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# Purinergic component of mechanosensory transduction is increased in a rat model of colitis

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**Wynn, Gregory, Bei Ma, Huai Zhen Ruan, and Geoffrey Burnstock.** Purinergic component of mechanosensory transduction is increased in a rat model of colitis. *Am J Physiol Gastrointest Liver Physiol* 287: G647–G657, 2004; 10.1152/ajpgi.00020.2004.—ATP contributes to mechanosensory transduction in the rat colorectum. $P2X_3$ receptors are present on dorsal root ganglia (DRG) neurons that supply this area of the gut. Previous studies have shown an increased role for ATP in inflamed tissues. We aimed to investigate whether an increased purinergic component exists during mechanosensory transduction in a rat model of colitis. An in vitro rat colorectal preparation was used to investigate whether distension increased ATP release and to evaluate the role of purinergic antagonists in distension-evoked sensory discharges in the pelvic nerve in normal and colitis preparations. DRG neuron purinoceptors were also studied. Distension-evoked responses in the colitis model were attenuated to a significantly greater extent by 2′,3′-*O*-trinitrophenyl-ATP and pyridoxyl 5-phosphate 6-azophenyl-2′,4′-disulfonic acid. Inflammation caused augmented distension-evoked sensory nerve excitation after application of ATP and α,β-methylene ATP. Single-fiber analysis confirmed that mean firing per unit was increased. Distension-evoked increases in ATP release from epithelial cells were substantially higher. The number of DRG neurons responding to ATP and the number of those staining for the $P2X_3$ receptor, particularly those containing calcitonin gene-related peptide, were increased. Adenosine, after ectoenzymatic breakdown of ATP, is involved to a lesser degree in the longer-lasting distension-evoked sensory discharge, suggesting reduced ATPase activity. It was therefore concluded that ATP has an enhanced role in mechanosensory transduction in the inflamed rat colorectum. The underlying mechanisms appear to involve increased distension-evoked release of ATP as well as an increase in the number of DRG neurons supplying the colorectum expressing $P2X_3$ receptors, especially those containing calcitonin gene-related peptide.

ATP; dorsal root ganglia; inflammation

GENETIC, ENVIRONMENTAL, MICROBIAL, and immunologic advances have increased our understanding of the complex pathophysiological processes involved in inflammatory bowel disease (IBD) (for review see Ref. 2). To the 4,000,000 sufferers worldwide, these advances have brought about important clinical improvements. The etiology, however, is unknown.

One area of interest is the relation between the enteric nervous system and the immune system. Pelvic denervation or vagotomy has been used to treat refractory IBD. Inflammation in one area of the gut may profoundly affect the function of distant areas, and one episode of inflammation may give rise to future structural and functional abnormalities of enteric nerves (16, 27). Indeed, there is good evidence that inflammation plays a role in the pathogenesis of irritable bowel syndrome (3).

Tissue concentrations of various gastrointestinal neurotransmitters are altered after inflammation (28), and the intriguing relation between patients with ulcerative colitis and nonsmokers or ex-smokers has been followed up with studies suggesting that nicotine, itself a parasympathetic agonist in the gut, can be used to induce clinical improvement (15). Clonidine, an $\alpha_2$-agonist, has shown therapeutic promise (1), and sympathectomy in rats reduces the severity of experimental colitis (37). Local anesthetic agents applied topically in ulcerative colitis patients have induced remission (4).

Sensory enteric nerves are important in transduction and transmission of painful stimuli and also in local and central reflexes that modulate gut function (21, 58). Neuropeptides such as substance P (SP), VIP, and CGRP are released from stimulated primary afferents via axon reflexes to influence local cellular function. In particular, these neuropeptides are released in response to noxious stimuli such as vanilloid receptor type 1 (VR1) activation, acidosis, or distension (47). SP immunoreactivity is increased in afferent neuronal pathways during intestinal inflammation in the rat, and VR1-null mice lose their ability to develop inflammatory thermal hyperalgesia (17). In accordance with this, loss of extrinsic sensory nerves in rats by neonatal capsaicin treatment worsens experimental inflammation in the gut (37), as does the application of CGRP antagonists (44). 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 Ref. 49), indicating a complex reciprocal relationship between sensory neurons and the inflammatory tissue in which they lie.

