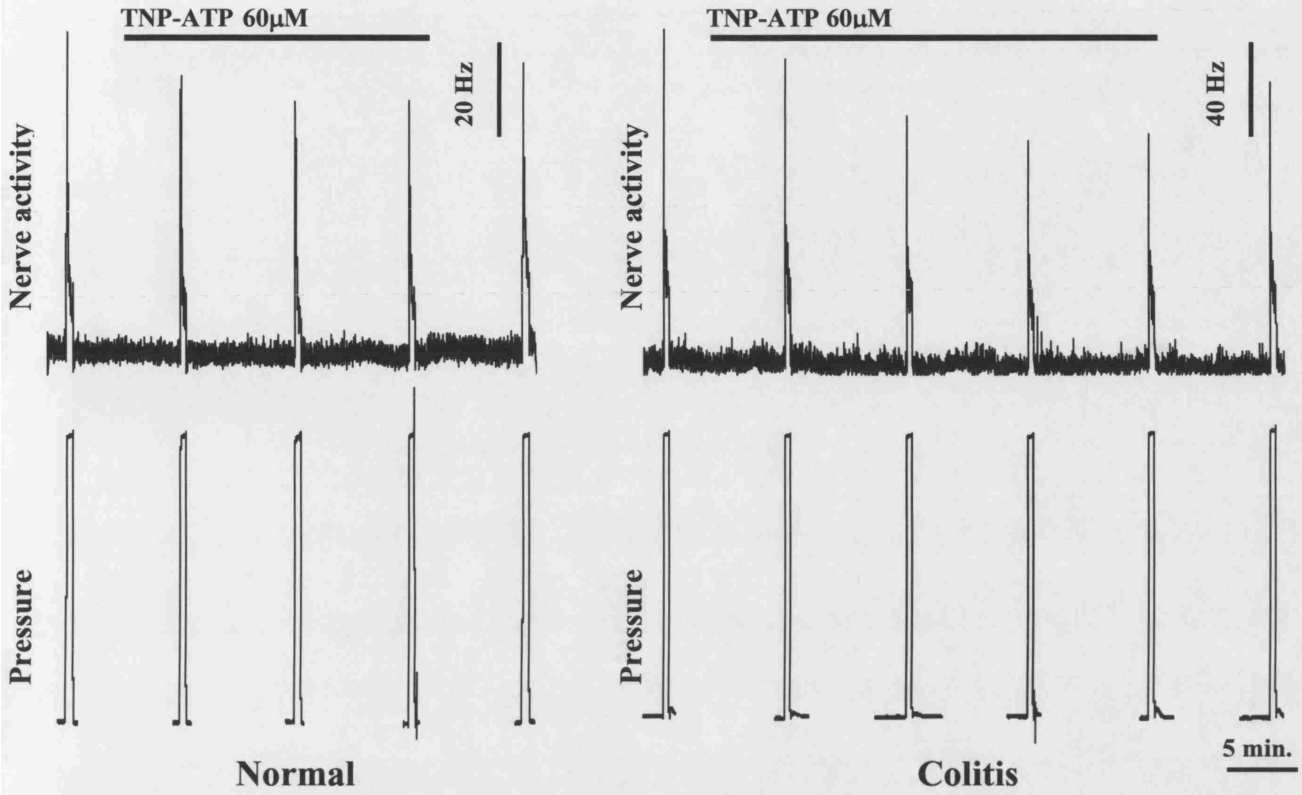


Fig. 4.3 Comparison of the pelvic nerve responses to distension (50 mmHg) in the presence of the P2X receptor antagonists TNP-ATP 60μM. The normal preparations are shown on the left. The colitis models on the right show smaller responses to distension in the presence of the antagonists. Note that each recording is not shown at the same scale.

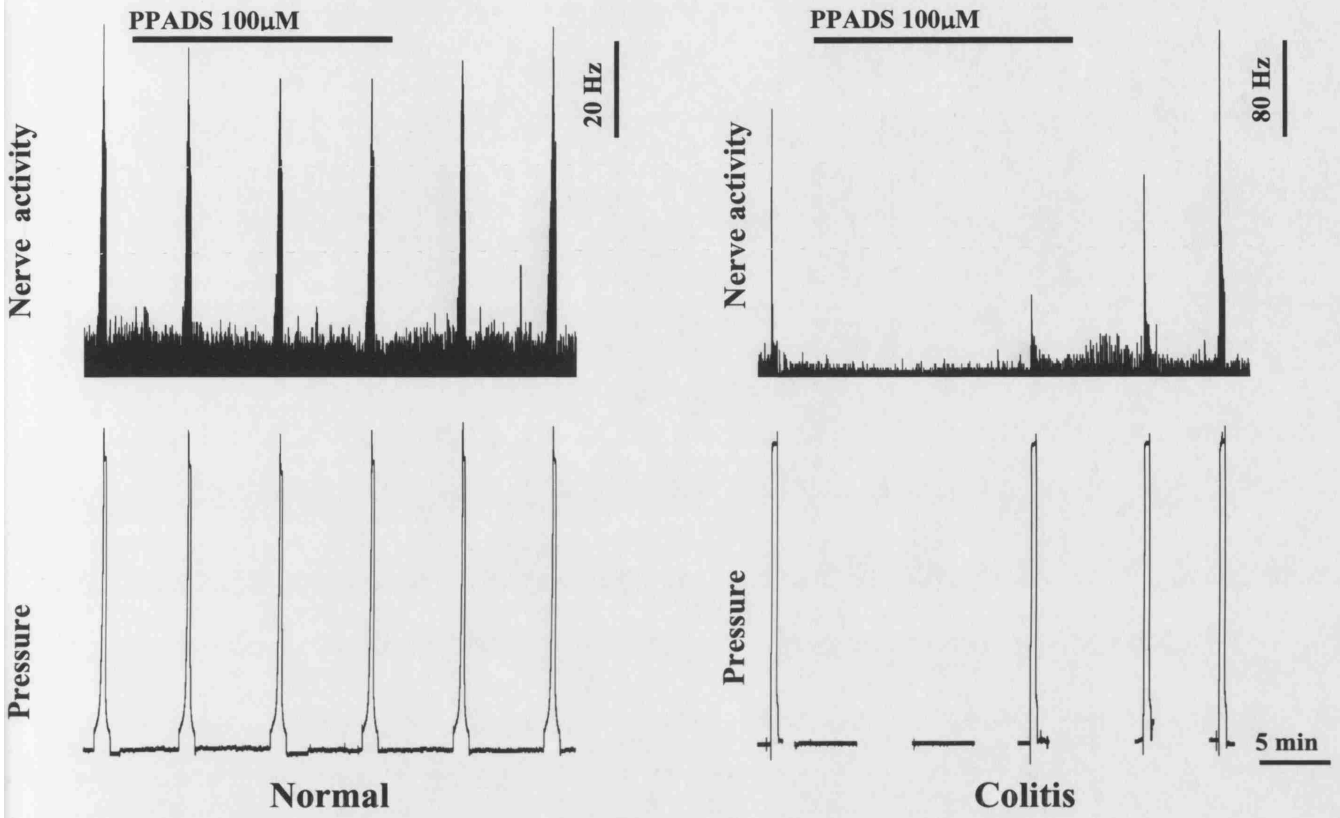


Fig. 4.4 Comparison of the pelvic nerve responses to distension (50 mmHg) in the presence of the P2X receptor antagonist PPADS 100μM. The normal preparations are shown on the left. The colitis models on the right show smaller responses to distension in the presence of the antagonists. Note that each recording is not shown at the same scale.

±6.59% in the 5 colitis preparations tested (P = 0.06). To allow any potential adenosine time to appear, colorectal distension was sustained for a longer period. In normal controls, the presence of 8p-SPT 100 μM reduced nerve activity throughout the course of a 90-second distension by 25.6 ±0.78% (n=5). In the colitis preparations, the presence of the adenosine antagonist resulted in a smaller effect overall on mean spike frequency (a reduction of 8.43 ±1.3%; n=5), but also progressively reduced the activity throughout the period of distension (see fig. 4.5). This ranged from 2.2 ±4.7% during the first 10 seconds to 17.3 ±4.2% between 80 and 90 seconds. Analysis of variance between the two responses confirmed that they were significantly different (P =0.005).

ATP release

From 4 inflamed colorectal preparations, 100 individual distensions ranging from 6 to 90 mmHg were performed. These were compared to similar distensions in normal controls, which were consistent with those described by Wynn et al. (2003). Fig. 4.6 shows the relationship between rising intraluminal pressure and concentration of ATP in the perfusate of each group. Intraluminal fluid was collected before each of the distensions (pressure approximately zero) and the background level of ATP measured from these samples in both groups remained low and stable regardless of intervening pressures. ATP levels at rest were higher in the colitis preparations (mean 0.352 ±0.018 pmol/ml) than the normal controls (0.154 ±0.004 pmol/ml). Post-distension samples collected from the inflamed colorectum yielded significantly greater concentrations of ATP than those collected from normal colorectal controls and this was consistently the case over every pressure group (analysis of variance; $P \leq 0.0001$). Compared to the normal colorectum where the distension-induced rise in ATP release became significant at pressures over 11 mmHg, the colitis preparations showed significant increases in ATP release at pressures below 10 mmHg (P = 0.033).

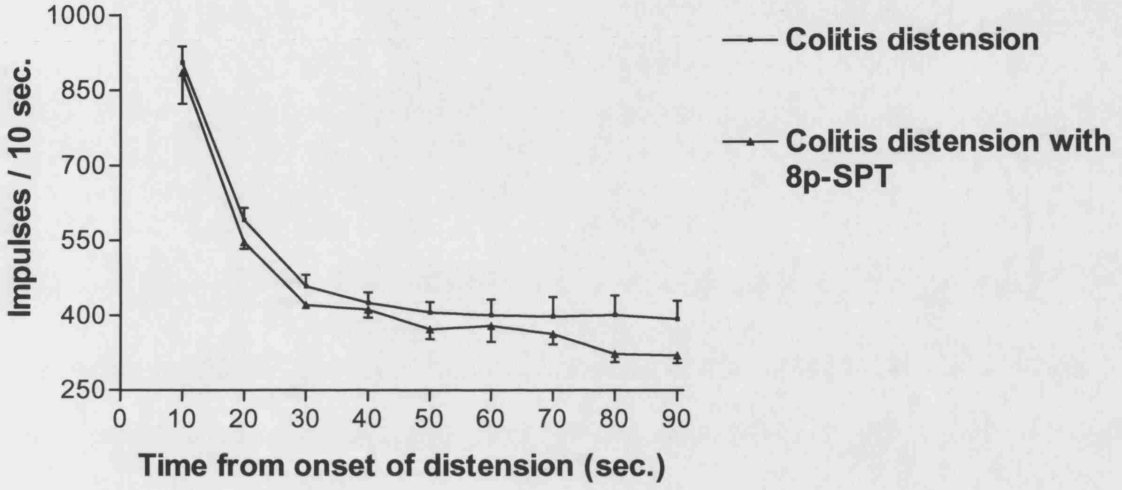


Fig. 4.5 Ten-second periods of nerve activity have been plotted for 90-second distensions in five colitis preparations, either with or without the general adenosine antagonist, 8p-SPT. When adenosine receptors are blocked, nerve activity is progressively reduced throughout the period of distension (P =0.005). This may indicate an increasing role for adenosine in the later part of distension-evoked nerve activity.

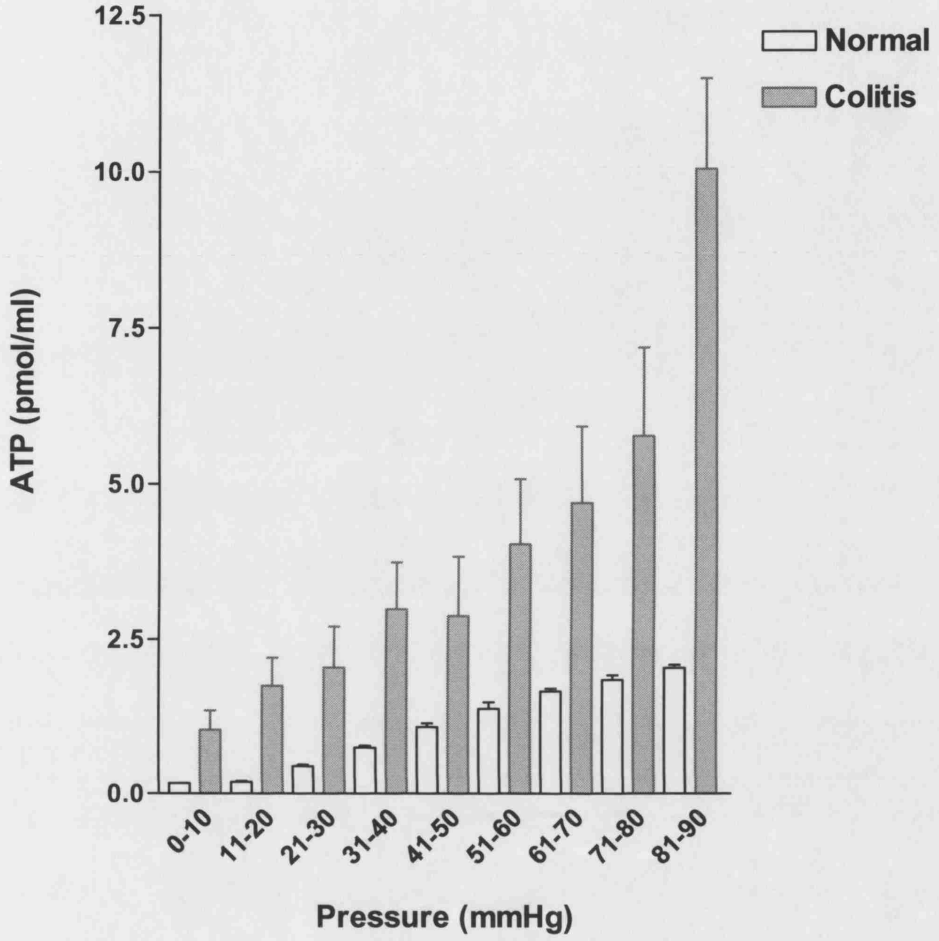


Fig. 4.6 Comparison of ATP concentration in luminal fluid samples from the normal and inflamed rat colorectum during distension. Each white column shows the mean ATP release (pmol/ml) ±SEM for the normal preparations and contrasts with the grey columns to their right that represent the colitis models. Columns are paired into the pressure groups shown (in mmHg).

