Purinergic Mechanisms Contribute to Mechanosensory Transduction in the Rat Colorectum

GREGORY WYNN, WEIFANG RONG, ZHENGHUA XIANG, and GEOFFREY BURNSTOCK
Autonomic Neuroscience Institute, Royal Free and University College Medical School, London, United Kingdom

Background & Aims: **Adenosine 5′-triphosphate plays a role in peripheral sensory mechanisms and, in particular, mechanosensory transduction in the urinary system. $P2X_3$ receptors are selectively expressed on small-diameter sensory neurons in the dorsal root ganglia; sensory neurons from dorsal root ganglia L1 and S1 supply the colorectum. This study investigated whether purinergic signaling contributes to mechanosensory transduction in the rat colorectum.** ***Methods:*** **A novel in vitro rat colorectal preparation was used to elucidate whether adenosine 5′-triphosphate is released from the mucosa in response to distention and to evaluate whether it contributes to sensory nerve discharge during distention.** ***Results:*** **$P2X_3$ receptor immunostaining was present on subpopulations of neurons in L1 and S1 dorsal root ganglia, which supply the rat colorectum. Distention of the colorectum led to pressure-dependent increases in adenosine 5′-triphosphate release from colorectal epithelial cells and also evoked pelvic nerve excitation, which was mimicked by application of adenosine 5′-triphosphate and α,β-methylene adenosine 5′-triphosphate. The sensory nerve discharges evoked by distention were potentiated by α,β-methylene adenosine 5′-triphosphate and ARL-67156, an adenosine triphosphatase inhibitor, and were attenuated by the selective $P2X_1$, $P2X_3$, and $P2X_{2/3}$ antagonist 2′,3′-*O*-trinitrophenyl-adenosine 5′-triphosphate and by the nonselective P2 antagonists pyridoxyl 5-phosphate 6-azophenyl-2′,4′-disulfonic acid and suramin. Adenosine, after ectoenzymatic breakdown of adenosine 5′-triphosphate, seems to be involved in the longer-lasting distention-evoked sensory discharge. Single-fiber analysis showed that high-threshold fibers were particularly affected by α,β-methylene adenosine 5′-triphosphate, suggesting a correlation between purinergic activation and nociceptive stimuli.** ***Conclusions:*** **Adenosine 5′-triphosphate contributes to mechanosensory transduction in the rat colorectum, and this is probably associated with pain.**

There is now well-established evidence for the role of adenosine 5′-triphosphate (ATP) as an extracellular signaling molecule in sensory transduction and, in particular, nociception.[1] Attention has focused on $P2X_2$ and $P2X_3$ receptors, 2 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,[2,3] and sensory neurons in culture response to P2X agonists with a pharmacological profile suggestive of $P2X_3$ involvement.[4,5] $P2X_3$ immunoreactivity is seen in the peripheral projections of sensory neurons in a variety of tissues, including the tongue,[6] the tooth pulp,[7] the bladder,[8] and the gut,[9] and also on the presynaptic membrane in inner lamina II of the spinal dorsal horn.[10,11] The $P2X_2$ receptor, also present in sensory ganglia, is pH sensitive[12] and, along with $P2X_3$ subunits, can form heteromultimers that yield ATP-activated currents similar to those found in sensory neurons.[13]

In vitro studies have shown the ability of P2X agonists and antagonists to change afferent nerve activity in models of pain: knee joint,[14] skin,[15] and bladder.[16] Injection of ATP and related agonists into the rat hindpaw results in dose-dependent nocifensive behavior and localized thermal hyperalgesia.[17] In the absence of selective $P2X_3$ receptor agonists and antagonists, $P2X_3$ knockout mice have been invaluable. These animals have shown bladder hyporeflexia and reduced inflammatory pain–related behavior.[8]

A working hypothesis of purine-mediated mechanosensory transduction has been proposed.[18,19] ATP released during distention 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 centers in the brain. Supporting evidence for this comes from demonstration of peripheral purinoceptors in sensory nerves and from studies that show ATP release after distention of the bladder[16,20] and

Abbreviations used in this paper: α,β-meATP, α,β-methylene adenosine 5′-triphosphate; 5-HT, 5-hydoxytryptamine; PPADS, pyridoxyl 5-phosphate 6-azophenyl-2′,4′-disulfonic acid; 8p-SPT, 8-para-sulfophenyl-theophylline; TNP-ATP, 2′,3′-*O*-trinitrophenyl–adenosine 5′-triphosphate.

0016-5085/03/$30.00
doi:10.1053/S0016-5085(03)01353-2

ureter.[21] Furthermore, recent studies show that pelvic afferent activity during bladder distention can be potentiated with P2X agonists and attenuated with P2X antagonists.[16,22,23]

The responses of afferent neurons to distention of the stomach and small intestine[24–26] and the colon[27–36] have been described. A study exploring the effects of ATP on mesenteric afferents of the jejunum in the anesthetized rat has shown excitatory effects,[37] but no experiments have recorded afferent nerve activity during distention in relation to purinergic signaling in the colon. In this study, we tested the hypothesis of purinergic mechanosensory transduction in the rat colorectum.

Methods

Animals

Experiments were performed with adult male and female Sprague–Dawley rats (240–320 g) that were allowed free access to food and water. Animals were killed by exposure to increasing levels of carbon dioxide and cervical dislocation in accordance with UK Home Office regulations covering Schedule One procedures.

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 the colon was stretched 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 O.C.T. compound (Agar Scientific, Stansted, UK) and frozen in isopentane precooled in liquid nitrogen. Tissue was sectioned at 12 μm by using a Reichert Jung CM1800 cryostat (Leica Microsystems, Wetzlar, Germany). Preparations were first washed 3 × 5 minutes in 0.01 mol/L PBS (pH 7.2), then they were incubated in 1.0% H_2O_2 for 30 minutes to block the endogenous peroxidase. Preincubation in 10% normal horse serum and 0.2% Triton X-100 in PBS for 30 minutes followed, then incubation occurred overnight at 4°C with $P2X_2$ and $P2X_3$ antibodies diluted 1:500 in antibody dilution solution (10% normal horse serum, 0.2% Triton X-100, and 0.4% sodium azide in PBS). Subsequently, tissues were incubated with biotinylated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch, Luton, UK) diluted 1:500 in antibody dilution solution for 1 hour at 37°C and then with streptavidin–horseradish peroxidase (Sigma, Poole, UK) diluted 1:1000 in PBS for 1 hour at 37°C. Finally, a nickel-intensified diaminobenzidine reaction was used to visualize immunoreactivity. All the incubations and reactions were separated by three 10-minute washes in PBS.

For double staining among $P2X_2$, $P2X_3$, and calbindin D-28k, the preparations were washed 3 × 5 minutes in PBS and then preincubated in antibody dilution solution for 30 minutes. 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, Bellinzona, Switzerland) diluted 1:5000 in antibody dilution solution. Subsequently, the preparations were incubated with Cy3-conjugated donkey anti-rabbit immunoglobulin G (Jackson) diluted 1:300 for P2X antibodies and fluorescein isothiocyanate–conjugated donkey anti-mouse immunoglobulin G (Jackson) diluted 1:200 for calbindin in antibody dilution solution for 1 hour at room temperature. All the incubations and reactions were separated by three 10-minute 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, Wetzlar, Germany).