A wide variety of signaling 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 sensitize primary afferent terminals to produce pain (8, 18). One of the molecules present during tissue injury, ATP, is a good candidate for signaling cellular damage, in that it is present intracellularly in millimolar concentrations. There is good evidence that ATP plays a role in nociception (for review see Ref. 10) and, in

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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 the $P2X_3$ receptor is selectively expressed on small-diameter sensory neurons in the dorsal root ganglia (DRG), which are known to supply, among other areas, the pelvic viscera (7, 13). The $P2X_2$ receptor, also present in these DRG, is pH sensitive (31) and, along with $P2X_3$ subunits, can form heteromultimers that yield ATP-activated currents similar to those found in sensory neurons (34). Metabotropic $P2Y_1$ and $P2Y_4$ receptors are also present on a subpopulation of DRG neurons that also express $P2X_3$ receptors (48). Behavioral studies in rats (23, 29) and humans (24) 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 (22), and $P2X_3$-null mice show reduced formalin-induced pain behavior (14, 50). SP and bradykinin (BK) potentiate currents mediated by $P2X_3$ and $P2X_{2/3}$ receptors expressed by *Xenopus* oocytes (43), and $P2X_3$ receptors are upregulated in colitis specimens obtained from patients with IBD (60) and in DRG neurons in models of chronic nerve injury (39).

A working hypothesis of purine-mediated mechanosensory transduction has been proposed (9, 11). 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 centers in the brain. Recent work in our laboratory has suggested that this mechanism contributes to afferent signaling during bladder distension in the mouse (46, 56) and colorectal distension in the rat (58). 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. The results were compared with 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 (26) before and after the induction of colitis for possible changes in $P2X_3$ and $P2X_{2/3}$ receptor expression and their electrophysiological responses to exogenous ATP.

## MATERIALS AND 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 killed by exposure to rising levels of carbon dioxide and cervical dislocation in accordance with UK Home Office regulations covering Schedule One procedures.

*Induction of colitis.* Experimental colitis was induced by administration of an intrarectal enema (8 cm from the anus) of 30% trinitrobenzenesulfonic acid (TNBS) in ethanol at a dose of 80 mg/kg body wt (36). The enemas were given through 6-Fr medical-grade polyurethane enteral feeding tubes while the rats were under light halothane anesthesia. Animals in the control group were given an equivalent enema of normal saline. This model of chronic inflammation was favored, because this most closely mimics 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, with the most severe period of inflammation starting at *day 5* (36). Animals were therefore killed 5–7 days later for the in vitro work and 10 days later for examination of the DRG. Assessment of colitis was based on body weight as well as macroscopic and microscopic features of the colorectum.

*Immunocytochemistry.* After death, the animals were perfused through the aorta with 60 ml of fixative (4% formaldehyde with 0.2% picric acid). The DRG were carefully dissected and placed in PBS. The tissue was embedded in OCT compound (BDH/Merck, Leicester, UK) and frozen in isopentane that had been precooled in liquid nitrogen in preparation for sectioning at 12 μm with a cryostat (model CM1800, Reichert Jung). Endogenous peroxidase was blocked by 30 min of incubation in 20% acetic acid containing 0.5% hydrogen peroxide. Nonspecific protein binding sites were blocked by 2 h of incubation in 10% normal horse serum containing 0.05% thimerosal (Merthiolate, Sigma, Poole, UK). DRG sections were incubated overnight at room temperature with $P2X_2$ or $P2X_3$ antibody (diluted to 2.5 μg/ml with 10% normal horse serum). The antibodies were raised in New Zealand White rabbits against a synthetic peptide corresponding to the COOH terminus of the cloned rat $P2X_2$ or $P2X_3$ receptor. After this incubation, all washes were performed using 0.05% Tween 20 (Sigma). The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch, Luton, UK) diluted at 1:500 for 2 h followed by incubation with ExtrAvidin peroxidase (Sigma) diluted 1:1,000 for 1 h. The tyramide signal amplification kit (NEN Life Science Products, Boston, MA) was applied for 8 min, followed by streptavidin fluorescein (Amersham Pharmacia Biotech, Bucks, UK) diluted 1:100 for 10 min. Omission of the primary antibody and preincubation with specific peptide were used as controls. The sections were mounted on gelatin-coated slides and observed under a Zeiss Axioplan microscope (Jena, Germany) at an excitation of 520 nm. Images were captured by a digital camera (Leica).