Recordings from DRG neurons

Those ganglia most important in relaying sensory information from the distal colon and rectum (L_1 and S_1) were compared to two other ganglia (L_2 and S_2) that are less important in this regard. DRG neurons respond to ATP with 3 different types of inward current and these are demonstrated in fig. 4.7. Transient responses (fig. 4.7a) correspond to $P2X_3$ activation, sustained responses (fig. 4.7b) correspond to activation of $P2X_2$ receptors and biphasic responses (fig. 4.7c) correspond to $P2X_{2/3}$ receptor activation. Table 4.2 shows the percentage of neurons in each group that were responsive to ATP in DRG L_1 and S_1 before and after induction of colitis. There was no significant increase in the number of cells responding with a sustained or biphasic current after inflammation, but the proportion of neurons responding with a transient current was raised in the colitis group. The percentage of cells that were unresponsive to ATP dropped after inflammation from 14% to just 4%. Similarly, in the L_2 and S_2 DRG (table 4.3), there was no difference in the sustained and biphasic responders, but a significant increase in the number of cells responding with a transient current in the colitis group. In the normal rats, over one in six neurons tested in L_2 and S_2 ganglia (17%) were unresponsive to ATP, however after inflammation, there were none.

Immunohistochemistry

The DRG from 4 normal rats were studied for immunoreactivity to $P2X_3$ and CGRP. Fig. 4.8 compares typical immunostaining of S_1 DRG in the normal rat (left hand column) with that of the colitis model (right hand column). Fig. 4.8a shows a subpopulation of neurons that are positive for CGRP in the normal rat. CGRP-staining cells in the colitis model are shown in fig. 4.8b. Similarly, a subpopulation of $P2X_3$ immunoreactive neurons in the normal rat DRG are shown in fig. 4.8c; those staining in

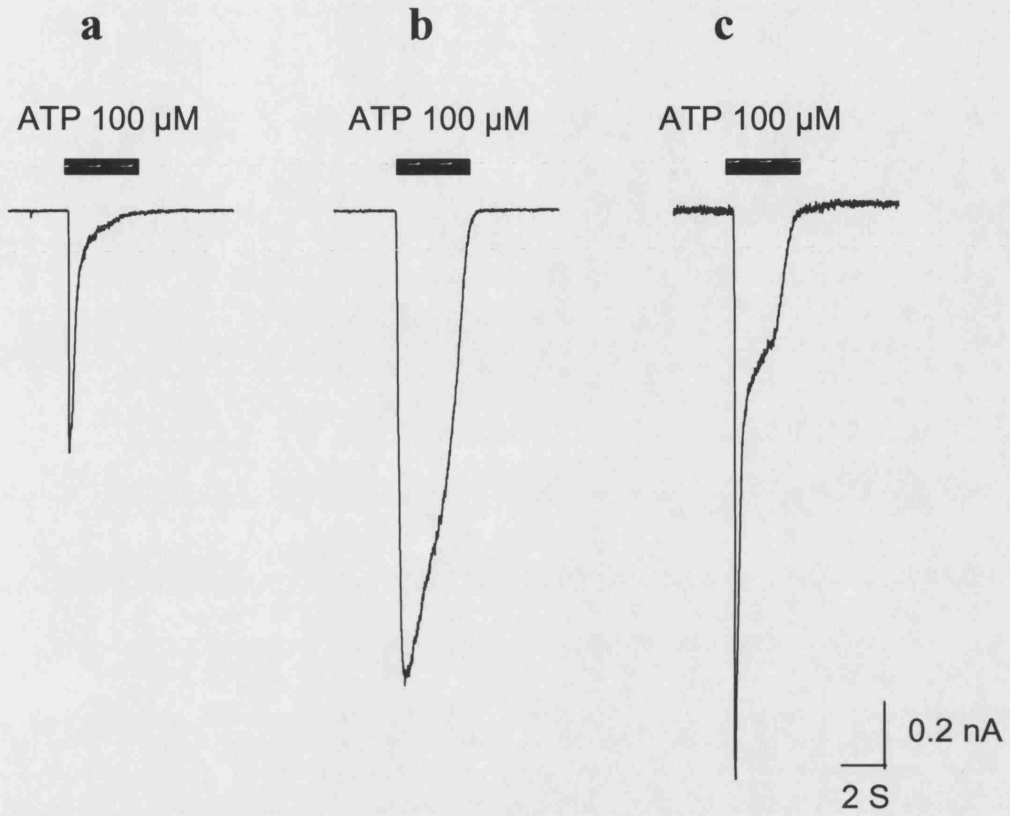


Fig. 4.7 Typical traces of the three different inward currents encountered when ATP is applied to DRG neurons. (a) shows a transient current that corresponds to activation of the $P2X_3$ receptor. (b) shows a sustained current that corresponds to activation of the $P2X_2$ receptor and (c) shows a biphasic current corresponding to the $P2X_{2/3}$ heteromultimeric receptor.

	n	Sustained	Transient	Biphasic	No response
Normal	28	21%	46%	18%	14%
Colitis	27	22%	56%	19%	4%

Table 4.2 The percentage of L_1 and S_1 DRG neurons that respond to ATP with either a sustained ($P2X_2$ receptor), transient ($P2X_3$ receptor) or biphasic ($P2X_{2/3}$) inward current before and after induction of colitis.

	n	Sustained	Transient	Biphasic	No response
Normal	23	22%	39%	22%	17%
Colitis	19	21%	58%	21%	0%

Table 4.3 The proportion of L_2 and S_2 DRG neurons responding to ATP show a similar distribution in the two experimental groups.

the inflammatory model now show staining of axons also (fig. 4.8d). Colocalisation (yellow staining) between CGRP and $P2X_3$ is shown in the normal state (fig. 4.8e) and after induction of colitis (fig. 4.8f). In the normal rat, the percentage of neurons staining for $P2X_3$ (33%) and CGRP (37%) was constant regardless of the level of the ganglion. In the 4 colitis preparations examined, the percentage of $P2X_3$ positive neurons had increased from 33.1 ±0.74% to 38.9 ±0.75% in L_1 and S_1 and from 32.3 ±0.71% to 40.5 ±0.83% in L_2 and S_2 (see table 4.4). Both these increases were statistically significant: $P \leq 0.0001$ and 0.0001 respectively. More CGRP positive neurons had also appeared in the inflammatory preparations: up from 36.8 ±0.79% to 41.9 ±0.71% in L_1 and S_1 and from 37.9 ±0.84% to 42.3 ±0.78% in L_2 and S_2 (see table 4.5). Again, these increases were highly statistically significant: $P = 0.0001$ and 0.0008 respectively.

Quantification of the colocalisation between $P2X_3$ and CGRP was carried out. Table 4.6 shows that the proportion of $P2X_3$ positive neurons that also stained for CGRP in L_1 and S_1 increased from 24.3 ±0.88% to 31.6 ±1.1% after inflammation, which was statistically significant ($P < 0.0001$). $P2X_3$/CGRP colocalisation was also increased in L_2 and S_2 ganglia (22.8 ±0.99% to 29.9 ±0.84% respectively; $P < 0.0001$). When CGRP neurons were studied, the percentage of L_1 and S_1 neurons that also stained for $P2X_3$ increased from 20.8 ±0.78% to 28.9 ±0.83% in the colitis preparations, which was again statistically significant ($P < 0.0001$). A similar increase was also seen in neurons in L_2 and S_2 (19.5 ±0.83% to 28.1 ±0.81%; $P < 0.0001$; see table 4.7).

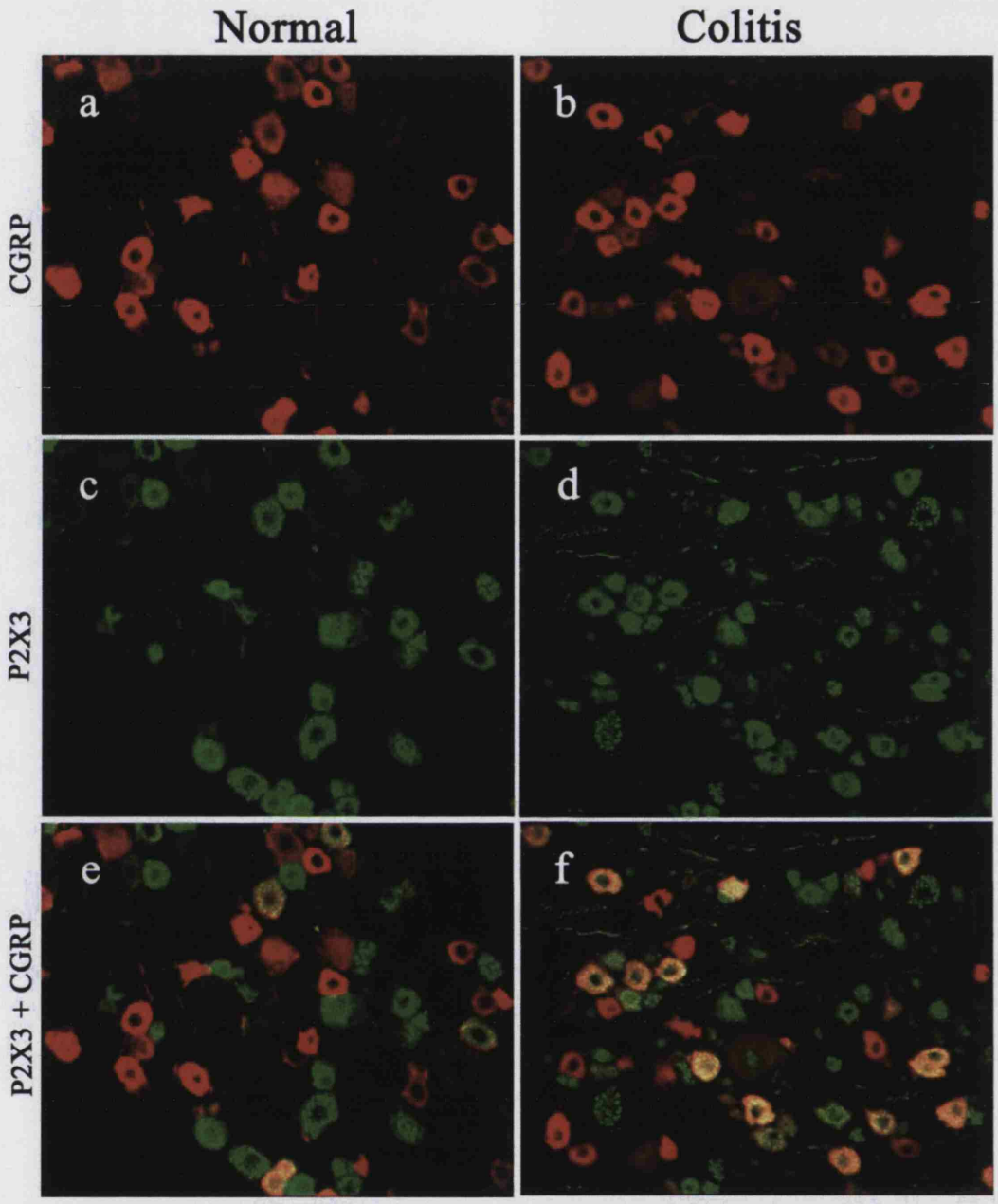


Fig. 4.8 CGRP (a and b; red) and $P2X_3$ (c and d; green) immunoreactivity in S_1 DRG in the normal rat (left hand column) and after induction of colitis (right hand column). There are nerve fibres as well as cell bodies that stain for $P2X_3$ after induction of colitis (d). Note the increased number of neurons showing colocalisation between CGRP and $P2X_3$ receptors in the colitis models (e and f; yellow).

DRG	n	% of neurons staining for $P2X_3$		*P* value
		Normal	Colitis	
L_1 and S_1	12	33.1 ±0.74%	38.9 ±0.75%	<0.0001
L_2 and S_2	12	32.3 ±0.71%	40.5 ±0.83%	<0.0001

Table 4.4 The percentage of DRG neurons that stain for the $P2X_3$ receptor before and after the induction of colitis.

DRG	n	% of neurons staining for CGRP		*P* value
		Normal	Colitis	
L_1 and S_1	12	36.8 ±0.79%	41.9 ±0.71%	0.0001
L_2 and S_2	12	37.9 ±0.84%	42.3 ±0.78%	0.0008

Table 4.5 The percentage of DRG neurons that stain for CGRP before and after the induction of colitis.

DRG	n	% of $P2X_3$ positive neurons that also stain for CGRP		*P* value
		Normal	Colitis	
L_1 and S_1	12	24.3 ±0.88%	31.6 ±1.1%	<0.0001
L_2 and S_2	12	22.8 ±0.99%	29.9 ±0.84%	<0.0001

Table 4.6 The percentage of $P2X_3$ positive DRG neurons that also stain for CGRP before and after induction of colitis.

DRG	n	% of CGRP positive neurons that also stain for $P2X_3$		*P* value
		Normal	Colitis	
L_1 and S_1	12	20.8 ±0.78%	28.9 ±0.83%	<0.0001
L_2 and S_2	12	19.5 ±0.83%	28.1 ±0.81%	<0.0001

Table 4.7 The percentage of CGRP positive DRG neurons that also stain for $P2X_3$ before and after induction of colitis.

Discussion

The present study has indicated that the purinergic contribution to mechanosensory transduction in the rat colorectum is increased in the inflammatory state. Distension-induced release of ATP is significantly elevated and $P2X_3$ receptor expression in DRG neurons is increased after induction of colitis. Furthermore, the afferent response to distension can be changed to a far greater degree by purinergic agonists and antagonists in colitis models compared to normal controls. To our knowledge, this is the first time an enhanced role for ATP has been described during colitis in response to a noxious stimulus.