Control experiments were performed with $P2X_2$ and $P2X_3$ antibodies reabsorbed with $P2X_2$ and $P2X_3$ peptides. No staining was observed in the preparations that were incubated with the antibody solutions reabsorbed with $P2X_2$ and $P2X_3$ peptides.

Adenosine 5′-Triphosphate Assay and Electrophysiology

The distal colon and rectum were dissected from the pelvis with attached pelvic nerve and placed in a 10-mL bath superfused with oxygenated Krebs solution (contents [mmol/L]: NaCl 120, KCl 5.9, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 15.4, $CaCl_2$ 2.5, and glucose 11.5). Both the proximal and distal ends of the 30-mm length of bowel were secured to 8.5Fr. 3-way cannulae, and then was 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 stabilize in the bath for 60 minutes before data were gathered.

For the ATP release experiments, the colon was distended to pressures between 1 and 90 mm Hg at random. Fluid was drained through a short, small-diameter tube with a calculated dead space of 50 μL (this volume was discarded before collection).

Samples were immediately frozen and collected for luminometry with the luciferin–luciferase assay.[21,38] For electrophysiology experiments, the pelvic nerve was carefully divided into small branches under the microscope, and multifiber afferent activity was recorded with a suction glass electrode (tip diameter, 50–100 μm) connected to a Neurolog headstage (NL 100; Digitimer Ltd., Hertfordshire, UK) and an alternating current amplifier (NL 104). Signals were amplified (10,000×), filtered (NL 125; bandpass 200–4000 Hz), and captured by a computer via a power 1401 analogue-to-digital interface and Spike 2 software (version 4.03; Cambridge Electronic Design, Cambridge, UK). Those branches that did not

yield a good response to distention were not used. Control distentions to 50 mm Hg with Krebs solution were repeated at 10-minute intervals until nerve responses were stable. Purinergic agonists and antagonists were applied either intraluminally or to the serosa and circulated until 2 similar responses to distention were obtained (usually after approximately 20 minutes). The frequency of distention-induced firing was then compared with that of controls.

The results for all experiments are presented as mean ± SEM. Data were compared by the Student *t* test unless otherwise stated, and differences were considered statistically significant at $P < 0.05$.

Chemicals

ARL-67156, ATP (disodium salt), α,β-methylene ATP (α,β-meATP; lithium salt), 5-hydroxytryptamine (5-HT), 8-para-sulfophenyl-theophylline (8p-SPT), pyridoxyl 5-phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS), and suramin (hexasodium salt) were obtained from Sigma. 2′,3′-*O*-Trinitrophenyl-ATP (TNP-ATP) was obtained from Molecular Probes (Leiden, The Netherlands). All chemicals were diluted in Krebs solution to the required concentrations before use.

Results

Immunohistochemistry

Many of the neurons in the L1 and S1 DRG in the rat show immunoreactivity for $P2X_3$ (Figure 1*A* and *B*). $P2X_2$ immunoreactivity was also shown in a subpopulation of these DRG neurons, and colocalization 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 (Figure 2*A*) and the submucous plexus (Figures 1*C* and 2*B*) of the rat colorectum. Similarly, immunostaining for the $P2X_2$ subunit in the myenteric plexus (Figure 2*C*) and submucous plexus (Figure 2*D*) shows many heavily stained cell bodies and axons. Calbindin staining colocalizes with both $P2X_3$ and $P2X_2$ immunostaining in a subpopulation of neurons in the submucous plexus (Figure 1*E* and *F*), respectively, but colocalization was not seen in the neurones in the myenteric plexus. Relatively strong nuclear staining for calbindin can be seen (Figure 1*D*), but other studies have confirmed that this is often a typical feature of calbindin immunoreactivity.[39]

Adenosine 5′-Triphosphate Release

From 4 male and 4 female rats, 139 separate gut distentions ranging from 4 to 90 mm Hg were performed. Figure 3 shows the relationship between increasing 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 distention, 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 highly statistically significant ($P \leq 0.001$). The distention-induced increase in ATP became significant at pressures >11 mm Hg. Experiments were repeated after removal of the colorectal mucosa. Table 1 compares the percentage increase in ATP release during various distention pressures in the 2 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.

Distention Responses of Colonic Pelvic Nerve Afferents

Typical responses of multifiber recordings from the pelvic nerve in response to distention are shown in Figure 4. Phasic distentions 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 multifiber responses to distention. In 5 preparations with the mucosa stripped, the colorectum was subjected to 30-second phasic distentions at pressures of 10, 20, 30, 40, 50, and 60 mm Hg. The responses were compared with those of 5 normal controls. There were reductions in mean nerve activity of 48.0% ± 5.6% (10 mm Hg), 26.9% ± 2.6% (20 mm Hg), 28.6% ± 2.3% (30 mm Hg), 26.5% ± 2.2% (40 mm Hg), 33.0% ± 2.6% (50 mm Hg), and 28.0% ± 2.2% (60 mm Hg). The overall mean reduction in afferent activity was 31.8% ± 2.9% ($P = 0.007$; analysis of variance).

Effect of Adenosine 5′-Triphosphate 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 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. Figure 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