*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 of $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution (HBSS; Life Technologies) with 10 mM HEPES buffer, pH 7.4, containing 1.5 mg/ml collagenase (class II, Worthington Biochemical) and 6 mg/ml bovine serum albumin (Sigma) at 37°C for 45 min. The ganglia were then incubated in 4 ml of HBSS containing 1 mg/ml trypsin (Sigma) at 37°C for 15 min. The solution was replaced with 1 ml of growth medium consisting 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 IU/ml penicillin, and 200 μg/ml streptomycin. The ganglia were dissociated into single neurons by gentle trituration and then centrifuged at 160 *g* for 5 min. The resulting pellet was resuspended in 0.8 ml of 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 of 5% $CO_2$ and used within 30 h. Whole cell voltage-clamp recordings were carried out at room temperature with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with membrane potential held at −60 mV. Data were acquired using pClamp software (version 6.1. Axon Instruments). Signals were filtered at 2 kHz (−3-dB frequency, Bessel filter, 80 dB/decade), digitized at 10–50 kHz (Digidata 1320A interface, Axon Instruments), and stored on the hard disk of a personal computer for viewing and analysis. Traces were acquired using Clampfit (pCLAMP software) and plotted using Origin7 (Microcal, Northampton, MA). External solution contained (in mM) 154 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, and 5.6 glucose, with pH adjusted to 7.4 with NaOH. Recording electrodes (resistance 2–4 MΩ) were filled with internal solution, which contained (in mM) 120 KCl, 10 HEPES, and 10 tripotassium citrate, with pH adjusted to 7.2 with KOH. Solutions of ATP were prepared using deionized water, stored frozen, and then diluted in extracellular bathing solution to the final concentration. They were applied rapidly through a manifold comprising three capillaries made of fused silica coated with polyimide with 250-μm ID (SGE, Milton Kegnes), connected to a single outlet made of the same tubing, which was placed ~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 s at 2-min intervals, which was sufficient for responses to be reproducible.

*ATP assay.* This in vitro protocol was based on previous studies of ATP release involving distension of the guinea pig ureter reported by Knight et al. (33). The distal colon and rectum were dissected from the pelvis with attached pelvic nerve and placed in a bath superfused with oxygenated Krebs solution (in mM: 120 NaCl, 5.9 KCl, 1.2 $NaH_2PO_4$, 1.2 $MgSO_4$, 15.4 $NaHCO_3$, 2.5 $CaCl_2$, and 11.5 glucose). Proximal and distal ends of the 30-mm length of bowel were secured to 8.5-Fr three-way cannulas, and the lumen was perfused with Krebs solution. Ports on the cannulas were connected to a pressure transducer, large and small drainage tubing, and infusion tubing, which were connected in turn to a syringe driver (model sp210iw, World Precision Instruments, Sarasota, FL). In all cases, the tissues were allowed to stabilize in the bath for 60 min before data were gathered.

The normal or inflamed colon was distended to pressures of 1–90 mmHg at random by opening the infusion tubing to a reservoir of Krebs solution that was positioned at various heights to achieve a range of intraluminal pressures almost instantaneously. The pressure was held for 5 s before the infusion tubing was clamped and drainage was allowed. Fluid was drained through a short, small-diameter tube with a calculated dead space of 50 μl (this volume being discarded before collection). Samples were immediately frozen in liquid nitrogen and collected for luminometry using the luciferin-luciferase assay (33).