These findings concur with studies in other models that have suggested an important purinergic component exists in sensory nerve signalling in inflammatory conditions. In an in-vitro skin-nerve model in the rat, there was an increase in the magnitude of α,β-meATP responsive nociceptors after inflammation induced with carageenan (Hamilton et al. 2001). ATP and α,β-meATP produce dose-dependent nocifensive behaviour when injected into the rat hindpaw and the effect of these agonists are greatly augmented after ultraviolet irradiation, prior injection with carageenan and immediately following prostaglandin E_2 treatment (Hamilton et al. 1999). The formalin rodent paw model has been used to demonstrate the antinociceptive effects of intrathecally administered P2X antagonists (Driessen et al. 1994; Tsuda et al. 1999). As well as bladder hyporeflexia, $P2X_3$-null mice have reduced inflammatory pain-related behaviour (Cockayne et al. 2000). When $P2X_3$ and $P2X_{2/3}$ receptors are expressed by Xenopus oocytes, their currents are potentiated by substance P and bradykinin (Paukert et al. 2001). Mechanosensory function in a model of oesophagitis was sensitised by α,β-meATP (Page et al. 2000) and another study suggested that $P2X_3$ receptors on intrinsic enteric neurons are increased in human inflammatory bowel disease (Yiangou et al. 2001).

Recordings from the pelvic nerve in the present study showed that background activity in the colitis models was significantly higher than in normal colorectal preparations. This finding was paralleled during distension, where individual units from inflamed colon fired at a higher frequency than in the controls at a given intraluminal pressure. Other studies have shown that intrinsic neurons in the guinea pig jejunum (Palmer et al. 1998) and dorsal horn neurons receiving input from the colon in the rat (Olivar et al. 2000) also exhibit enhanced excitability following enteric inflammation. In this study, we have demonstrated that the afferent excitation in response to exogenous ATP is greater in colitis models than in the normal colorectum. Serosal application of the agents gave more predictable responses than mucosal application and the reasons for this have been discussed previously (Wynn et al. 2003). Briefly, passive permeation of hydrophilic molecules and ions across the gastrointestinal epithelium is conducted in the most part by tight junctions that allow selective absorption. The colon has a very high transepithelial electrical resistance (10^6 ohms/cm^2) and hydrophilic molecules with a Stokes radius greater than about 11.5 Å are excluded (Madara 1989). This may explain why luminal application of ATP did not always result in afferent excitation. Purinergic agonists also augment distension-induced afferent discharge to a greater degree in the inflammatory state, whereas the P2 antagonists PPADS and TNP-ATP reduced this activity (37.2 and 34.5% respectively) after induction of colitis compared to normal controls (23.4 and 26.2% respectively). There was wide variation in the colitis groups (range: 18.9% - 74.8% and 27.2% - 45.6% for PPADS and TNP-ATP respectively) possibly representing variable degrees of inflammation in different rats. Further work needs to be carried out to investigate the effect of purinergic agonists and antagonists in relation to an objective measure of severity of colitis (perhaps myeloperoxidase activity).

ATP is metabolised by ectonucleotidases to adenosine by the progressive removal of phosphate groups. In this study, inhibiting the breakdown of ATP increased the frequency of action potentials during the early part of the distension-evoked response in the normal colorectum by about 12%. In the colitis group, enzyme inhibition had an even greater effect, increasing early activity by 26%. Reduced degradation of ATP could explain the augmented response in both scenarios by prolonging the availability of ATP. However, in the colitis models there are higher levels of endogenous ATP release throughout the distension period and this would simply multiply the effective signalling available even if there was no effect on enzyme activity. We have not specifically investigated whether ATPases are up- or down-regulated in colitis, but P1 (adenosine) receptor antagonists had a smaller influence in the colitis models than on normal preparations. So, proportionally, adenosine plays a smaller role in the longer lasting nerve activity during inflammation and this might be due to less efficient enzymatic breakdown of ATP.

Another possible mechanism for the augmented purinergic component during inflammation is an increase in the local concentration of the signalling molecule itself. ATP is released from endothelial cells subjected to shear stress (Bodin et al. 1991), from urothelial cells during bladder (Ferguson et al. 1997; Vlaskovska et al. 2001) and ureteric distension (Knight et al. 2002). There is good evidence that the mechanism of release is by vesicular exocytosis (Bodin and Burnstock 2001). Endothelial cells increase their release in acute inflammation (Bodin and Burnstock 1998) and in the bladder stretch-activated ATP release is increased in interstitial cystitis (Sun et al. 2001) and coincides with an augmented component of purinergic neurotransmission in this condition (for review see Burnstock 2002). The present study demonstrates that in the normal rat colorectum, there is a strong relationship between intraluminal pressure and amounts of ATP measured from the perfusate, but these amounts were significantly

increased in inflammatory models. Background samples collected between distensions also showed higher levels of ATP than normal controls. ATP is released in other painful pathological conditions also. Tumour cells are known to contain exceptionally high levels of ATP (Maehara et al. 1987) and in sympathetic reflex dystrophy surgical sympathectomy, sympathetic ganglion blockers and guanethidine are all more effective at relieving pain than adrenergic antagonists, suggesting a role for the release of co-transmitters such as ATP (Hannington-Kiff 1974; Bonezzi et al. 1994; Yasuda and Schroeder 1994). ATP exists within cells in millimolar concentrations and therefore any significant cellular damage is also likely to increase local release. In a model of post-operative pain in the rat, the P2X antagonist PPADS given before surgery significantly attenuated mechanical allodynia caused by the incision and c-Fos protein expression was also reduced in the dorsal horn of the spinal cord (Tsuda et al. 2001). ATP activates visceral sensory nerves in a dose-dependent way (Kirkup et al. 1999; Rong et al. 2002), so it follows that in situations where release of ATP is greater, there is augmented activation of purinergic nerves. One study has reported a reduction in ATP concentrations in homogenised colitis specimens in rats (Zingarelli et al. 1998). However, this was not a measure of ATP release, but of total tissue content, possibly indicating a depletion of intracellular stores due to increased release.

In an attempt to understand the mechanism(s) underlying increased purinergic mechanosensory transduction in the inflamed colorectum, we have shown differences in the electrophysiological responses of DRG neurons to ATP after induction of colitis. These neurons respond to ATP with transient, persistent or biphasic inward currents and these responses can be attributed to $P2X_3$, $P2X_2$ and $P2X_{2/3}$ receptors respectively (Zhong et al. 2001). In the present study, inflammation increased the number of DRG neurons responding to ATP with a transient inward current and this correlates with the immunocytochemical findings that more cells expressed $P2X_3$ receptors. The increased

responsiveness was present in all the DRG cells studied, but was most apparent in L_2 and S_2 DRG, where 17% of neurons were unresponsive in the normal rat, but every cell became responsive after inflammation. This is consistent with other studies, where induction of inflammation in the rat hindpaw gave rise to a two to three-fold increase in ATP-activated currents and altered the voltage dependence of P2X receptors of neurons in the DRG (Xu and Huang 2002).

Increased responses to ATP in DRG neurons are likely to come about by an increase in $P2X_3$ receptor expression. In this study we have clearly demonstrated that after induction of colitis, $P2X_3$ receptor expression is increased in the DRG that are known to supply the rat distal colon and rectum. Interestingly, this $P2X_3$ up-regulation occurs in adjacent DRG also, suggesting that inflammation in one area of the gut may affect sensory traffic from other areas. This idea correlates with evidence suggesting that there are profound physiological disturbances in areas of the gut distant from the site of inflammation (Manousos and Salem 1965; Rao et al. 1987; Jacobson et al. 1997). In other experimental conditions where sensory nerves detect injury there are also changes to expression of $P2X_3$ receptors. Following a chronic constriction injury to the rat sciatic nerve, the number of $P2X_3$ positive small and medium diameter neurons increased in the DRG compared to sham operated animals (Novakovic et al. 1999). Studies using tight ligation of a spinal nerve (Kage et al. 2002) or axotomy (Bradbury et al. 1998) report a reduction in $P2X_3$ expression in the relevant DRG. Tsuzuki et al. (2001) demonstrated that axotomised neurons reduced the expression of $P2X_3$ mRNA, while adjacent neurons that were spared, increased their expression. Together with the knowledge that $P2X_3$ receptors accumulate proximal the site of nerve ligation, indicating receptor transport to the periphery (Vulchanova et al. 1998), this gives indirect evidence that $P2X_3$ receptors located on the peripheral terminals of colorectal

afferents are up-regulated during colitis. $P2X_3$ receptors may be increased too in the intrinsic nervous system of the colorectum during inflammation (Yiangou et al. 2001).

The $P2X_3$ receptor is normally found largely in non-peptidergic sensory neurons that bind the lectin IB4; however, a minority of $P2X_3$-positive neurons do also contain the neuropeptide CGRP (Bradbury et al. 1998). This study has provided data suggesting that after an inflammatory insult the proportion of CGRP-containing neurons that express $P2X_3$ increases significantly. CGRP is released from extrinsic enteric neurons by a variety of noxious stimuli including VR1 receptor activation, distension and acidosis (Roza and Reeh 2001). CGRP released in response to inflammation is thought to provide tissue protection by increasing blood flow to damaged areas (Eysselein et al. 1992) and rats treated with CGRP antagonists develop more severe colitis after TNBS enema (Reinshagen et al. 1998). These data suggest that purinergic signalling may play a more important role in regulating these peptidergic neurons during colitis, amplifying their role in the inflammatory process or vice versa. Changes in both the number of $P2X_3$ receptors per neuron and a change in the number and type of neurons expressing $P2X_3$ receptors suggests a possible mechanism underlying the increased responses to purinergic stimuli seen during the inflammatory state. It may, at first, appear confusing that purinergic antagonists are more powerful in the colitis models and at the same time, there is increased bioavailability of ATP and up-regulation of $P2X_3$ receptors. However, if more neurons are expressing $P2X_3$, then the relative proportion of units being blocked is correspondingly increased.

Previous work in our laboratory has shown that removal of the mucosa abolishes the relationship between ATP release and colorectal intraluminal pressure, whilst significantly reducing pelvic nerve afferent activity to distension (Wynn et al. 2003). ATP released during noxious CRD and the purinergic component of graded visceral afferent activation are increased during colitis. It seems likely that a combination of

raised endogenous ATP levels and up-regulation and/or sensitisation of peripheral $P2X_3$ receptors located on enteric sensory nerves during colitis is responsible for the augmented purinergic component of the afferent responses. It is possible that inflammation has an inhibitory effect on ATPases, but further work needs to be done to show this clearly. Enteric sensory nerves are influenced by a wide range of inflammatory mediators: bradykinin, prostaglandins, histamine and cytokines such as Il-1β and Il-6 are examples (Sharket and Kroese 2001). Many of the properties of ATP suggest it may play a similar role to some of these mediators. ATP causes pain when injected into the base of blisters (Bleehan et al. 1976) and this pain is increased in states of inflammation (Hamilton et al. 2000). It is interesting that the $P2X_3$ and $P2X_2$ subunits found on nociceptive sensory neurons will form cation channels together, one being implicated in pain and the other being pH sensitive. It follows that the $P2X_2$ receptor should be activated by tissue environments where acidosis is present i.e. during inflammation. Mechanisms that become more important in pathophysiological conditions are useful in helping us to understand the aetiology of a disease. It seems likely that visceral afferent neurons play a role in the pathophysiology of IBD and many cases of functional bowel disorders, such as irritable bowel syndrome (IBS) have an inflammatory episode as the trigger for sensory neuron dysfunction (Rodriguez and Ruigomez 1999; Barbara et al. 2002). ATP may act as one of the signalling molecules during the initiation of pain and in particular, contribute to the communication of tissue damage and inflammation. Visceral afferent neurons are known to undergo almost continual remodelling and plasticity in response to their local environment and the ongoing need for the mucosa to renew itself (Stead et al. 1991). If abnormal or prolonged sensitisation of purinoceptors occurred due to an inflammatory visceral insult, then this mechanism might contribute to some of the symptoms seen in functional bowel disorders, such as abdominal pain and bloating. Selective antagonists

that are pharmacologically active in-vivo will need to be developed before $P2X_3$ and/or $P2X_{2/3}$ receptors can be fully tested for their potential therapeutic benefit in patients.

CHAPTER FIVE

ADENOSINE 5'-TRIPHOSPHATE AND ITS RELATIONSHIP WITH OTHER MEDIATORS THAT ACTIVATE PELVIC NERVE AFFERENT NEURONS IN THE RAT COLORECTUM

Introduction

Evidence of a role for purinergic signalling in sensory neurons is well established (Burnstock 2000). $P2X_2$ and $P2X_3$ receptors, two members of the larger P2X family of ligand-gated cation channels, are important in this process because both are expressed in sensory ganglia. $P2X_3$ receptors are selectively expressed largely on a subpopulation of small diameter sensory neurons in dorsal root ganglia (DRG) that label for the isolectin IB4 (Chen et al., 1995; Bradbury et al. 1998). $P2X_2$ receptors, also present in DRG, are pH sensitive (King et al. 1996) and along with $P2X_3$ subunits can form heteromultimers that yield adenosine 5'-triphoshate (ATP)-activated currents similar to those found in sensory neurons (Lewis et al. 1995). Metabotropic $P2Y_1$ and $P2Y_4$ receptors have been demonstrated on a subpopulation of DRG neurons that also express $P2X_3$ receptors (Ruan and Burnstock 2003). In addition, the $P2Y_2$ and $P2Y_4$ ligand uridine 5'-triphosphate (UTP) can activate a large proportion of capsaicin-sensitive DRG neurons that also respond to the $P2X_1$ and $P2X_3$ receptor agonist α,β-methylene ATP (α,β-meATP) (Stucky et al. 2003), indicating that P2Y and P2X receptors are coexpressed.