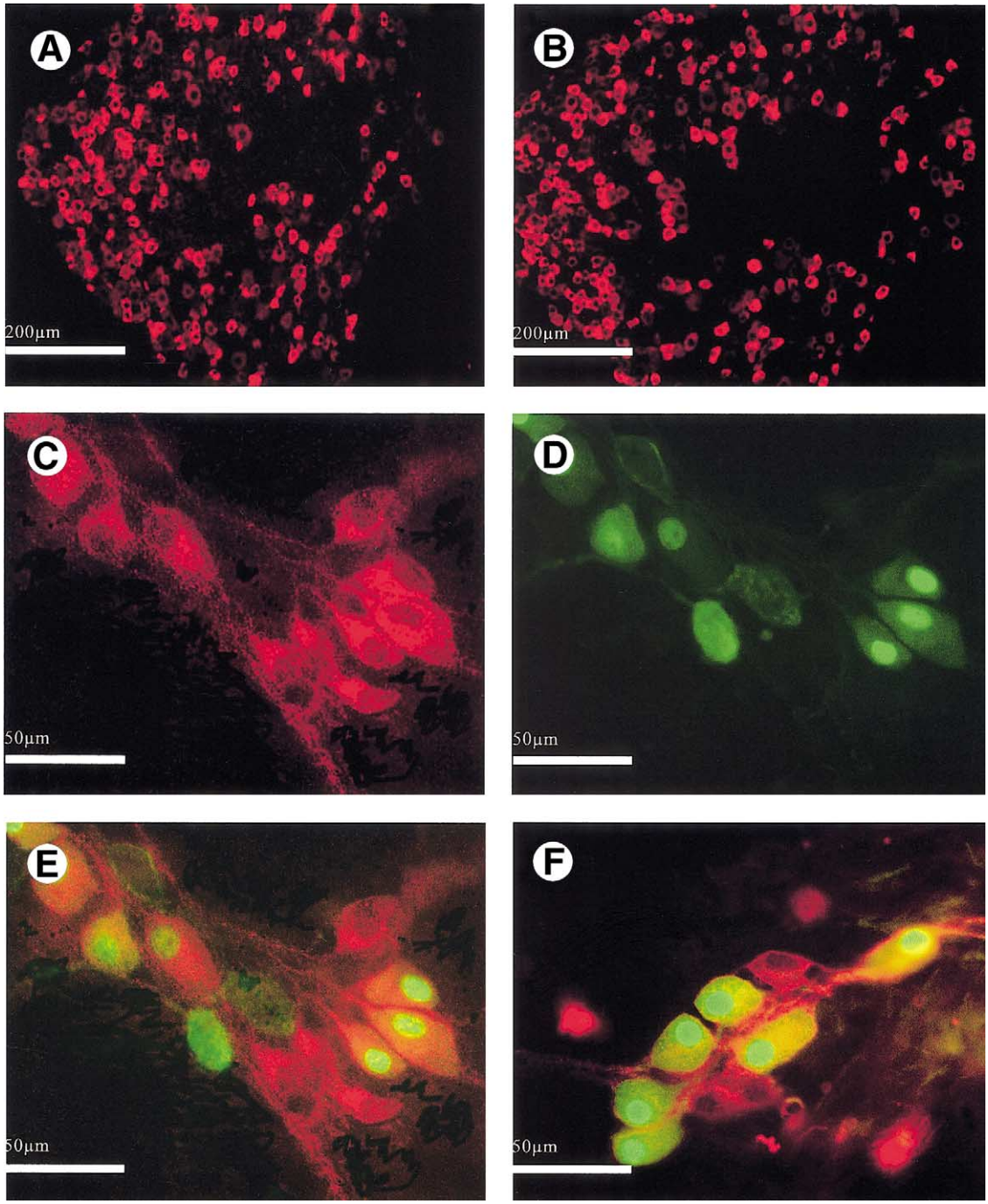

Figure 1. DRG L1 and S1 supply the distal colon and rectum, and these ganglia show immunoreactivity with $P2X_3$ in many of their cells (*A* and *B*, respectively). Neurons in the submucous plexus show immunolabeling of $P2X_3$ receptors (*C*) and calbindin (*D*), and colocalization occurs in a subpopulation of these cells (*E*). Neurons in the submucous plexus are immunopositive for $P2X_2$ receptors, and they also colocalize with calbindin in a subpopulation of cells (*F*).

below threshold for ATP (100 μmol/L) and also produced a greater response than ATP at the same 1 mmol/L concentration (Figure 6). The latency of evoked responses was similar for α,β-meATP and ATP. At a concentration of 100 μmol/L, 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 Distention

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 mmol/L; n = 4), 22.6% ± 4.2% (3 mmol/L; n = 8), and 25.2% ± 1.3% (5 mmol/L; n =

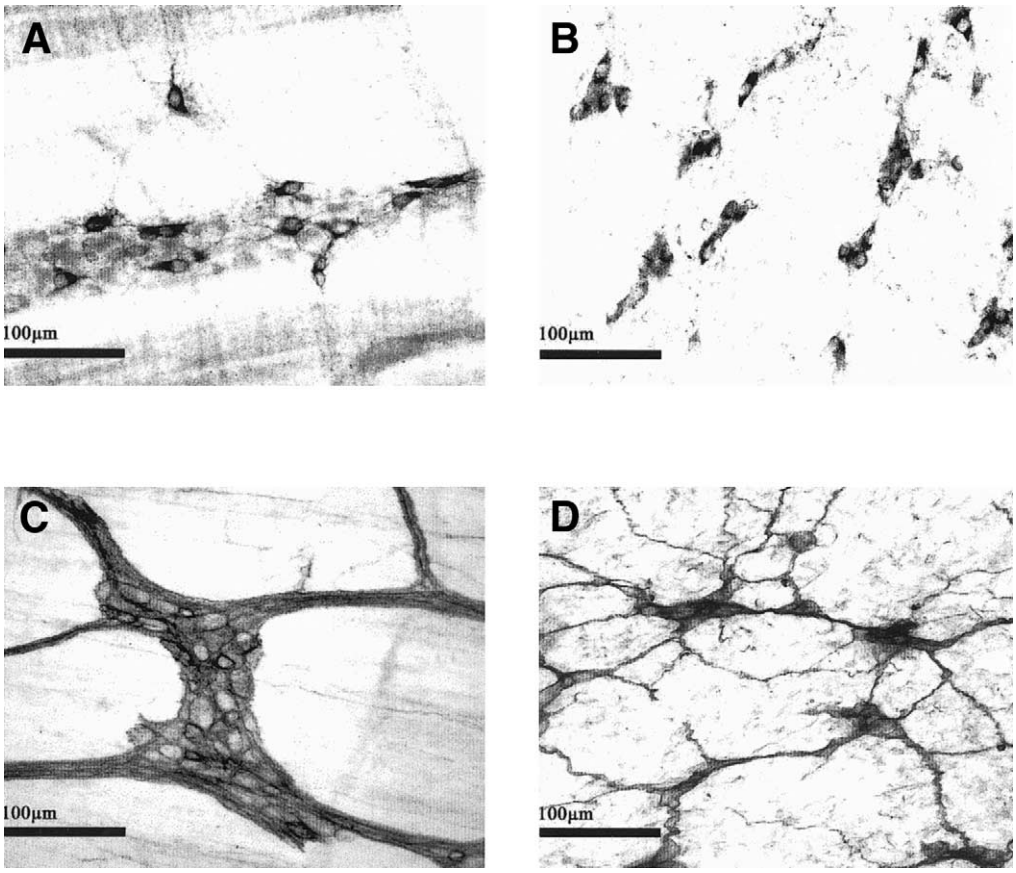

Figure 2. Immunoreactivity to $P2X_3$ is seen in a subpopulation of neurons in the rat colorectal myenteric plexus (*A*) and submucous plexus (*B*). More neurons stain for $P2X_2$ receptors in the myenteric plexus (*C*), and nerve fibers and cell bodies show positive immunoreactivity to $P2X_2$ in the submucous plexus (*D*).

9). The α,β-meATP increased the distention-induced response at lower concentrations: 21.4% ± 0.8% (100 μmol/L; n = 4) and 24.8% ± 3.1% (1 mmol/L; n = 5). Figure 7 shows an example of distention-induced afferent discharge before and during serosal application of α,β-meATP.

Experiments on 7 preparations showed that the nonselective P2 receptor antagonists, PPADS 100 μmol/L and suramin 300 μmol/L, reduced peak firing in response to distention by 24.7% ± 2.1% and 23.4% ± 2.4%, respectively. Figure 8 shows the effect of the selective $P2X_1$, $P2X_3$, and $P2X_{2/3}$ heteromultimer antagonist TNP-ATP (60 μmol/L). The mean reduction in

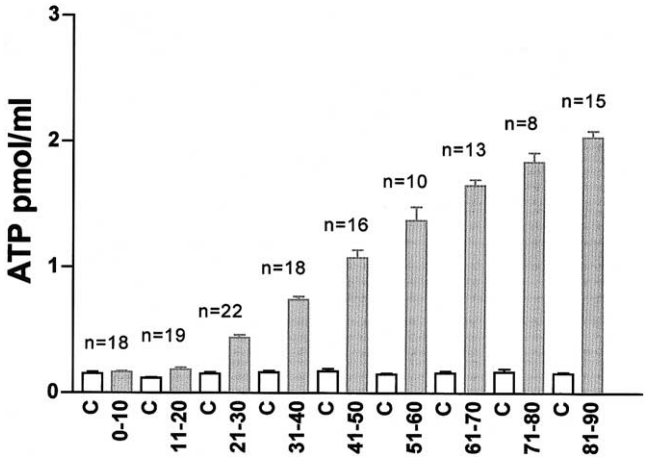

Figure 3. ATP concentration of luminal fluid samples from the rat colorectum during distention. Each column shows the mean ATP release (pmol/mL) ± SEM for each of the pressure groups listed (mm Hg). Control samples were collected before each distention (C). The numbers above the columns refer to the number of distentions in each pressure group.