*Pelvic nerve electrophysiology.* The experimental apparatus was set up in a manner similar to that described for the ATP release studies. In addition, as described previously (58), the attached pelvic nerve was carefully divided into small branches under the microscope, and multifiber afferent activity was recorded using a suction glass electrode (50- to 100-μm tip diameter) connected to a Neurolog head stage (model NL 100, Digitimer) and an alternating-current amplifier (model NL 104). Signals were amplified (×10,000), filtered (model NL 125, bandpass 200–4,000 Hz), and captured by a computer via a power 1401 analog-to-digital interface and Spike 2 software (version 4.03, Cambridge Electronic Design). 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 50 mmHg for 30-s periods. During set-up, control distensions to 50 mmHg with Krebs solution were repeated at 10-min intervals until nerve responses were stable. This pressure is known to represent a noxious stimulus in in vivo studies (38). Purinergic agonists and antagonists were applied intraluminally or to the serosa, and peak firing rates of the nerve were compared with controls.

*Chemicals.* ARL-67156, ATP (disodium salt), α,β-methylene ATP (lithium salt, α,β-MeATP), 8-(*p*-sulfophenyl)theophylline (8-SPT), pyridoxyl 5-phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS), and TNBS were obtained from Sigma, and 2′,3′-*O*-trinitrophenyl-ATP (TNP-ATP) was obtained from Molecular Probes (Leiden, The Netherlands). All chemicals were diluted in Krebs solution for experimental use.

*Statistical analysis.* Values are means ± SE. Data were compared by Student's *t*-test or by ANOVA, and differences were considered statistically significant at $P < 0.05$.

## 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 death. In 22 controls, the mean body weight was 269 ± 4.57 g, which was not significantly different from that of 22 preenema 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 hemorrhage. 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 (hematoxylin 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 <10% of the surface area of the lumen.

All pelvic nerve experiments were carried out on *day 5,* except for three experiments, which were performed on *day 6* (serosal ATP, α,β-MeATP, and PPADS application), and two experiments, which were carried out 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 normal and inflamed colorectum.* With intraluminal pressure at 0 in 14 suitable colitis preparations, background activity in the pelvic nerve was compared with activity in 14 normal controls over a 100-s period. Single-unit analysis by Spike 2 software 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/s. In the model of colitis, this value increased to 0.457 ± 0.074 impulses/s ($P = 0.018$). In recordings from normal and inflamed colorectum, phasic distensions in the rat typically produce a sudden burst of spikes that settle to a stable level after 30–60 s, and responses show good reproducibility, even after short recovery periods. Single-unit analysis of pelvic nerve recordings from the colitis preparations, during 30-s phasic distensions with Krebs solution to 50 mmHg, revealed a mean firing rate per unit of 3.39 ± 0.267 impulses/s. In normal colorectum, distensions to the same pressure evoked a mean firing rate of only 1.95 ± 0.113 impulses/s ($P < 0.0001$). Figure 1 compares background and distension-evoked spike frequency in recordings from a normal and a colitis preparation. These examples were selected, because single-unit analysis demonstrated that each preparation contained the same number of fibers; therefore, a meaningful comparison could be made of the multifiber activity in each preparation.

*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 inflamed preparations, similar results were obtained. In contrast, application of ATP or α,β-MeATP to the serosal surface of the colitis model or the normal colon evoked consistent, rapid responses with a mean latency in the controls that was not significantly different from that in the colitis preparations: 13.7 ± 0.85 and 14.6 ± 1.21 s, respectively. Table 1 compares the multifiber responses elicited by a bolus application of ATP or α,β-MeATP. In the normal colorectum and the colitis model, the mean percent 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,