A working hypothesis of purine-mediated mechanosensory transduction has been proposed (Burnstock 1999 and 2001). ATP released during distension from epithelial cells lining tubes (such as ureter or gut), and sacs (such as bladder), acts on $P2X_3$ and/or $P2X_{2/3}$ receptors on a subepithelial sensory nerve plexus to initiate impulses that are relayed to the central nervous system via extrinsic spinal afferents

such as the pelvic nerve. Work in our laboratory has suggested that this mechanism contributes to afferent signalling during distension of the mouse bladder (Cockayne et al. 2000; Vlaskovska et al. 2001; Rong et al. 2002) and guinea pig ureter (Knight et al. 2002; Rong et al. 2003) and colorectal distension in the rat (Wynn et al. 2003). In the latter study, we demonstrated that ATP release from colorectal epithelial cells was pressure-dependent and that exogenous ATP applied to the serosal surface activated the same neurons in the pelvic nerve that were stimulated by noxious distension. In addition, about 25% of the distension-evoked pelvic nerve discharge could be blocked with P2X receptor antagonists. It is clear that other agents therefore play an important role in mechanosensory transduction during distension in the normal colorectum. However, the relative importance of these agents may change in different physiological and pathophysiological conditions.

The gastrointestinal mucosa is known to release a whole host of different mediators in a wide range of situations. 5-hydroxytryptamine (5-HT) is released from enterochromaffin cells in response to luminal contents and mechanical stimuli (Gershon 2003) and other enteroendocrine cells release cholecystokinin, secretin, somatostatin and corticotrophin-releasing factor (Bueno et al. 1997). In addition to ATP and 5-HT, adenosine, interleukin-6 (Sitaraman et al. 2001) and vasoactive intestinal peptide (Dimaline and Dockray 1978) are released from colonic epithelial cells and recently, PGE_2 release from mouse colon epithelial cells has been demonstrated in response to distension (Roza and Reeh 2001). During inflammation, bradykinin (BK), prostaglandins (PGs), leukotrienes, substance P (SP), histamine, serine proteases, cytokines and growth factors can all stimulate enteric nerves either directly or indirectly via specific membrane receptors (Holzer 2001). It is in pathophysiological states such as this that complex interactions governing the response to tissue injury are critical. Many studies have implicated ATP as particularly important in the inflammatory setting

(Driessen et al. 1994; Hamilton 1999 and 2001; Tsuda et al. 1999; Cockayne et al. 2000; Page et al. 2000; Paukert et al. 2001; Yiangou et al. 2001) and this correlates with the fact that ATP is present intracellularly in millimolar concentrations making it a good candidate for signalling significant tissue damage. In fact, recent work in our laboratory has indicated that release of ATP from the colorectal mucosa and sensory nerve discharges evoked by ATP are significantly increased during colitis (Wynn et al. 2004). It is possible that ATP may interact with some of these mediators and/or modulate their effect on extrinsic enteric neurons.

The present study was designed to investigate the correlation of ATP-sensitive pelvic nerve fibres with sensitivity to a range of other important agents that are known to stimulate extrinsic afferent neurons in the gut. We used an established in-vitro preparation that was designed to investigate pelvic nerve activation from the rat colorectum (see Wynn et al. 2003). In this study, we found that intraluminal administration of mediators did not stimulate the pelvic nerve in a consistent way and this is explained by the relative impermeability of the colorectal mucosa. In contrast, we applied mediators to the highly permeable serosal surface to provide an indirect measure of subepithelial nerve plexus stimulation. This model studies the changes in net afferent traffic in spinal nerves when the tissue concentration of one or more mediators rise. It does not tell us about the precise mechanisms via which neurons in the enteric nervous system are stimulated but does give us a measure of extrinsic afferent signalling to the central nervous system of which pain must be a component.

Results

Pelvic nerve responses to ATP and other agents

Figure 5.1 demonstrates typical examples of pelvic nerve activity in response to bolus applications of a variety of neuroactive mediators applied to the serosal surface of

the colorectum at the concentrations shown. Each individual trace is of about 2 minutes duration and represents the increase in spike frequency during a multifibre recording. ATP and 5-HT produced very similar frequency plots, as did BK and SP. Capsaicin activated the extrinsic afferents with the shortest latency (5.47 ±1.7 sec., see fig. 5.2) and reached peak activity after a mean period of just 11.8 ±1.3 sec. (see fig. 5.3). This was typically followed by a sharp fall in the frequency of spikes and a period of desensitisation where nerve activity fell to almost zero but recovered after several minutes, depending on the dose used. Concentrations of 50 μM or more usually ensured that nerve activity never fully recovered. Fig. 5.2 shows that there was no significant difference in the pelvic nerve activation latencies of capsaicin, ATP, 5-HT, BK, SP or protons, but PGE_2 took longer to excite the nerve above baseline levels and to reach its maximum firing frequency (fig. 5.3).

Single unit analysis (using Spike 2 software) revealed how many individual units were being recorded and which units were responding to particular agents (and distension). Previous analysis had shown that resting activity in the same unit did not vary by more than 10%. This allowed an assumption that any activity of over 10% of the mean resting frequency of a unit during the first 60 sec. after application of an agent would represent a response. 44 different experiments were carried out in 26 in-vitro preparations and a total of 674 individual units were analysed. Each recording had an average of 15 units. Because the number of units per recording can affect the accuracy of the results, the responses to ATP were compared in those recordings that contained less than 12 units and those that contained more than this. In the smaller recordings, 71 ±0.05% of units responded to ATP. In the larger recordings, 66 ±0.03% responded to ATP. The difference between the two groups was not significant (P =0.384). Table 5.1 shows the percentage of units that responded to the various stimuli. Phasic distension to 50 mmHg activated 95% of units and the rank order of agents activating the most units

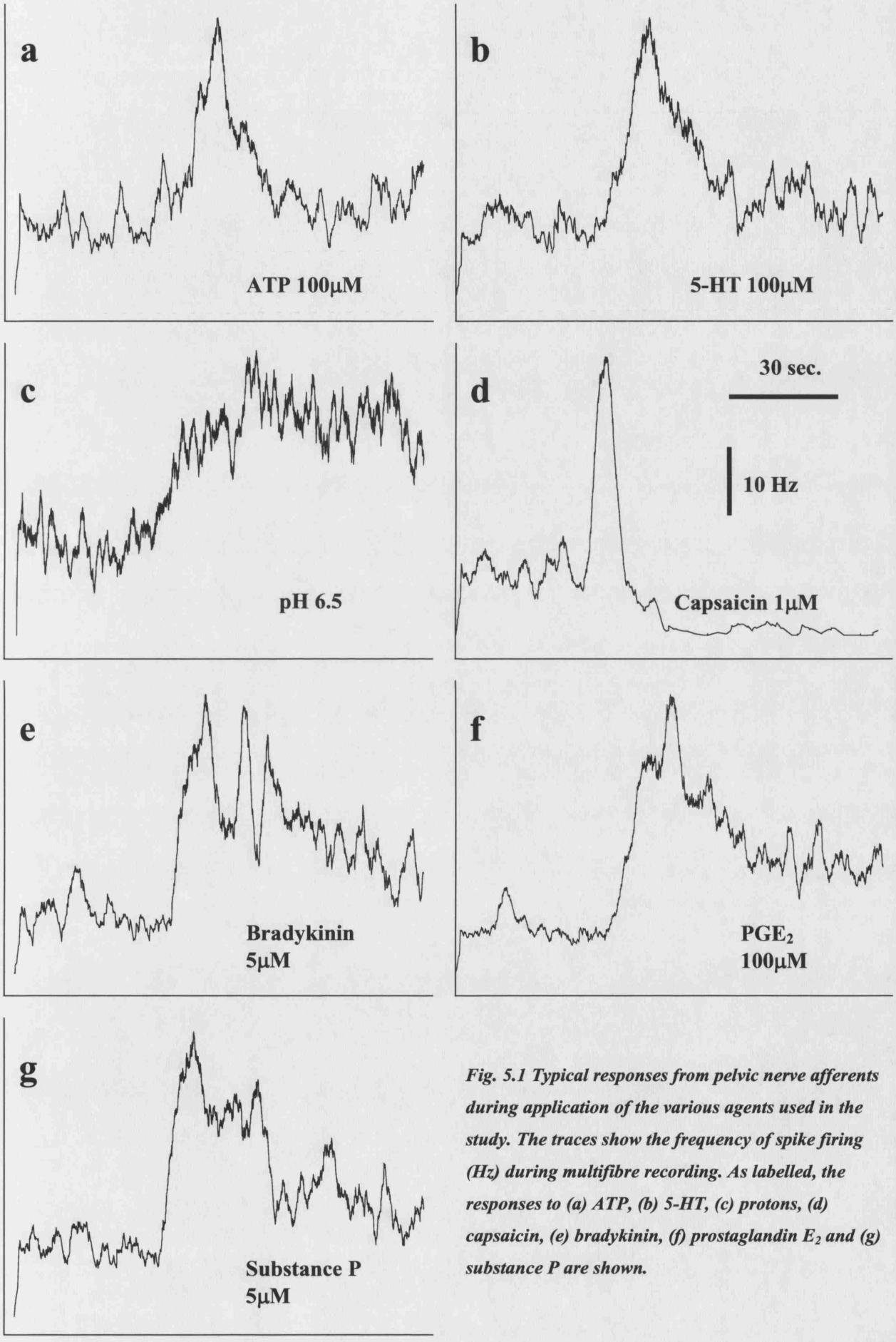


Fig. 5.1 Typical responses from pelvic nerve afferents during application of the various agents used in the study. The traces show the frequency of spike firing (Hz) during multifibre recording. As labelled, the responses to (a) ATP, (b) 5-HT, (c) protons, (d) capsaicin, (e) bradykinin, (f) prostaglandin E_2 and (g) substance P are shown.

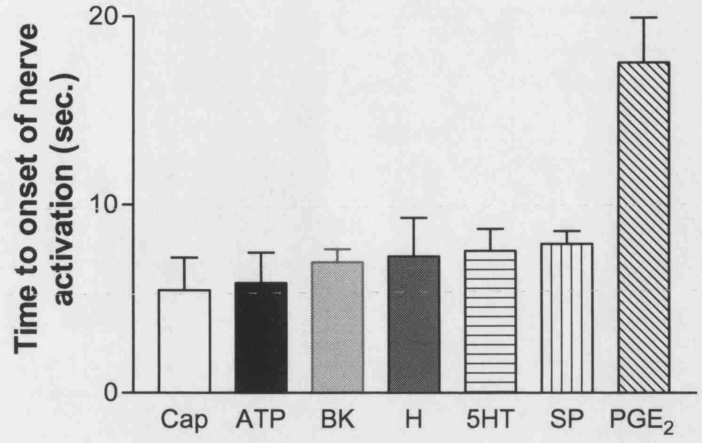


Fig. 5.2 The latencies of activation of the agents applied to the serosal surface of the colorectum were similar except for prostaglandin E_2.

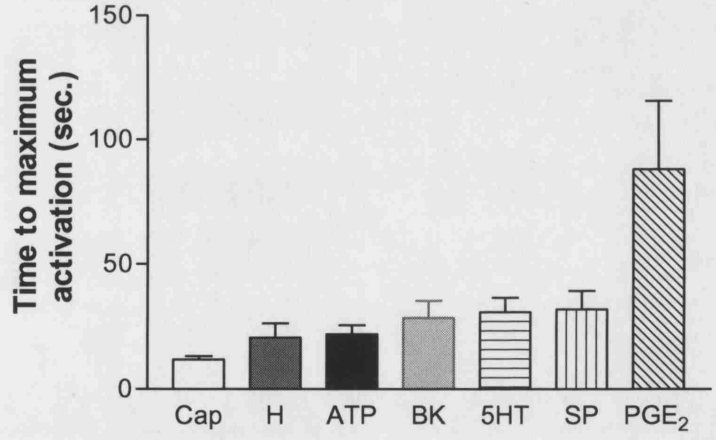


Fig. 5.3 The time taken to reach peak spike frequency. Capsaicin was fastest and prostaglandin substantially slower than the others (cap, capsaicin; H, protons).

after this was: BK ≥ 5-HT > SP > Protons > ATP > Capsaicin > PGE_2.

When ATP was applied in the same experiment as other agents, it was possible to establish which units each agent activated. Table 5.2 illustrates this. The most common agent (that was tested) to activate ATP-responsive units was 5-HT (61% of units). 90% of all units responded to either ATP or 5-HT but about a sixth of these responded to 5-HT only and a further sixth to ATP only giving a two-thirds overlap between the populations. BK activated the largest percentage of units overall (78%) but over a fifth of these (21%) were unresponsive to ATP. The percentage of units responding to ATP as well as substance P, capsaicin and a lowered pH was very similar (just over half) and the proportion responding to ATP only was similar for these agents also. The main difference between these three agents was their ability to activate units that were unresponsive to ATP. SP achieved this in 21% of cases, protons in 16% and capsaicin in just 8%. In fact, 77% of all units either responded to both ATP and capsaicin or to neither, giving the greatest degree of correlation between ATP and any of the agents applied. In the units that responded to both ATP and capsaicin, ATP was able to stimulate activity after the application of capsaicin 1 μM had reduced the background firing to almost zero (fig. 5.4). This excitation of capsaicin-sensitive units occurred in all the units that were initially responsive to the control bolus of ATP, although the frequency of spikes in these units was substantially reduced during the desensitisation period. PGE_2 activated the smallest percentage of units overall (54%) and alongside SP had the lowest activity correlation with ATP (65%).

In two experiments, all the agents were applied in turn with at least half an hour washout between each one. Only 5% of units were activated by all seven agents as well as distension and no units were activated by a single agent alone. Raising the concentration of the agent increased the frequency of spikes in individual units but didn't significantly alter the latency of activation or the percentage of units responding.