Table 1. ATP Release From the Rat Colorectum: Comparison Between Intact Bowel and Bowel With Mucosa Removed

	% Increase in ATP release during distention	
Pressure (*mm Hg*)	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

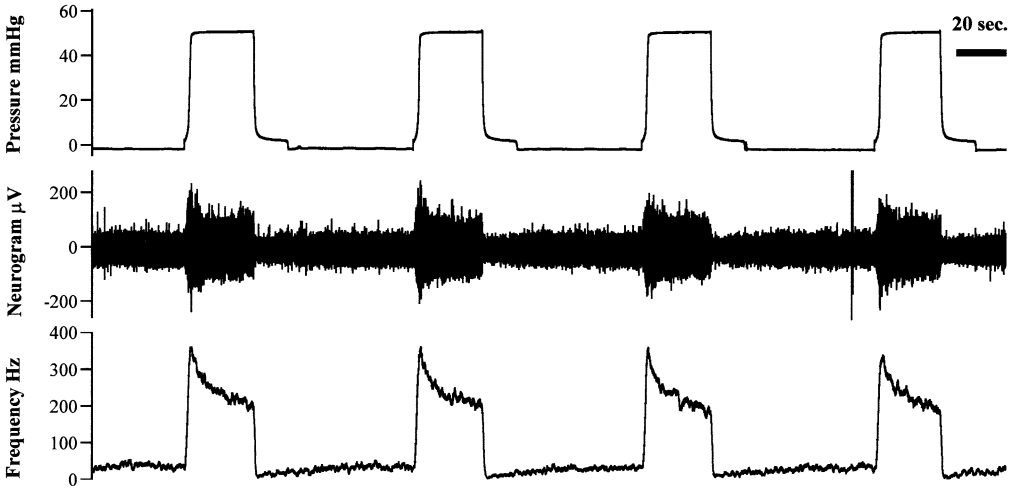

Figure 4. Repeated phasic distentions to 50 mm Hg in the rat colorectum. (*Top*) Intraluminal pressure (mm Hg); (*middle*) pelvic afferent nerve activity (μV); (*bottom*) frequency of spikes (Hz).

sensory nerve discharge in the presence of TNP-ATP was 26.2% ± 3.3% (n = 11). To exclude the possibility that the observed reduction in afferent activity was due to a nonspecific effect of the antagonists, separate experiments were performed. 5-HT 1 mmol/L was applied to the colorectal serosa before and after circulation of PPADS 100 μmol/L. 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 adenosine triphosphatase inhibitor ARL-67156. Mean activity was measured for every 10-second period during the distention, and these were compared with controls. Activity was augmented during the early phase by up to 17.2% ± 3.8% and was reduced during the late phase of distention by as much as 12.9% ± 5.2% during adenosine triphosphatase inhibition (Figure 9). Analysis of variance of the 2 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 distention responses, the nonselective adenosine antagonist 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 μmol/L was circulated for 20 minutes. No effect was seen on the nerve response to serosal α,β-meATP. For the distention response, sustaining intraluminal pressure at 50 mm Hg for 2 minutes allowed assessment of afferent activity over 10-second intervals. Figure 10 shows that although activity was reduced in all intervals (mean, 25.6% ± 0.78%), no period was especially affected.

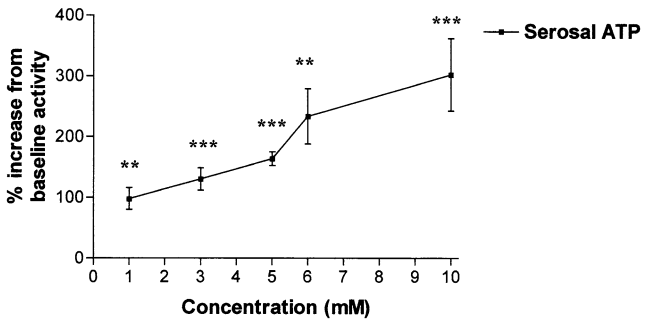

Figure 5. Percentage increase from baseline activity of the pelvic nerve after administration of different concentrations of ATP to the colorectal serosa.

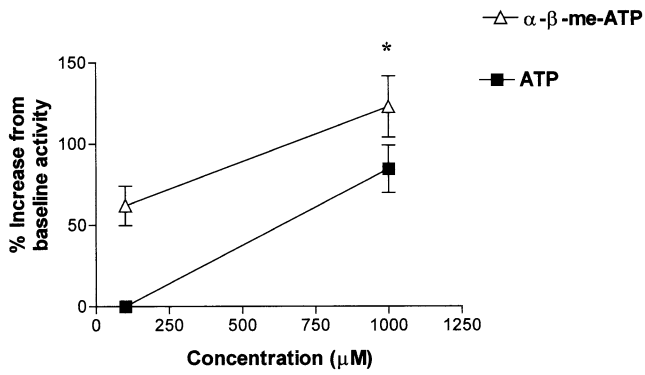

Figure 6. Comparison between the magnitude of the responses of pelvic nerve afferents to ATP and α,β-meATP when applied to the colorectal serosa. For each concentration, the percentage increase from baseline activity is shown and plotted as mean ± SEM (*P = 0.05 for the 1 mmol/L group).