Stimulus	Number of units studied	Units responding to stimulus
Distension	188	178 (95%)
Bradykinin	219	171 (78%)
5-HT	131	101 (77%)
Substance P	95	70 (73%)
Protons	68	47 (69%)
ATP	674	440 (65%)
Capsaicin	89	53 (60%)
PGE_2	72	39 (54%)

Table 5.1 The percentage of pelvic nerve fibres that are responsive to the various agents applied to the colorectal serosa. The high proportion of units responding to distension shows that these fibres were primarily concerned with afferent signalling from the colorectum.

Agent	Number of units studied	Units responding to both ATP and agent	Units responding to neither ATP nor agent	Units responding to agent but not to ATP	Units responding to ATP but not to agent
5-HT	131	80 (61%)	13 (10%)	21 (16%)	17 (13%)
Bradykinin	219	125 (57%)	38 (17%)	46 (21%)	10 (5%)
Protons	68	36 (53%)	12 (18%)	11 (16%)	9 (13%)
Capsaicin	89	46 (52%)	22 (25%)	7 (8%)	14 (15%)
Substance P	95	50 (52%)	12 (13%)	20 (21%)	13 (14%)
PGE_2	72	31 (43%)	16 (22%)	8 (11%)	17 (24%)

Table 5.2 The degree of overlap between the pelvic nerve units responding to ATP and the populations of units activated by the various agents applied to the colorectum. The similarity in responsiveness between the ATP-sensitive fibres and the other populations can be calculated by adding the percentages of the "both" and "neither" columns to give an idea of activity correlation.

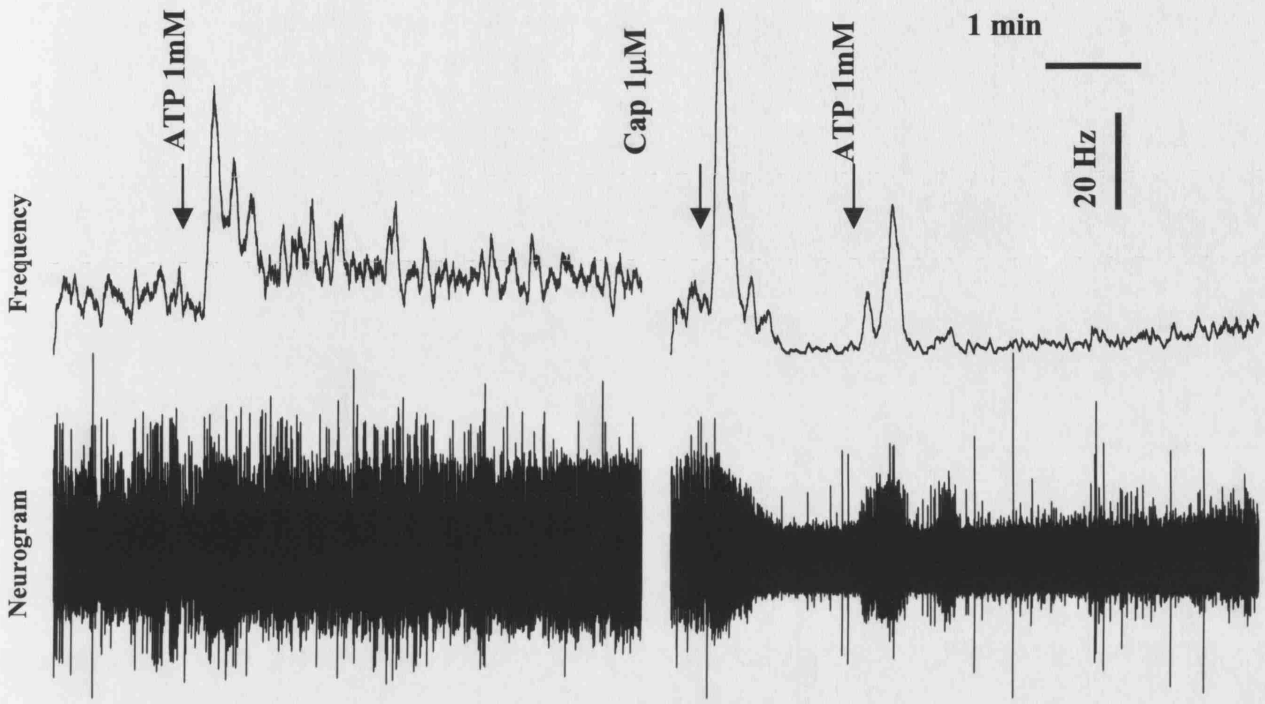


Fig. 5.4 Two continuous traces from the same experiment are shown. On the left, the pelvic nerve response to application of ATP 1 mM to the colorectum. On the right (after 30 minutes washout) the response to capsaicin 1 μM is shown, followed shortly afterwards by a repeat application of ATP 1 mM. The ATP-sensitive fibres are still responsive, but activity is reduced. Top trace: spike frequency (Hz), bottom trace: neurogram.

Interactions between ATP and other agents

The influence of ATP on the activation of colorectal extrinsic afferents by the various agents was investigated by applying the transmitters alone or together with ATP. A low concentration of either ATP or the agent to be tested was applied to the colorectal serosa and after a 30-minute washout, a high dose of the mediator not used initially was applied. Both were then applied simultaneously in the same concentrations following a further lengthy washout period. The concentrations (and therefore the order of application) were then reversed.

When ATP (50 μM or 1 mM) was applied simultaneously with either BK (100 nM or 5 μM), PGE_2 (1 μM or 10 μM) or SP (1 μM or 10 μM), all had non-additive effects. The size of the combined response was always greater than either of the individual responses but smaller than the sum of the two. This was the case regardless of the order of application or concentration. In contrast, the combined responses of ATP with either 5-HT, capsaicin or lowered pH were greater than the sum of their individual responses and examples of these three experiments are shown in figs. 5.5a, b and c respectively. 5-HT (5 μM or 100 μM) applied with ATP (1mM or 50 μM respectively) resulted in an overall increase in spike frequency of 19.4 ±9.8% (n=8) over the sum of the individual components and this was statistically significant (P =0.05; see fig. 5.6a). There was no significant difference between the two experimental conditions relating to the concentrations of the agents: 5-HT 5 μM and ATP 1mM gave an increase of 17.7 ±10.2% whereas ATP 50 μM added to 5-HT 100 μM resulted in an increase of 21.0 ±16.7%. When low concentration capsaicin (100 nM) was applied together with ATP 1mM (a high dose of capsaicin could not be used due to its neurotoxicity), the frequency of spikes was increased by 25.9 ±8.7% (n=8) compared with the sum of the individual responses (P =0.018; fig. 5.6b). Application of ATP in the presence of a

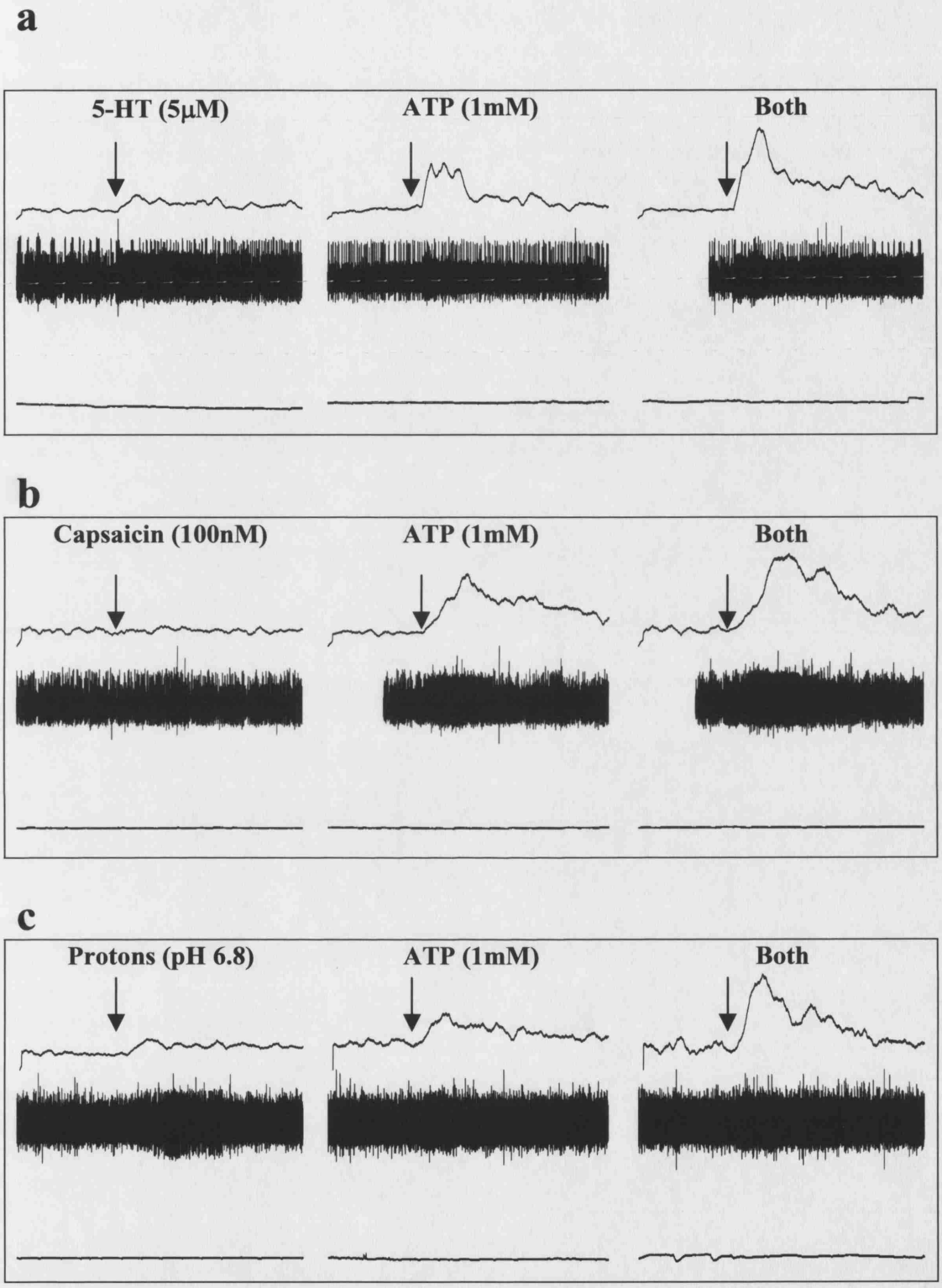


Fig. 5.5 Pelvic nerve recordings are shown during the application of 5-HT (a), capsaicin (b) and protons (c). For each experiment, three traces are shown: the top trace shows the frequency of activity, the middle trace the neurogram and the bottom line shows the pressure, which remained at zero throughout. The figures show the application of low concentration agent (left hand traces), followed by ATP 1 mM (middle traces) and then the co-application of both (right hand traces) showing greater activity than the sum of the two individual responses. The three figures (a), (b) and (c) are not shown at the same scale.

lowered pH gave the most striking results. At pH 6.8, the response to ATP 1mM was increased by 40.3 ±3.1% (n=4; see fig. 5c) above the sum of the individual responses (P <0.0001), whereas at pH 6.5 this increase had risen to 72.6 ±4.1% (n=4; P <0.0001; see fig. 5.6d). Changing the concentration of ATP altered the magnitude of the response to the control bolus and the combined response at a given pH, but the degree of increase above the sum of the individual responses could only be affected by the concentration of protons.

Analysis of single units contributing to the increased responses seen by combined application

By studying the spike frequency of individual units during the application of one or more agents, it was possible to evaluate which units were contributing to the increased response of the nerve as a whole. The vast majority of units that contributed to a greater combined response were activated by both agents, but this was not exclusively the case. In the experiments involving 5-HT, 24% of units increased their firing frequency sufficiently in the presence of a combined bolus of ATP and 5-HT to surpass the responses of both single boluses put together. Of these, three quarters were activated by both single applications and a quarter of them by ATP only. None of the units studied were activated by 5-HT only and then went on to contribute to the increased response. 31% of units contributed to the combined increased response seen with ATP and capsaicin. All of these units were activated by both single applications. In the experiments involving ATP and protons, the larger combined response was mediated by those units responding to both agents or to protons alone; units exclusively activated by ATP did not contribute. The contribution of units activated by both ATP and protons was only slightly greater at pH 6.5 (34%) than pH 6.8 (32%). However,

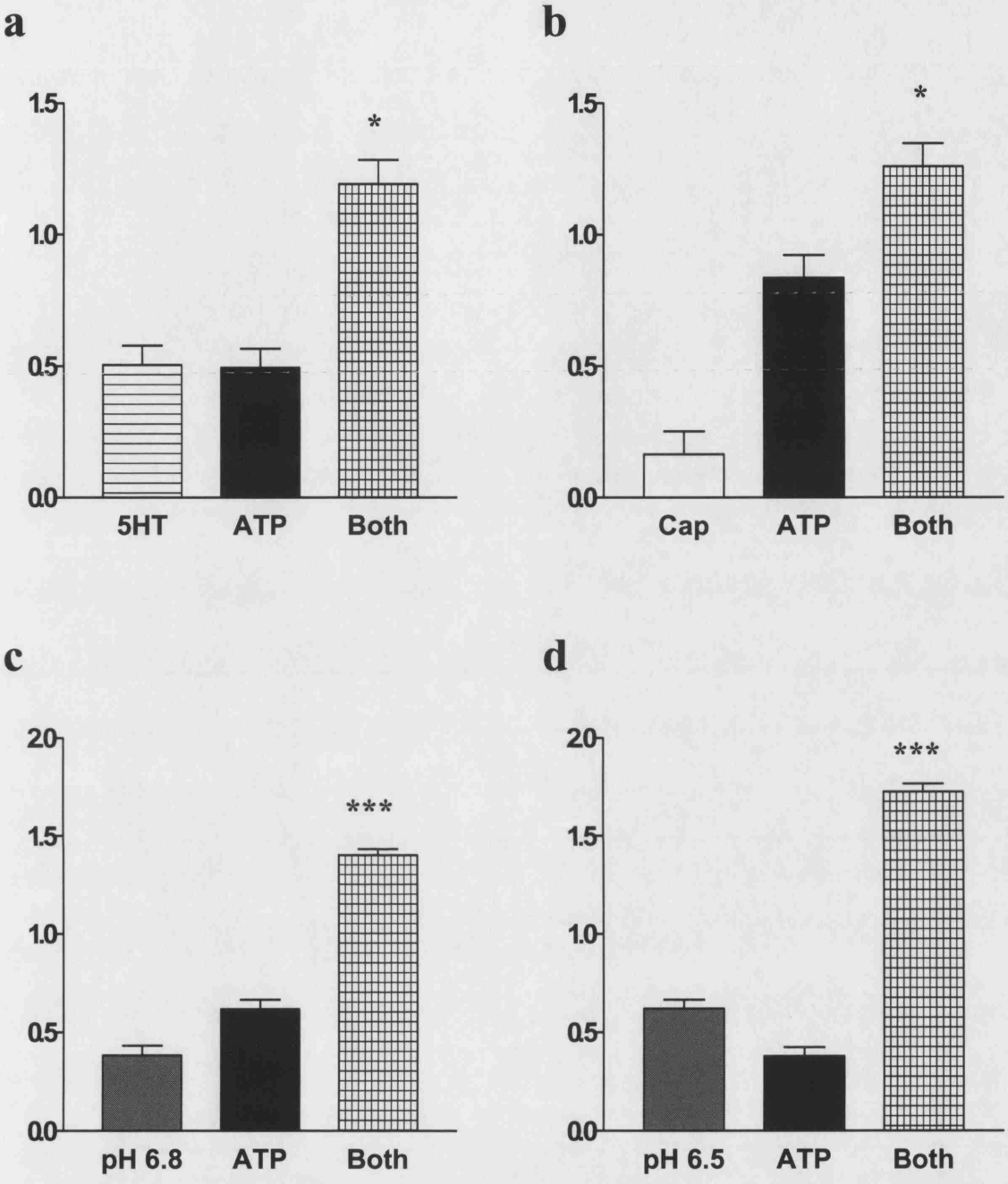


Fig. 5.6 (a) Co-application of 5-HT (5 μM or 100 μM) and ATP (1mM or 50 μM respectively) resulted in greater nerve activity than the sum of the individual responses suggesting synergism (combined data for high and low concentrations is shown). This was also the case for low concentration capsaicin (cap) 100 nM and ATP 1mM (b). Responses to ATP 1mM are greater at pH 6.8 (c) and even more so at pH 6.5 (d). For each graph, the relative proportion of the sum of the individual responses is shown, so the first two bars add up to 1.0. The third bar (on the right) then demonstrates the degree of increased activity when the two agents are applied together.

those responding to protons alone contributed to a greater extent at the lower pH: a further 12% of units at pH 6.8 but a further 21% at pH 6.5. This amounted to a total of 44% of units at pH 6.8 and 55% of units at pH 6.5 that made a contribution to the increased response to a combined bolus of ATP and protons.

Discussion

This study has demonstrated that ATP, protons, capsaicin, 5-HT, BK, SP and PGE_2 can activate colorectal extrinsic afferents in the rat pelvic nerve and examples have been given of the typical characteristics of each response. For the first time, a large number of spinal afferent nerve fibres have been analysed with particular reference to ATP, documenting important data relating to the overlap of responsiveness between ATP and other agents. Previous studies have estimated that about 16% of neurons in the pelvic nerve are responsive to colorectal distension (Sengupta and Gebhart 1994). However, analysis of the recordings from the particular part of the pelvic nerve selected for our study showed that 95% of units were responsive to colorectal distension to 50 mmHg, thus providing data from neurons that are specifically involved with the processing of noxious events from the colorectum. The study has presented data suggesting that ATP may influence other signalling systems that involve 5-HT and capsaicin-sensitive neurons and that the pH of the tissue is likely to influence the response of ATP-sensitive neurons. The study has selected a representative sample of the mediators that are known to stimulate extrinsic enteric neurons and forms an incomplete picture of visceral afferent stimulation. However, the agents have been chosen because they demonstrate the diversity of mediators that are active in the colorectum, all of which have been implicated in visceral pain.

In the experiments where all the agents were applied in turn, no units were activated by a single agent and only a tiny proportion were activated by all the agents. Along with the different percentages of units being stimulated by the different agents, this clearly shows that there are marked variations in the sensitivities of visceral neurons. BK and 5-HT were able to stimulate the greatest number of afferent units in this study (78 and 77% respectively) and these figures are slightly higher than previous studies have suggested. Longhurst et al. (1984) calculated that 65% of visceral afferent fibres responded to BK, although these recordings were made from splanchnic nerves in the cat and were from a wider source than the colorectum. Hicks et al. (2002) reported that 56% of lumbar splanchnic nerve fibres from the rat colorectum responded to 5-HT and this figure rose to over 60% in the splanchnic nerve of the cat (Lew and Longhurst 1986). In studies looking at SP, 50% of spinal fibres (as opposed to just 10% of vagal fibres) innervating the rat stomach contained SP (Green and Dockray 1988) and of the spinal afferents in the cat that innervated the gut, 57% of A-fibres and 50% of C-fibres were SP positive (Lew and Longhurst 1986). In rat DRG neurons that supply the pelvic viscera, 70% were capsaicin sensitive (Yoshimura et al. 2003) compared to just 30% of vagal fibres innervating the ferret gastrointestinal tract (Blackshaw et al. 2000). In another study, PGE_2 was found to excite 45% of myenteric neurons in the guinea-pig colon (Kelles et al. 2002). When evaluating the number of afferent neurons responding to ATP, again, studies looking at both receptor expression and activation of neurons can be used. $P2X_3$ receptors are expressed on about a third of DRG neurons, nearly all of them small diameter (Bradbury et al. 1998), but there are also other populations of DRG neurons that express $P2X_{2/3}$, $P2X_2$ and $P2X_1$ receptors (Petruska et al. 2000) as well as P2Y metabotropic receptors (Ruan and Burnstock 2003; Moriyama et al. 2003). In DRG neurons of the mouse, 54% of C-fibres and a further 12% of Aδ-fibres (a total of 66%) were responsive to the P2Y receptor agonist, UTP and many of these were also

responsive to the selective P2X agonist α,β-methylene ATP, indicating coexpression of P2X and P2Y receptors on many of these cells (Stucky et al. 2003). The 65% of units responsive to ATP in this study may reflect the diversity of purinoceptors on spinal afferent neurons supplying the colorectum. Further electrophysiological experiments with more selective agonists are required to distinguish between these receptor types. On the whole, the percentages of units being activated by the various agents in the present study are consistent with previous experiments. Where there are discrepancies, this might occur due to subtle variations in the receptor expression between the innervation of specific visceral organs, relating to differing function or to the method used to apply the agents. Other studies have used intra-arterial injection (Lew and Longhurst 1986; Longhurst et al. 1984), whereas we used a bolus application to the organ bath and this in itself may preferentially activate those neurons that supply the serosal surface of the colorectum. It must be remembered that the method used to discriminate units in the pelvic nerve in this study may play a part in these differences (see methods). It is also likely that some of the agents used would affect smooth muscle tone and indirectly cause activation of mechanosensitive visceral afferents. However, to elucidate the precise indirect mechanisms responsible for stimulation of extrinsic afferent neurons was beyond the scope of this study. What these experiments do show is the end result of spinal nerve activity in response to rises in tissue concentrations of the various agents.

The large proportion of units responsive to 5-HT in this study underlines the importance of this agent in the gut. 5-HT is released from enterochromaffin cells (for review see Gershon 2003) and excites extrinsic afferents that supply the colorectum (Hicks et al. 2002). More specifically, both 5-HT_3 (Morteau et al. 1994; Kozlowski et al. 2000) and 5-HT_4 (Schikowski et al. 2002) receptors are involved in signalling colorectal distension. Purinergic signalling is also involved in colorectal distension

(Wynn et al. 2003) and with 61% of units responding to both 5-HT and ATP, it is likely that these two systems are functionally cooperative. Co-application of both agents in this experiment resulted in a moderate increase in multifibre activity compared to the sum of the individual responses and although this was not great, the difference did reach statistical significance. Studies on intrinsic enteric sensory neurons have suggested that currents induced by co-administration of both 5-HT and ATP were only as large as the currents induced by the individual agents, although many of these neurons were shown to co-express receptors for both (Zhou and Galligan 1998; Barajas-Lopez et al. 2002). It is quite possible that extrinsic afferents behave in a different way, as it is these neurons that must signal noxious stimulation such as colorectal distension to the central nervous system. Analysis of the firing of individual units in this study suggested that a quarter of the fibres responding to the agents applied together contributed to the synergism and more interestingly, a quarter of these (6% of the total) were not initially responsive to 5-HT. This implies that either these units did not express 5-HT receptors, although their ATP-evoked activity was augmented by 5-HT, or that the application of ATP lowered the threshold of activation of 5-HT receptors that were unresponsive to the initial bolus.

Just over half the units responsive to ATP in this study also responded to capsaicin and 77% of units responded in the same way to both agents, showing a good deal of functional correlation. Capsaicin is important because it is a potent agonist at the VR1 receptor, which is expressed on small diameter extrinsic enteric neurons and activated by noxious stimuli such as heat (>42°C), acidosis and several lipoxygenase products (Tominaga et al. 1998). Stimulation of capsaicin-sensitive nerves in the rat stomach induces mast cell degranulation with subsequent hyperaemia (Wallace et al. 1992) and capsaicin-sensitive nerves have been shown to exert a protective role during inflammation by the release of peripheral and central neuropeptides such as SP and CGRP (Green and Dockray 1988; Reinshagen et al. 1998; McVey and Vigna 2001). In

fact, NK1 knock-out mice fail to develop neurogenic inflammation that is VR1 dependent (Laird et al. 2000). This study has demonstrated that co-application of low concentration capsaicin with ATP can increase the activity in the pelvic nerve by 25.9% more than the sum of the individual responses. If capsaicin-sensitive neurons are important in the response to tissue injury, then it follows that ATP, itself released during tissue injury and noxious stimuli, should interact with the VR1 receptor. Previous studies have shown that ATP can enhance the effect of capsaicin on rat VR1-gated currents by acting as an allosteric factor (Kwak et al. 2000). Recent evidence suggests that application of ATP to capsaicin-sensitive sensory neurons increases VR1-dependent SP release (Nakatsuka et al. 2001; Huang et al. 2003). Also, ATP-induced thermal hyperalgesia is abolished in VR1 knock-out mice and this mechanism may involve the $P2Y_2$ receptor (Moriyama et al. 2003). In addition to demonstrating that a non-desensitising bolus of capsaicin applied with ATP can act synergistically, we have also shown that ATP can still activate pelvic nerve afferents during the period of desensitisation following a large concentration bolus of capsaicin. This is in contrast with a previous study that showed the lingual nerve was unable to respond to α,β-methylene ATP after a desensitising bolus of 10 μM capsaicin (Rong et al. 2000). We used a concentration of 1 μM in this study, which, although reduced background firing to almost zero, may not have been enough to render the neurons completely insensitive. Higher concentrations (50 μM and above) were certainly able to completely desensitise the nerve fibres to ATP and other agents. This demonstrates an interesting aspect of the VR1 receptor. At a critical range of tissue concentrations, ligands may alter the sensitivity of the neuron to depolarise and this sensitivity may impact upon the degree to which other mediators can stimulate these neurons in any given physiological situation. These capsaicin-sensitive nerve fibres may protect against stimulation from other mediators that is of a magnitude which may not actually be helpful.

The pH of tissues drops during inflammation and protons are known to sensitise and activate nociceptive neurons (Reeh and Steen 1996). Some proton-gated ion channels or acid-sensing ion channels (ASICs) are exclusively expressed on primary afferent neurons (Chen et al. 1998) and $P2X_2$ and $P2X_{2/3}$ receptors are pH sensitive (King et al. 1996; Dunn et al. 2001). In this study, protons were able to activate 53% of units that were responsive to ATP, a very similar proportion to capsaicin. At pH 6.8, the activities of these units and a further 12% that responded to protons but not ATP, were over 40% higher than the sum of the responses to ATP and protons alone. At pH 6.5, this percentage increase in activity had risen to 72.6%. As the pH was lowered, an increasing percentage of units that were only responsive to protons contributed to the increased activity (12% at pH 6.8 and 21% at pH 6.5), suggesting two scenarios. Firstly, that the pH sensitive purinoceptors that were initially not activated became so as the concentration of protons increased. This seems unlikely, as some sort of response would have been expected in these units at the concentrations of ATP used (1mM). A more likely scenario is that when the pH is reduced, the presence of ATP is able to lower the threshold of VR1 receptors and therefore allow more of them to be activated by increasing concentrations of protons. Although ASICs undoubtedly play an important nociceptive role in an inflammatory environment, the VR1 receptor is key because it mediates an important protective response through the release of neuropeptides as well as transmitting other nociceptive information.

The role of ATP in regard to extrinsic afferents in inflammatory tissues is primarily as a signalling molecule, directly activating sensory neurons via P2X and P2Y receptors. The two different classes of receptors might mediate nociceptive function and its role in neurogenic inflammation respectively. We have also discussed a role for ATP as a neuromodulator acting at other receptors, facilitating their actions. Many of these mechanisms indicate that when there is a noxious stimulus that threatens tissue,

extrinsic enteric sensory nerves are critical in communicating important nociceptive information to the central nervous system to influence other body systems and behaviour, in addition to orchestrating an ongoing local response that leads to protective vasoactive, secretory and immunological functions that eventually aid the healing process. There is now good evidence that ATP plays a role in both these processes.