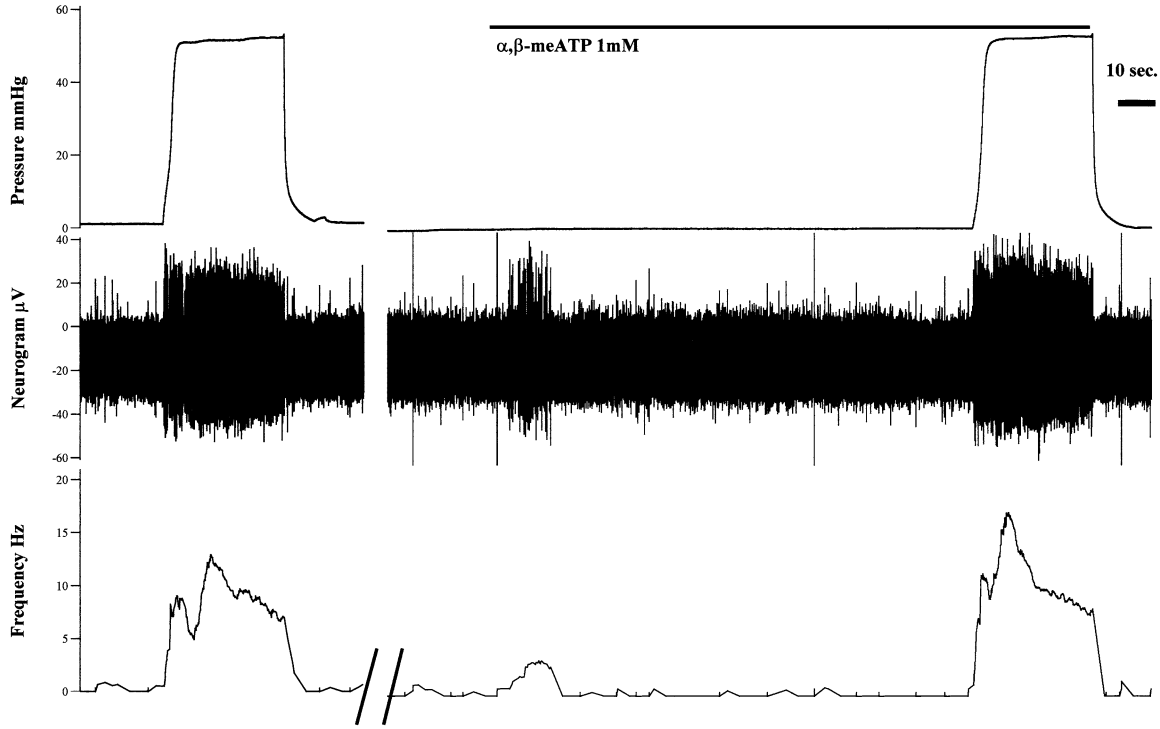

Figure 7. Example of administration of α,β-meATP 1 mmol/L to the rat colorectal serosa, producing a burst of activity and increasing the response to subsequent distention. Control distention is shown on the left.

Single-Unit Analysis

Figure 11 shows a single unit that was activated by both ATP and distention. The response was dose dependent. Computer analysis of 15 suitable multiunit recordings of distention responses showed 137 individual units. Of these, 106 (77%) were low-threshold fibers with a mean threshold of activation of 6.83 ± 0.29 mm Hg, and 31 units (23%) were high-threshold fibers with a mean threshold of activation of 23.46 ± 2.03 mm Hg. Of those units that responded to a distention pressure of 50 mm Hg, 78% responded to α,β-meATP. Three units were initially silent in response to distention but could be sensitized by α,β-meATP; 1 responded to distention at a low threshold and 2 at a high threshold after treatment with α,β-meATP. Most of both low- and high-threshold fibers could be activated by α,β-meATP (77% and 82%, respectively). Of those high-threshold fibers that were activated by α,β-meATP, 86% contributed to the increased responses to distention, whereas the same could be said of only 46% of low-threshold fibers.

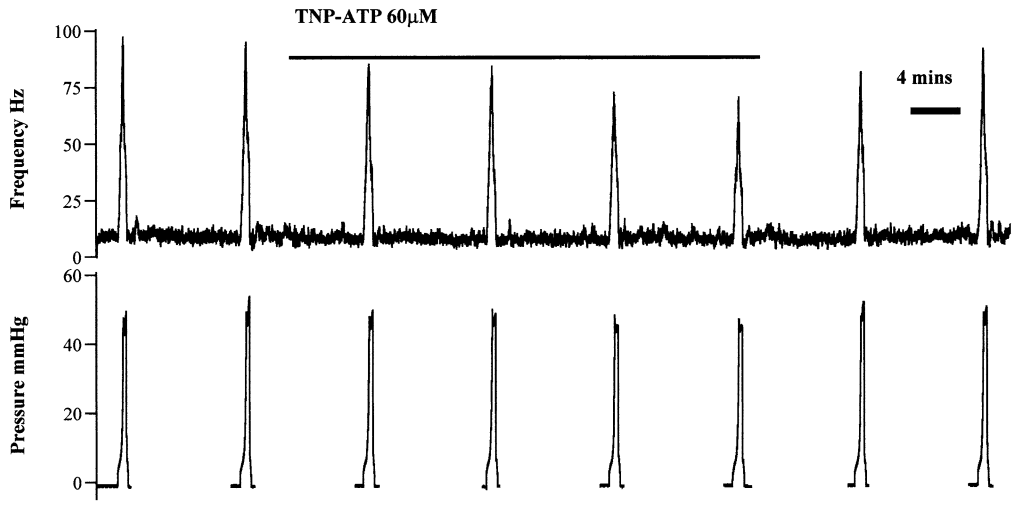

Figure 8. Multiunit recording from the pelvic nerve in response to distention, showing inhibition of peak afferent activity during administration of the $P2X_1$, $P2X_3$, and $P2X_{2/3}$ antagonist TNP-ATP 60 μmol/L. Recovery is seen with washout. (*Top*) Pressure (mm Hg); (*middle*) nerve activity (μV); (*bottom*) frequency of spikes (Hz).

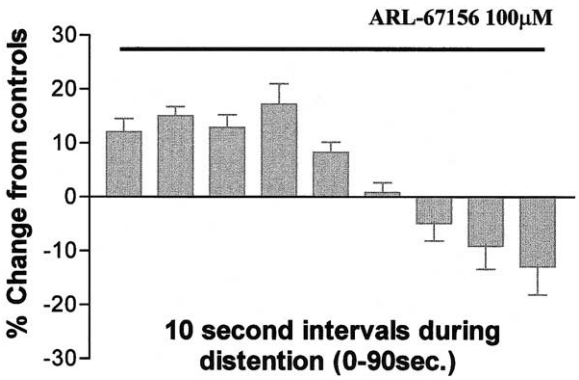

Figure 9. Pelvic nerve activity during distention was compared before and after application of the adenosine triphosphatase inhibitor ARL-67156. The graph shows the percentage change from controls over 10-second intervals.

In the presence of PPADS, all of the high-threshold fibers reduced their frequency of firing, whereas only 63% of low-threshold fibers were inhibited.

Figure 12 shows that of the low-threshold fibers that responded to α,β-meATP, the mean threshold of activation was similar before and after application of the agonist (7.60 ± 0.42 mm Hg vs. 6.62 ± 0.49 mm Hg). In contrast, the mean onset of activation of high-threshold fibers was significantly reduced by α,β-meATP from 28.07 ± 3.36 mm Hg to 15.14 ± 3.10 mm Hg ($P = 0.0013$). PPADS was able to significantly increase the threshold of activation in both low-threshold (6.56 ± 0.42 mm Hg to 10.42 ± 1.19 mm Hg; $P = 0.0006$) and high-threshold (19.51 ± 2.08 mm Hg to 28.98 ± 4.16 mm Hg; $P = 0.0092$) fibers.

Discussion

A hypothesis of purinergic mechanosensory transduction in visceral organs has been proposed.[18,19] This hypothesis states that endogenous ATP is released from epithelial cells in response to stretch and acts on $P2X_3$ or $P2X_{2/3}$ receptors to excite extrinsic afferent nerve fibers. This mechanism has already been shown to occur in the bladder[8,16,22,23] and ureter[21]; P2X antagonists reduced distention-induced sensory nerve discharge in both organs by approximately 40%, indicating that other signaling systems also contribute. This study presents data for the first time that suggest that a similar mechanism may operate in the colorectum. We have shown 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 that these same fibers are also responsive to noxious colorectal distention. In the presence of P2X antagonists, the pelvic nerve response to distention is reduced by approximately 25%. P2X agonists can also sensitize high-threshold mechanosensitive units. These data give firm evidence that endogenous ATP released during noxious colorectal distention can activate and sensitize P2X receptors in the wall of the rat colorectum, i.e., ATP acts as both a signaling 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 contributes to this mechanism only in part and that other signaling systems must be present. Ongoing work in this laboratory with a model of colitis indicates a greater role for ATP during inflammation.