CHAPTER SIX

GENERAL DISCUSSION

Overview

The work carried out during this period of research was directed initially towards the stated hypothesis of purinergic mechanosensory transduction. Having answered some of the questions posed by this hypothesis in the normal colorectum, the project proceeded to deal with some of the same questions applied to a pathophysiological state, in this case, a model of colitis. However, the broader context of the research was to further our understanding of how ATP affects sensory nerves in the gut. This could only be achieved if some attempt was made to study other mediators that are known to activate sensory enteric neurons and how they relate to ATP and other purinergic compounds. This was addressed in the third experimental chapter where analysis of single units from the pelvic nerve revealed both interactions between various mediators and an estimate of the proportion of fibres responding to each one. The following discussion will state to what extent the work has achieved its aims set out in the introduction and some ideas for further experiments. There will be some discussion on how this work relates to other similar research and how the advancing field of purinergic signalling in relation to visceral pain might be of use to clinical medicine in the future.

Firstly, there are some general considerations about the apparatus and methodology. My in-vitro preparation was extremely reliable and the experiments easily repeatable, removing many of the external variables that could have led to errors if working on different preparations on different days. However, the colorectal distensions used were rather non-physiological in their characteristics and this is critical, as some of the main conclusions of the project are based around the results obtained during these distension experiments. Phasic distensions, which were used frequently in the work,

allow intraluminal pressure to rise very quickly and it is not clear whether this occurs under natural conditions. Nevertheless, the intraluminal pressures used have been demonstrated to be noxious in the rat (Ness and Gebhart 1988). Similar in-vitro preparations of bladder, ureter and tongue have yielded good results in the past (Vlaskovska et al. 2001; Knight et al. 2002; Rong et al. 2000 and 2002) and care was taken to adhere to many of the principles set out by these experiments, in particular the handling of the tissues and in the case of nerve recordings, the filtering and capture of electrical impulses.

To what extent have I achieved my objectives?

The objective of this project was to examine whether the hypothesis of purinergic mechanosensory transduction (Burnstock 1999 and 2001) applies to the colorectum, as well as to investigate the wider role of purinergic compounds in relation to sensory nerve signalling in the gut. The individual aspects of the hypothesis have been studied in turn to try to piece together evidence for or against it. The components to be considered are: ATP release from gut epithelial cells during distension, involvement of $P2X_3$ or $P2X_{2/3}$ receptors in subepithelial sensory nerves and their excitation by ATP and subsequent activation of central pain pathways. In each of these fundamental elements of the project, strong evidence has accumulated that enables us to build up a picture of the mechanisms involved in purinergic sensory transmission in the colorecum. These will be discussed in turn.

A convincing correlation was obtained between ATP concentration in the perfusate after colorectal distension and the extent of peak intraluminal pressure. These experiments attempted to recreate the effect of uniform circumferential stretch of the gut wall similar to that encountered in-vivo with normal bowel contents.Other methods of

simulating stretch of the colorectal wall might be considered even less physiological: applying tension to cultured monolayers of enterocytes or stretching flat strips of bowel wall. Currently under development is a needle probe that directly measures concentration of ATP when embedded into tissues. This may give more accurate values of ATP concentration in relation to more physiological distensions, but would be difficult to use in experiments that, by their very nature, involve movement.

After removal of the majority of the mucosa from the colorectal specimens, the increase in measured ATP after distension was not significantly different from basal levels. Undoubtedly, routine histology confirmed some mucosal cells were left behind due to tight adhesions to underlying tissues, but this was not enough to alter the result and it is likely that the epithelial cells within the colorectal wall were responsible for the release of ATP during distension. This is in accordance with studies suggesting that other important mediators that are involved in sensory nerve signalling, such as 5-HT, are released from the mucosa in response to specific stimuli (Zhu et al. 2001).

$P2X_3$ receptors have been identified on neurons in the dorsal root ganglia that project to the epithelium of the colorectum (Wynn et al. 2004) and strong evidence is accumulating for the presence of $P2X_3$ receptors in intrinsic enteric sensory neurons that also project to the subepithelial region in the rat gut (Xiang and Burnstock 2004a,b). Intrinsic sensory neurons have also been shown to express $P2X_3$ receptors in the mouse gut (Bian et al. 2003). These results are consistent with the hypothesis proposed by Burnstock (2001) that $P2X_3$ (and/or $P2X_{2/3}$) receptors on subepithelial sensory nerve endings mediate both intrinsic reflexes and nociception via extrinsic nerves.

The evidence presented from the colitis model suggests that increased local concentrations of ATP and a lower pH that activates $P2X_2$ receptors may sensitise neurons responsible for transmitting information to the central nervous system and as a result, magnify afferent traffic during background activity and distension in this

situation. Up-regulation of $P2X_3$ receptors on the cell bodies of sensory nerves after inflammation may represent communication between neuronal and non-neuronal cells within the DRG after a tissue insult. Our finding that $P2X_3$ receptors were up-regulated in both the DRG cells involved in signalling from the area of inflammation and those not directly involved, may suggest that there is a more generalised neuronal response to injury from a specific location. In other words, the whole of the gut must react when a single part of it is damaged. This makes sense, because luminal contents stimulating proximal regions of the gut are likely to stimulate more distal parts later on and in this way the gut may act as an integrated organ.

Software allowing single unit analysis of the multifibre recordings from the pelvic nerve was invaluable to the study. One function of this analysis was to look at the responses of single units to various stimuli. We were able to show that individual fibres were responsive to distension as well as to ATP and that increasing the concentration of ATP resulted in a corresponding increase in spike frequency. This strongly suggests that individual neurons can gauge both the intensity of a noxious mechanical stimulus in addition to the concentration of ATP at their peripheral terminal. If endogenous ATP is to be proposed as a transmitter in mechanosensory transduction in the rat colorectum, then this single piece of evidence is critical. What we have not shown directly is activation of the same cells that expressed $P2X_2$ or $P2X_3$ receptors within the submucosal plexus; our preparation could only study extrinsic neurons. However, the bottom line is that neurons communicating to central regions respond to both noxious mechanical stimuli and ATP.

Perhaps the most difficult aspect of this work was to give conclusive evidence for the involvement of pain pathways. An in-vitro preparation can only ever give indirect evidence for the involvement of pathways that eventually terminate within pain centres in the brain. In-vivo studies and behavioural animal studies are required to give

convincing evidence for this. A good deal of previous work has indicated that purinergic signalling, especially involving $P2X_3$ receptors, is integral to the processing of pain from both somatic and visceral sources (for review see Burnstock 2000). However, in this study, question marks were raised about the destination of the pelvic nerve fibres that were responsive to both ATP and to noxious distension. Could we have been recording from the afferent limb of spinal reflexes, not directly involved in transmission to higher centres? Experimental design dictated that the pelvic nerve was cut just distal to the DRG, ensuring that all recordings represented spinal pathways with a good response to colorectal distension. Previous studies have shown an important role for the pelvic nerve in the rat in transmitting painful sensations (Ness and Gebhart 1988). As discussed above, we have shown that ATP can stimulate the same pelvic nerve fibres as a noxious mechanical stimulus. In addition to this, we have shown that the more selective agonist for $P2X_3$ receptors, α,β-meATP, was able to preferentially lower the activation threshold of high-threshold fibres, without affecting the activation threshold of low-threshold fibres. By their very nature, high-threshold neurons are activated by distension pressures that are in the noxious range. The mean threshold of activation of high-threshold units in our preparation was 23.46 mmHg and we know from previous behavioural studies that colorectal distension over 30 mmHg is noxious in the rat (Ness et al. 1991) and pseudoaffective pressor, tachycardic and visceromotor reflexes that precede this occur at 20-25 mmHg (Ness and Gebhart 1988). This gives indirect evidence that at colorectal pressures that activate high-threshold neurons, rats feel pain. Moreover, α,β-meATP, via $P2X_3$ or $P2X_{2/3}$ receptors, can cause greater activation in these units by lowering their thresholds. Although we cannot conclusively prove from our experimental preparation that the neurons we were recording from were directly involved in pain pathways, there is ample other evidence to suggest this is more than likely.

Future directions

In this section, I will discuss work that would be interesting and relevant to the project if I were to continue in the future.

Experiments could be set up to investigate the mechanism of ATP release from the rat colorectum. Previous experiments in the guinea pig ureter have demonstrated reduced ATP release during distension using the inhibitors of exocytosis, brefeldin A and monensin (Knight et al. 2002). This would be relatively straight-forward to carry out and could be extended to the colitis model, where it would be interesting to assess what proportion of the increase in ATP concentrations in the perfusate after inflammation was dependent on direct cellular damage and how much was due to increased controlled release by exocytosis. Both basal levels and post-distension levels could be analysed.

In the present study, we have demonstrated that both the number of DRG neurons responding to ATP and those staining for the $P2X_3$ receptor, particularly those containing CGRP, were increased after a period of colitis. These neurons are perhaps the most important in relation to the transmission of pain to the central nervous system and as a result, it will be fascinating to see how these receptors and others (in particular P2Y receptors) change after other types of noxious stimuli. One experimental condition that might reveal interesting results would be a model of acute and chronic large bowel obstruction. We know that both P2X and P2Y receptors are expressed by a subpopulation of DRG neurons (unpublished observations). With the use of more selective agonists and antagonists, it might be possible to find out more about the interactions of these receptors and their functional significance. It would also be of benefit to our wider understanding of the role of purinergic signalling in sensory

mechanisms in the gut to find out the extent of P2X as well as P2Y expression in other neuron groups. Intestinofugal fibres are important for sensorimotor reflexes and their cell bodies reside in the myenteric ganglia, while their axons synapse in the PVG. Both these sites would be prime candidates for further immunohistochemical analysis in relation to intracellular electrophysiology in response to tissue distortion or damage. We have demonstrated $P2X_2$ and $P2X_3$ receptors on intrinsic sensory neurons in the submucosal plexus of the rat colorectum. It is not clear yet whether these receptors play a role in nociception or are an incidental finding relating to other sensory functions. Previous analysis of human IBD specimens revealed an increase in $P2X_3$ immunoreactivity in the myenteric plexus compared to controls but this was not demonstrated in the submucous plexus. Future experiments should examine what happens to these receptors after inflammation in the rat and whether their responses to ATP and other agonists change.

In order to demonstrate more clearly that the DRG cells being studied (either immunocytochemically or electrophysiologically) related to the functional observations in pelvic nerve afferents, we needed to show that both experiments were studying the same neurons. This could be achieved by retrogradely labelling the DRG neurons from the colorectum. We know from previous work that axons in the pelvic nerve of the rat have their cell bodies in the DRG (Sengupta and Gebhart 1994). We also know which DRG are most important for sensory processing from this area of the gut (Hicks et al. 2002). However, by labelling neurons from the wall of the colorectum, only those DRG cells directly involved from this area would have been considered in the results. This would have proved the presence of $P2X_3$ receptors on colorectal extrinsic afferent neurons and in the colitis experiments, by focussing on the relevant neuron only, this would have increased the sensitivity of the results and refined the conclusions.

The project used single unit analysis in order to identify those pelvic nerve fibres that were responsive to various mediators and as a result, made a useful estimation of the cross-reactivity of individual neurons to these. What was not undertaken was a dose response study for each mediator to give a rank order of neuronal sensitivity. This would have added qualitative data to the quantitative data presented. Single unit analysis was also used to calculate threshold of activation of individual units. A useful and interesting analysis would be to repeat this for the colitis preparations in order to show whether the high threshold units had been affected in the same way as in the presence of α,β-meATP in the normal preparations (i.e. a reduction in threshold of activation). An alternative (and more time-consuming) method of analysing single units is to record directly from individual neurons within the pelvic nerve. This potentially could give more accurate results, but would need an altogether different experimental set-up and range of skills. However, this method would allow verification of computer-assisted single unit analysis.

The most powerful way of demonstrating delivery of noxious stimuli is in behavioural studies. In-vivo experiments can show visceromotor reflexes that are known to accompany pain. This project was based around an in-vitro preparation and as such, had to rely mainly on indirect evidence for the initiation and transmission of pain (as discussed above). In order to strengthen the evidence for the involvement of purinergic signalling in the transmission of pain from the rat colorectum, it would be helpful to repeat some of the experiments in anaesthetised animals. In this way we could measure known indicators of pain such as tachycardic and pressor reflexes to evaluate the effect of purinergic agonists and antagonists. Ethical approval would have to be sought and there would be some technical difficulties, not least the delivery and metabolism of the active agents. We do not, to date, have adequate antagonists for the $P2X_3$ receptor that are active in-vivo. However, these experiments could be attempted in

$P2X_3$ knock-out mice. Similarly, colorectal distension delivered to mobile, awake animals would allow us to compare the different responses in $P2X_3$ knock-outs and their corresponding wild-types. Behavioural studies in rats could involve colorectal distension-induced passive avoidance behaviour in the presence or absence of purinergic agonists or antagonists that were active in-vivo. In the future, it will be interesting to see how P2Y receptors fit into the jigsaw, using both effective antagonists and P2Y knock-out mice.

How does this work relate to other research in its field?

Distension-induced ATP release has been shown in the bladder (Vlaskovska et al. 2001; Ferguson et al. 1997) and ureter (Knight et al. 2002). All these experiments used the highly sensitive luciferin-luciferase assay, as was the case in our study. In the guinea pig ureter, similar to our own findings in the rat colorectum, release continued to increase at very high pressures. In the mouse bladder, maximum release was achieved at 20mmHg and we can only assume that pressure-release relationships are likely to be species-specific. More importantly, we have shown that release can be increased after induction of colitis and this correlates with findings that urinary ATP levels are significantly elevated in patients with interstitial cystitis (Sun et al. 2001). This is a painful condition that leads to hypersensitivity to bladder distension and increased frequency of voiding.

Studies on the pelvic nerve in the rat by Sengupta and Gebhart (1994) have laid a solid knowledge base for our experiments. By recording from single fibres within the S1 dorsal root, they identified that 16% of them were responsive to colorectal distension and these responses remained stable despite repeated distensions. This corresponds to our own experiences, where pelvic nerve fibres that had a good response to colorectal

distension were not always easy to find. Once present though, responses were repeatable and dependable for many hours. Other similarities exist between these two studies. The number of units responding to colorectal distension at low and high thresholds was almost identical in both studies (23% and 77% respectively). After application of bradykinin to our preparation, single unit analysis by Spike 2 software revealed that 171 out of 219 (78%) fibres in the pelvic nerve responded; Sengupta and Gebhart tested just 9 fibres, but found 7 of them responsive to bradykinin (78%). These results provide examples as to the reliability of computer-assisted single unit analysis, on which a critical proportion of our results are based.