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 2 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 this 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.

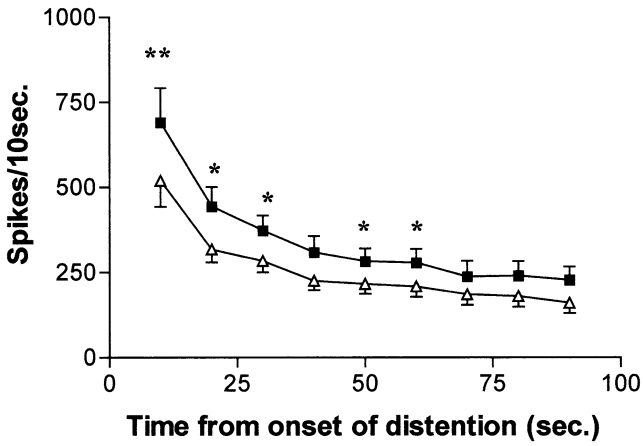

Figure 10. The effect of the general adenosine antagonist 8p-SPT 100 μmol/L on pelvic nerve activity in response to distention. ■, Control distention; △, Sp-SPT distention. Each data point represents the average activity for the preceding 10 seconds. Statistical significance was assessed by the paired Student *t* test; $*P < 0.05$; $**P < 0.01$.

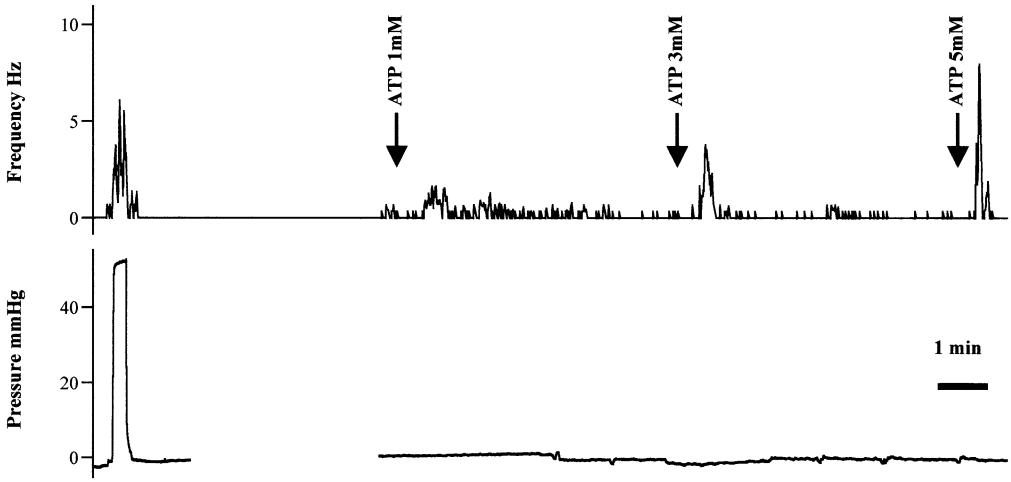

Figure 11. Single-unit analysis shows that fibers responding to distention are also activated by ATP in a dose-dependent way. (*Top*) Frequency of single-unit firing (Hz); (*bottom*) pressure (mm Hg).

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.[40] However, another study comparing staining in the guinea-pig ileum and distal colon observed that colocalization of $P2X_3$ and NeuN occurred in approximately 25% of submucous plexus neurons in the colon.[41] It was suggested that $P2X_3$ receptors were expressed in intrinsic primary afferent neurons in this part of the gastrointestinal tract. Both studies implicate $P2X_2$ receptors with intrinsic primary afferent neurons. $P2X_3$ staining has also been described on intrinsic nerves in human colon.[9] In this study, we have shown colocalization 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 show $P2X_3$ 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 L1 and S1 DRG neurons, which are known to supply the distal colon and rectum in the rat.[42] Further, experiments performed to investigate the effects of spinal nerve ligation have shown that $P2X_3$ receptor subunits accumulate just proximal to the site of ligation, indicating that these receptors are transported to the periphery.[11] 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.

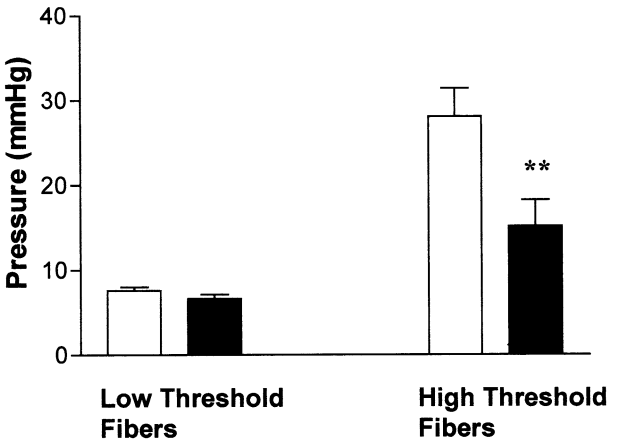

Figure 12. The effect of α,β-meATP on the threshold of activation of single units responding to colorectal distention: comparison between low- and high-threshold fibers. □, Control threshold; ■, threshold after α-β-me-ATP. Statistical significance was assessed by the paired Student *t* test; $^{**}P < 0.01$.

ATP is released from endothelial cells subjected to shear stress,[43] and there is good evidence that the mechanism of release is by vesicular exocytosis.[44] ATP is also released from urothelial cells during bladder distention,[16,20] and experiments have shown that release from the distended ureter is abolished after removal of the urothelial cells that line the lumen.[21] In the rat colorectum, as in the urinary system, there is also a pressure-dependent ATP release, and this is disturbed after mucosal ablation. ATP release was significantly increased at intraluminal pressures of >11 mm Hg. These data suggest that ATP is released in response to normal physiological distention and continues to be released proportionately into the noxious range, which is estimated to be approximately 30 mm Hg in the rat.[45] 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 showed a 32% reduction in pelvic nerve activity during distention 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.[46] 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 approximately 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 mostly conducted by tight junctions that allow selective absorption. The colon has a transepithelial electrical resistance much higher (10^6 Ω/cm^2) than that of the small intestine (10^2 Ω/cm^2), and hydrophilic molecules with a Stokes radius greater than approximately 11.5 Å are excluded.[47] 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 fibers that responded to distention, 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 in which α,β-meATP has been reported to be more potent than ATP.[37] When the colon was superfused with ATP, the multifiber afferent activity increased by 100%–300% above baseline. Of those fibers that were activated by distention pressures of 50 mm Hg, 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.[48] In this study, the general P1 (adenosine) receptor antagonist 8p-SPT reduced the sensory nerve discharge to ATP by 27.5%. Similarly, the distention-evoked afferent excitation was reduced by approximately a third, indicating that endogenous adenosine also contributes to this response. Adenosine is likely to appear as the result of rapid breakdown of ATP by ectonucleotidases. By preventing ATP breakdown, the weak adenosine triphosphatase inhibitor ARL-67156 enhanced the response to distention early on but reduced it in the later stages, supporting the idea that adenosine contributes to the longer-lasting distention-evoked sensory discharge.

The pelvic nerve is important in colonic nociception in rats.[49] Approximately 16% of the estimated 1600 pelvic nerve afferents in the rat are responsive to colorectal distention.[34] At low intraluminal pressures, reflexes involving both the enteric nervous system and extrinsic pathways to lower brain centers maintain physiological mechanisms. As pressure increases, low-threshold fibers increase their activity, and high-threshold fibers are recruited. Colorectal distention >30 mm Hg is noxious in the rat,[45] and pseudoaffective pressor, tachycardic, and visceromotor reflexes that precede this occur at 20–25 mm Hg.[49] It is interesting to note that in this study, the mean threshold of activation of high-threshold units was similar to this value (23.46 mm Hg).

Pelvic nerve afferents are activated by noxious colorectal distention, but in the presence of ATP or α,β-meATP, this activation can be potentiated. A smaller response to distention is achieved by blocking P2X receptors with PPADS or TNP-ATP, suggesting that a proportion of the afferent outflow involves purinergic signaling. Other mediators are likely to act alongside ATP in this process by directly opening ion channels at the nerve terminal (endogenous VR1 ligands, protons, and 5-HT), by sensitizing the terminal to other stimuli (prostaglandin E_2, bradykinin, substance P and histamine), or by altering receptor expression or their ligand-binding characteristics.[50–54] In this study, ATP and α,β-meATP were shown to alter the threshold of activation of low- and high-threshold fibers, and some fibers, which had no background activity and were unresponsive to distention, were activated by α,β-meATP and subsequently responded to distention, providing evidence that colorectal afferents can be sensitized 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 signaling molecules may occur in the transition between normal and pathologic conditions. In fact, there is good evidence to indicate an enhanced role for ATP in inflammation and states of hyperalgesia.[8,9,17,55–60] ATP would be a good candidate for signaling cellular damage in this context, because it is present intracellularly at millimolar concentrations. Work in this laboratory is currently being undertaken on purinergic signaling in a model of colitis 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 be unraveled, not only to further our quest for selective analgesics, but also because receptor mechanisms may well play a significant role in the peripheral component of functional bowel disorders.

References

1. Burnstock G. P2X receptors in sensory neurones. Br J Anaesth 2000;84:476–488.
2. Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN. A P2X purinoceptor expressed by a subset of sensory neurons. Nature 1995;377:428–431.
3. Bradbury EJ, Burnstock G, McMahon SB. The expression of $P2X_3$ purinoceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. Mol Cell Neurosci 1998;12:256–268.
4. Cook SP, Vulchanova L, Hargreaves KM, Elde R, McCleskey EW. Distinct ATP receptors on pain-sensing and stretch-sensing neurons. Nature 1997;387:505–508.
5. Dunn PM, Zhong Y, Burnstock G. P2X receptors in peripheral neurones. Prog Neurobiol 2001;65:107–134.
6. Bo X, Alavi A, Xiang Z, Oglesby I, Ford A, Burnstock G. Localization of ATP-gated $P2X_2$ and $P2X_3$ receptor immunoreactive nerves in rat taste buds. Neuroreport 1999;10:1107–1111.
7. Alavi AM, Dubyak GR, Burnstock G. Immunohistochemical evidence for ATP receptors in human dental pulp. J Dental Res 2001;80:476–483.
8. Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford APDW. Urinary bladder hyporeflexia and reduced pain-related behaviour in $P2X_3$-deficient mice. Nature 2000;407:1011–1015.
9. Yiangou Y, Facer P, Baecker PA, Ford AP, Knowles CH, Chan CL, Williams NS, Anand P. ATP-gated ion channel $P2X_3$ is increased in human inflammatory bowel disease. Neurogastroenterol Motil 2001;13:365–369.
10. Llewellyn-Smith IJ, Song ZM, Costa M, Bredt DS, Snyder SH. Ultrastructural localization of nitric oxide synthase immunoreactivity in guinea-pig enteric neurons. Brain Res 1992;577:337–342.
11. Vulchanova L, Riedl MS, Shuster SJ, Stone LS, Hargreaves KM, Buell G, Surprenant A, North RA, Elde R. $P2X_3$ is expressed by DRG neurons that terminate in inner lamina II. Eur J Neurosci 1998;10:3470–3478.
12. King BF, Ziganshina LE, Pintor J, Burnstock G. Full sensitivity of $P2X_2$ purinoceptor to ATP revealed by changing extracellular pH. Br J Pharmacol 1996;117:1371–1373.
13. Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A. Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. Nature 1995;377:432–435.
14. Dowd E, McQueen DS, Chessell IP, Humphrey PPA. P2X receptor-mediated excitation of nociceptive afferents in the normal and arthritic rat knee joint. Br J Pharmacol 1998;125:341–346.
15. Hamilton SG, McMahon SB, Lewin GR. Selective activation of nociceptors by ATP and the P2X-agonist $\alpha\mu\beta$-methylene ATP in the adult rat. (abstr) Soc Neurosci Abstr 2003;25:683–685.
16. Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne DA, Ford APDW, Burnstock G. $P2X_3$ knockout mice reveal a major sensory role for urothelially released ATP. J Neurosci 2001;21:5670–5677.
17. Hamilton SG, Wade A, McMahon SB. The effects of inflammation and inflammatory mediators on nociceptive behaviour induced by ATP analogues in the rat. Br J Pharmacol 1999;126:326–332.
18. Burnstock G. Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. J Anat 1999;194:335–342.
19. Burnstock G. Purine-mediated signalling in pain and visceral perception. Trends Pharmacol Sci 2001;22:182–188.
20. Ferguson DR, Kennedy I, Burton TJ. ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes—a possible sensory mechanism? J Physiol 1997;505:503–511 (Pt 2).
21. Knight GE, Bodin P, de Groat WC, Burnstock G. ATP is released from guinea pig ureter epithelium on distension. Am J Physiol 2002;282:F281–F288.
22. Namasivayam S, Eardley I, Morrison JF. Purinergic sensory neurotransmission in the urinary bladder: an in vitro study in the rat. BJU Int 1999;84:854–860.
23. Rong W, Spyer M, Burnstock G. Activation and sensitisation of low and high threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder. J Physiol 2002;541:591–600.
24. Pan HL, Longhurst JC. Ischaemia-sensitive sympathetic afferents innervating the gastrointestinal tract function as nociceptors in cats. J Physiol 1996;492:841–850 (Pt 3).
25. Kunze WA, Furness JB, Bertrand PP, Bornstein JC. Intracellular recording from myenteric neurons of the guinea-pig ileum that respond to stretch. J Physiol 1998;506:827–842 (Pt 3).
26. Thornton PD, Bornstein JC. Slow excitatory synaptic potentials evoked by distension in myenteric descending interneurones of guinea-pig ileum. J Physiol 2002;539:589–602.
27. Blumberg H, Haupt P, Jänig W, Kohler W. Encoding of visceral noxious stimuli in the discharge patterns of visceral afferent fibres from the colon. Pflugers Arch 1983;398:33–40.
28. Haupt P, Janig W, Kohler W. Response pattern of visceral afferent fibres, supplying the colon, upon chemical and mechanical stimuli. Pflugers Arch 1983;398:41–47.
29. Kreulen DL, Peters S. Non-cholinergic transmission in a sympathetic ganglion of the guinea-pig elicited by colon distension. J Physiol 1986;374:315–334.
30. Bahns E, Halsband U, Jänig W. Responses of sacral visceral afferents from the lower urinary tract, colon and anus to mechanical stimulation. Pflugers Arch 1987;410:296–303.
31. Ness TJ, Gebhart GF. Characterization of neuronal responses to noxious visceral and somatic stimuli in the medial lumbosacral spinal cord of the rat. J Neurophysiol 1987;57:1867–1892.
32. Ness TJ, Gebhart GF. Characterization of neurons responsive to noxious colorectal distension in the T13-L2 spinal cord of the rat. J Neurophysiol 1988;60:1419–1438.
33. Janig W, Koltzenburg M. Receptive properties of sacral primary afferent neurons supplying the colon. J Neurophysiol 1991;65:1067–1077.
34. Sengupta JN, Gebhart GF. Characterisation of mechanosensitive pelvic nerve afferent fibers innervating the colon of the rat. J Neurophysiol 1994;71:2046–2060.
35. Lynn PA, Blackshaw LA. In vitro recordings of afferent fibres with receptive fields in the serosa, muscle and mucosa of rat colon. J Physiol 1999;518:271–282 (Pt 1).
36. Kalmari J, Niissalo S, Konttinen YT, Pertovaara A. Modulation of visceral nociceptive responses of rat spinal dorsal horn neurons by sympathectomy. Neuroreport 2001;12:797–801.
37. Kirkup AJ, Booth CE, Chessell IP, Humphrey PP, Grundy D. Excitatory effect of P2X receptor activation on mesenteric afferent nerves in the anaesthetised rat. J Physiol 1999;520:551–563.
38. Taylor AL, Kudlow BA, Marrs KL, Gruenert DC, Guggino WB, Schwiebert EM. Bioluminescence detection of ATP release mechanisms in epithelia. Am J Physiol 1998;275:C1391–C1406.
39. German DC, Ng MC, Liang CL, McMahon A, Iacopino AM. Cal-