Our in-vitro preparation was similar to that used by previous investigators (Rong et al. 2000 and 2002) and was known to be robust and provide reproducible results. The effect of purinergic compounds on afferent nerves from various tissues has been examined. Behavioural studies (Bland-Ward et al. 1997; Cockayne et al. 2000), in-vivo studies in anaesthetised animals (Dowd et al. 1998; Kirkup et al. 1998 and 1999) and in-vitro tissue-nerve preparations (Hamilton et al. 1999 and 2001; Lynn and Blackshaw 1999; Rong et al. 2000 and 2002; Page et al. 2000; Vlaskovska et al. 2001) have all advanced our knowledge of purinergic signalling in peripheral sensory nerves. These studies have examined afferent responses to both visceral and somatic tissues during application of purinergic agonists and antagonists. ATP and α,β-meATP were able to activate the (general sensory) lingual nerve (Rong et al. 2000) and cutaneous sensory neurons in the rat (Hamilton et al. 2001). In addition, after inflammation in the latter study, the magnitude of the response to α,β-meATP and the number of fibres activated both increased. Similarly, in our studies, the number of DRG neurons expressing the $P2X_3$ receptor increased after induction of colitis, as did the proportion of neurons responding with a transient inward current. In an in-vitro bladder-pelvic nerve study in the mouse, Rong et al. (2002) demonstrated that the afferent response to distension

could be potentiated by intravesical P2X agonists and attenuated by similar application of P2X antagonists. Furthermore, single unit analysis revealed that high threshold mechanosensitive fibres in particular could be sensitised by α,β-meATP, as demonstrated by our studies in the colorectum. In accordance with this, loss of the $P2X_3$ receptor was examined in a similar in-vitro mouse bladder-pelvic nerve preparation (Vlaskovska et al. 2001). The knock-out mice had an attenuated afferent response to bladder distension and larger bladder capacities. In another in-vitro study, α,β-meATP was also responsible for sensitising the wall of the oesophagus to mechanical stimulation, but only after inflammation (Page et al. 2000). These results all suggest a pivotal role for the $P2X_3$ receptor in particular, in mechanosensory transduction. Our studies in the colorectum of the rat agree with the general principles that the activation of this receptor (and possibly others also) contributes to the sensory traffic to the central nervous system and this is particularly important in high threshold fibres that are known to be associated with noxious stimuli. Purinergic activation seems to be up-regulated in states of inflammation, as demonstrated by the oesophagitis and skin-nerve experiments described above. The colitis preparation in our study also clearly shows this. Our experiments have indicated that a combination of cooperative factors such as increased ATP release, sensitisation of individual neurons in addition to increased numbers of excitable neurons leads to the increased mechanosensory afferent response.

Some investigators have chosen to work with anaesthetised animals, as this is assumed to demonstrate a closer resemblance to living tissues than an in-vitro preparation. Of particular importance are the studies showing excitation of mesenteric afferent nerves from the jejunum by ATP and α,β-meATP (Kirkup et al. 1999) and adenosine (Kirkup et al. 1998). These are the only other studies to date to document extrinsic enteric nerve activation by purinergic agonists. However, this work did not relate to mechanosensory transduction. One other in-vivo study in the normal and

inflamed rat knee joint has demonstrated neuronal sensitivity to ATP and α,β-meATP, but in contrast to our experimental results, activity was not increased after induction of arthritis (Dowd et al. 1998).

Some behavioural studies are of note. One showed that signs of overt nociception in the rat such as hindpaw lifting and licking were increased after injection with α,β-meATP and these were dose-related (Bland-Ward et al. 1997). Pre-treatment of the paw with capsaicin abolished this response and the authors concluded that P2X receptors on capsaicin-sensitive neurons mediated behaviour indicative of acute nociception. Single unit analysis in our experiments has shown that neurons in the pelvic nerve that respond to capsaicin are also likely to respond to ATP and that the concentration of capsaicin might be important for its interactions with ATP. Another study assessed the behaviour of $P2X_3$ knock-out mice (Cockayne et al. 2000). No deficit in sensorimotor performance could be determined and as in the previous study in the rat, injection of ATP into the wild-type paw revealed nociceptive behavioural responses. These were significantly reduced (but not abolished) in the knock-outs. Interestingly, during formalin-induced inflammation, the null-mutant mice had significantly attenuated pain-related behaviour. They were also noted to have large bladder capacities and reduced voiding frequencies. These observations suggest that purinergic signalling involving the $P2X_3$ receptor contributes to the sensory arm of the voiding reflex and also inflammatory pain. The latter discovery is in accordance with many other studies including our own. We have not specifically investigated the role of purines in colorectal defaecation reflexes, but as mentioned above, this would be a legitimate area for future study. In an important study examining the abdominal constrictions produced by acetic acid administration in mice, PPADS, suramin and in particular, TNP-ATP reduced this nociceptive behaviour (Honore et al. 2002).

Studies that have centred on DRG neurons have shown altered $P2X_3$ expression after injury or inflammation. Using in-situ hybridisation, DRG $P2X_3$ mRNA is decreased in injured peripheral nerves and increased in uninjured nerves following nerve ligation (Tsuzuki et al. 2001). This is consistent with evidence showing that axotomy decreases $P2X_3$ receptor protein and function (Ding et al. 2000). In contrast, chronic constriction injury to peripheral nerves result in increased numbers of $P2X_3$-positive DRG neurons (Novakovic et al. 1999). One study used the phoshorylation of specific kinases within DRG neurons to indicate noxious stimulation. Peripheral injection of α,β-meATP was able to induce this phosphorylation in the inflamed rat hindpaw but not its normal equivalent. There was good correlation between $P2X_3$-positive neurons and those that showed this kinase-related change (Dai et al. 2002).

There is a recurring and important theme to many of these experiments that agrees with the main conclusions of the current thesis. Not only is ATP and its related compounds that activate $P2X_3$ and $P2X_{2/3}$ receptors involved in nociception, but their effect is enhanced by inflammation and chronic nerve injury. Emerging studies have extended the potential scope for this field by suggesting that $P2X_7$ antagonists can induce nociception in a rat model of peripheral inflammation (Dell'Antonio et al. 2002). In addition, altered purinergic signalling in inflammatory states inevitably has an influence on the motor control of functional organs such as the gut. Chronic inflammation of the mouse ileum lead to loss of smooth muscle inhibition by purines (De Man et al. 2003) and in studies of $P2X_3$ and $P2X_2$ knockout mice, ileal contractions were reduced, whereas colonic contractions were increased in the $P2X_2$ knockouts only (Galligan et al. 2003).

What is the clinical significance of these findings?

Application of purinergic compounds to clinical problems has only just started to take off. There is now a real possibility that purinergic agonists and antagonists that survive in-vivo will be useful in a wide array of pathological conditions. ATP is a molecule that has an early evolutionary presence and what is most exciting is that these potential therapeutic compounds will strike at the very heart of many physiological processes, providing the clinician with powerful tools in the fight against disease.

It is not the remit of this section to review the current experimental promise within the purinergic field (for review see Burnstock 2002). However, some drugs are already being used with success. One of the first purinergic compounds to be of benefit was adenosine, which when injected intravenously, is useful for the treatment of supraventricular tachycardia. One of the side effects of this therapy is bronchoconstriction and so it is not surprising that aminophylline, which is metabolised to theophylline (a P1 receptor antagonist) is used in the treatment of asthma and chronic obstructive airways disease. Clopidogrel, a powerful platelet $P2Y_{12}$ receptor antagonist, is now widely used, alone or in conjunction with aspirin, as an antithrombotic drug in the treatment of ischaemic heart disease and stroke (CAPRIE Steering committee 1996; Yusuf et al. 2001).

As discussed in previous sections, purinergic signalling plays a significant role in neurotransmission in the gut. ATP is a cotransmitter in NANC nerves responsible for the inhibitory phase in peristalsis; it participates in synaptic transmission in both the myenteric and submucosal ganglia and is involved in vascular and secretory control. $P2X_2$ and $P2X_3$ immunoreactivity is seen on both intrinsic and extrinsic enteric neurons. Sensory ganglia that supply the gut have combinations of these two receptor types: nodose ganglia have $P2X_2$ and $P2X_{2/3}$ heteromutimers whereas DRG have $P2X_3$ and $P2X_{2/3}$ (Dunn et al. 2001). P2Y receptors are co-expressed on subpopulations of these

cells (Ruan et al. 2003). We also know that the $P2X_3$ positive DRG neurons (being particularly activated by high gut pressures) are increased in number after inflammation and this is likely to contribute to increased noxious afferent activity to the central nervous system, as spinal pathways from the gut are vital in nociception (Ness and Gebhart 1988). With this basis, we can now begin to see therapeutic promise. With the development of $P2X_3$ and/or $P2X_{2/3}$ antagonists that are stable in-vivo, inflammatory pain could be attenuated. Furthermore, early pharmacological intervention during inflammation could prevent subsequent detrimental neuroplastic changes more proximally. There is good evidence now that a large proportion of functional bowel disease such as irritable bowel syndrome (IBS) has an inflammatory trigger (Barbara et al. 2002). The mechanisms for this are still obscure but one possibility is that receptor changes that occur to sensory enteric neurons during the inflammatory phase are not properly reversed after resolution (it is interesting that many of the symptoms of IBS are similar to acute enteritis). If this theory were true with relation to $P2X_3$ receptors then on going hypersensitivity to normal gut pressures would result. In fact, evidence from patients with IBS shows that this is the case (Ritchie 1973; Prior et al. 1990). To confirm or refute this theory we would need to do two things. Firstly, to study DRG neurons from a population of rats after a prolonged recovery period from colitis to see if a proportion of them still had up-regulation of $P2X_3$ receptors and secondly, to see if IBS patients responded to treatment with $P2X_3$ antagonists. In doing so, we would need to be careful that by blocking afferent neurotransmission through the $P2X_3$ receptors, no useful axon reflex activity was abolished that might contribute to the normal processes of inflammatory healing. There also might be more generalised considerations to blocking $P2X_3$ receptors due to its location on sensory nerves elsewhere in the body and its preponderance for forming heteromultimers with $P2X_2$ receptors. For example, although the $P2X_2$ subunit appears most dominant, there is good evidence that these

receptors play an important role in chemoreceptor function in the carotid body and thus has implications for the control of breathing (Rong et al. 2003).

Conclusions

Purinergic signalling is no longer a hypothetical concept in the field of mechanosensory transduction. We now have good evidence that endogenous ATP is released from colorectal enterocytes in response to stretch and that sensory enteric neurons can be activated as a result of this mechanism. There is strong supporting evidence that these processes are involved in the pathways that contribute to the perception of pain. Although some compounds are in the process of being evaluated (Jarvis et al. 2002), we must await further pharmacological progress in order to fully test compounds that will be active in-vivo, but this is surely only a matter of time. At that point, we may be on the frontier of a new generation of treatments for inflammatory pain and related functional conditions.

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ABBREVIATIONS

5-HT	5-hydoxytryptamine
8p-SPT	8-para-sulfophenyl-thoephylline
α,β-meATP	α,β-methylene ATP
ACh	acetylcholine
ATP	adenosine 5'-triphoshate
BK	bradykinin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CRD	colorectal distension
DAB	diaminobenzidine
DRG	dorsal root ganglia
EJP	excitatory junction potential
FITC	Fluorescene isothyocyanate
GABA	gamma aminobutyric acid
HBSS	Hanks balanced salt solution
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IFAN	intestinofugal afferent neuron
IJP	inhibitory junction potential
Il	interleukin
IPAN	intrinsic primary afferent neuron
mRNA	messenger ribonucleic acid

nA	nano amps
NANC	non-adrenergic non-cholinergic
NO	nitric oxide
PBS	phosphate buffered saline
PD-IBS	post-dysenteric irritable bowel syndrome
PGE_2	prostaglandin E_2
PNS	peripheral nervous system
PPADS	pyridoxyl 5-phosphate 6-azophenyl-2',4'-disulfonic acid
SP	substance P
TNBS	trinitrobenzenesulfonic acid
TNP-ATP	2',3'-O-trinitrophenyl-ATP
UC	ulcerative colitis
UTP	uridine 5'-triphosphate
VIP	vasointestinal peptide
VR1	vanilloid receptor type 1

ACKNOWLEDGEMENTS

The work presented in this thesis was only possible with the considerable assistance of many people. Professor Marc Winslet initially introduced me to the Autonomic Neuroscience Institute and ensured that a link to clinical surgery at the Royal Free Hospital was maintained and that the work carried out was of relevance to my career progression. I was extraordinarily lucky to catch Professor Geoff Burnstock, the world leader in the field of purinergic research, in one of his final years of supervising postgraduate students. The burning enthusiasm in his eyes would transform even the most miserable of experimental days into a success and without him the thesis would have been extremely difficult to complete. My thanks to Weifang Rong who patiently taught me how to perform electrophysiology from the pelvic nerve and helped with analysis from the Spike2 software. My appreciation goes to Gillian Knight and Phil Dunn for their expert knowledge and advice regarding the experimental work and also to Chrystalla Orphanides for her tireless editorial assistance. A special thankyou to Annie, Geoff Burnstock's late secretary, who was much loved in the department and will be sorely missed. She bound the whole department together.

All the work completed during the period between January 2001 and September 2003 which contributed to this thesis was performed by myself at the Autonomic Neuroscience Institute, Royal Free Hospital, London. The exception being the immunohistochemistry and the DRG electrophysiology in chapter 2 which was completed by Huai Zhen Ruan and Bei Ma respectively.

The work was partly funded by grants from the Hamamelis Trust, Special Trustees of the Royal Free Hospital and Royal College of Surgeons of England. The remainder was kindly provided by the Autonomic Neuroscience Institute or from my own funds.