bindin-D28k in nerve cell nuclei. Neuroscience 1997;81:735–743.
40. Nassauw LV, Brouns I, Adriaensen D, Burnstock G, Timmermans JP. Neurochemical identification of enteric neurons expressing $P2X_3$ receptors in the guinea-pig ileum. Histochem Cell Biol 2002;118:193–203.
41. Poole DP, Castelucci P, Robbins HL, Chiocchetti R, Furness JB. The distribution of P2X3 purine receptor subunits in the guinea pig enteric nervous system. Auton Neurosci 2002;101:39–47.
42. Hicks GA, Coldwell JR, Schindler M, Ward PA, Jenkins D, Lynn PA, Humphrey PP, Blackshaw LA. Excitation of rat colonic afferent fibres by 5-HT(3) receptors. J Physiol 2002;544:861–869.
43. Bodin P, Bailey DJ, Burnstock G. Increased flow-induced ATP release from isolated vascular endothelial but not smooth muscle cells. Br J Pharmacol 1991;103:1203–1205.
44. Bodin P, Burnstock G. Evidence that release of ATP from endothelial cells during increased shear stress is vesicular. J Cardiovasc Pharmacol 2001;38:900–908.
45. Ness TJ, Randich A, Gebhart GF. Further behavioral evidence that colorectal distension is a 'noxious' visceral stimulus in rats. Neurosci Lett 1991;131:113–116.
46. Bertrand PP, Bornstein JC. ATP as a putative sensory mediator: activation of intrinsic sensory neurons of the myenteric plexus via P2X receptors. J Neurosci 2002;22:4767–4775.
47. Madara JL. Loosening tight junctions: Lessons from the intestine. J Clin Invest 1989;83:1089–1094.
48. Kirkup AJ, Eastwood C, Grundy D, Chessell IP, Humphrey PP. Characterization of adenosine receptors evoking excitation of mesenteric afferents in the rat. Br J Pharmacol 1998;125:1352–1360.
49. Ness TJ, Gebhart GF. Colorectal distension as a noxious visceral stimulus: physiologic and pharmacologic characterization of pseudaffective reflexes in the rat. Brain Res 1988;450:153–169.
50. Bueno L, Fioramonti J, Delvaux M, Frexinos J. Mediators and pharmacology of visceral sensitivity: from basic to clinical investigations. Gastroenterology 1997;112:1714–1743.
51. Bueno L, Fioramonti J, Garcia-Villar R. Pathobiology of visceral pain: molecular mechanisms and therapeutic implications. III. Visceral afferent pathways: a source of new therapeutic targets for abdominal pain. Am J Physiol Gastrointest Liver Physiol 2000; 278:G670–G676.
52. Gebhart GF. Pathobiology of visceral pain: molecular mechanisms and therapeutic implications IV. Visceral afferent contributions to the pathobiology of visceral pain. Am J Physiol Gastrointest Liver Physiol 2000;278:G834–G838.
53. Kirkup AJ, Brunsden AM, Grundy D. Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. Am J Physiol Gastrointest Liver Physiol 2001; 280:G787–G794.
54. Holzer P. Gastrointestinal afferents as targets of novel drugs for the treatment of functional bowel disorders and visceral pain. Eur J Pharmacol 2001;429:177–193.
55. Hamilton SG, Warburton J, Bhattachajee A, Ward J, McMahon SB. ATP in human skin elicits a dose-related pain response which is potentiated under conditions of hyperalgesia. Brain 2000;123: 1238–1246.
56. Souslova V, Cesare P, Ding Y, Akopian AN, Stanfa L, Suzuki R, Carpenter K, Dickenson A, Boyce S, Hill R, Nebenius-Oosthuizen D, Smith AJH, Kidd E, Wood JN. Warm-coding deficits and aberrant inflammatory pain in mice lacking $P2X_3$ receptors. Nature 2000;407:1015–1017.
57. Tsuda M, Koizumi S, Kita A, Shigemoto Y, Ueno S, Inoue K. Mechanical allodynia caused by intraplantar injection of P2X receptor agonist in rats: involvement of heteromeric $P2X_{2/3}$ receptor signaling in capsaicin-insensitive primary afferent neurons. J Neurosci 2000;20:1–5 RC90.
58. Jarvis MF, Wismer CT, Schweitzer E, Yu H, van Biesen T, Lynch KJ, Burgard EC, Kowaluk EA. Modulation of BzATP and formalin induced nociception: attenuation by the P2X receptor antagonist, TNP-ATP and enhancement by the $P2X_3$ allosteric modulator, cibacron blue. Br J Pharmacol 2001;132:259–269.
59. Paukert M, Osteroth R, Geisler HS, Brandle U, Glowatzki E, Ruppersberg JP, Grunder S. Inflammatory mediators potentiate ATP-gated channels through the $P2X_3$ subunit. J Biol Chem 2001; 276:21077–21082.
60. Hamilton SG, McMahon SB, Lewin GR. Selective activation of nociceptors by P2X receptor agonists in normal and inflamed rat skin. J Physiol 2001;534:437–445.

Received February 27, 2003. Accepted July 24, 2003.

Address requests for reprints to: Geoffrey Burnstock, Ph.D., Autonomic Neuroscience Institute, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, United Kingdom. e-mail: g.burnstock@ucl.ac.uk; fax: (020) 7830-2949.

Supported by the Hamamelis Trust, Special Trustees of the Royal Free Hospital, and Royal College of Surgeons of England.