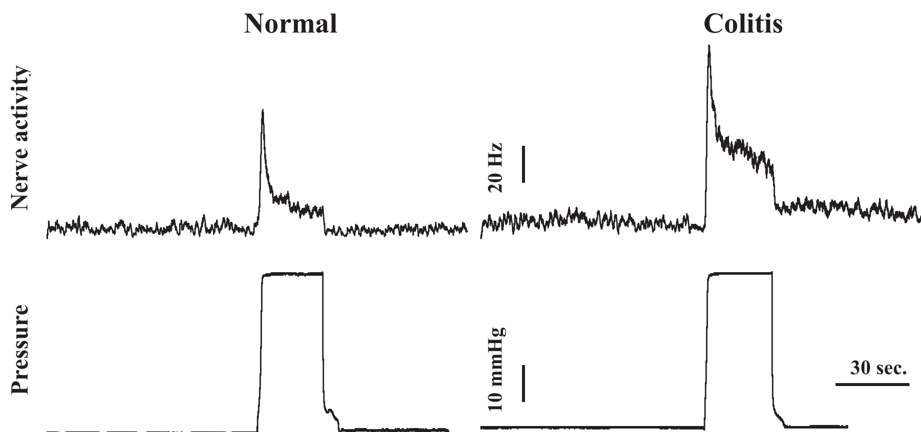


Fig. 1. Sample recordings from the pelvic nerve in a normal colorectal preparation and a colitis model. Single-unit analysis confirmed that both preparations have the same number of active nerve fibers. Background activity and response to 50-mmHg distension are increased in the colitis model, demonstrating a greater firing rate per unit.

showed substantially greater-magnitude responses for equivalent concentrations of agonist.

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

*Purinergic contribution to the afferent response to distension.* In at least eight normal animals and eight colitis preparations, the effect of serosal ATP application during distension to an intraluminal pressure of 50 mmHg was investigated. In the normal colorectum, the presence of ATP increased peak distension-induced activity in the pelvic nerve compared with control distensions with Krebs solution (Fig. 2), 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. Distension of the colitic colorectum in the presence of α,β-MeATP also increased the afferent response at 100 μM and 1 mM (28.1 ± 1.58 and 37.6 ± 3.84%, respectively, $n$ = 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 above, α,β-MeATP was more potent than ATP. Distension of the normal colorectum in the presence of the nonspecific P2X receptor antagonist PPADS (100 μM) 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 (60 μM) produced a 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 are compared in Fig. 3.

The metabolism of ATP to adenosine and their effect on the pelvic nerve response to distension at 50 mmHg were studied using the ATPase inhibitor ARL-67156 and the general P1 (adenosine) antagonist 8-SPT. In six normal preparations, the presence of the ATPase inhibitor resulted in a mean increase in nerve activity of 12.0 ± 2.45% over the first 10 s of colorectal distension. However, this value increased to 26.1 ± 6.59% in the five colitis preparations tested ($P$ = 0.06). To allow time for any potential adenosine to appear, colorectal distension was sustained for a longer period. In normal controls, 100 μM 8-SPT reduced nerve activity throughout the course of a 90-s 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 (Fig. 4): from 2.2 ± 4.7% during the first 10 s to 17.3 ± 4.2% between 80 and 90 s. ANOVA between the two responses confirmed that they were significantly different ($P$ = 0.005).

Table 1. *Magnitude of pelvic nerve responses to bolus doses of ATP and α,β-MeATP in normal and inflamed colorectum*

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

Values (means ± SE of number of samples in parentheses) represent percent increase in frequency of spikes from baseline activity. α,β-MeATP, α,β-methylene ATP.

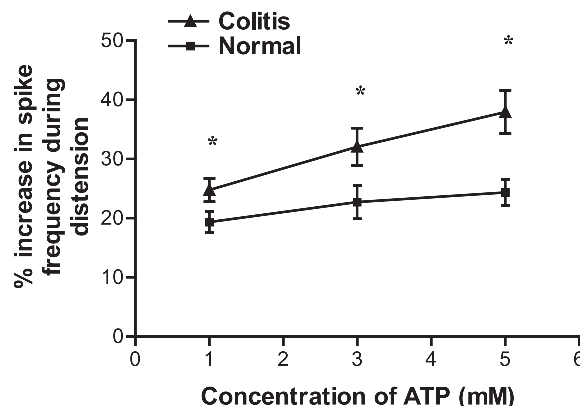


Fig. 2. Augmentation of pelvic nerve response to colorectal distension (50 mmHg) in the presence of ATP is dose dependent. In colitis models, this potentiation is increased to a greater extent. *$P$ ≤ 0.05.

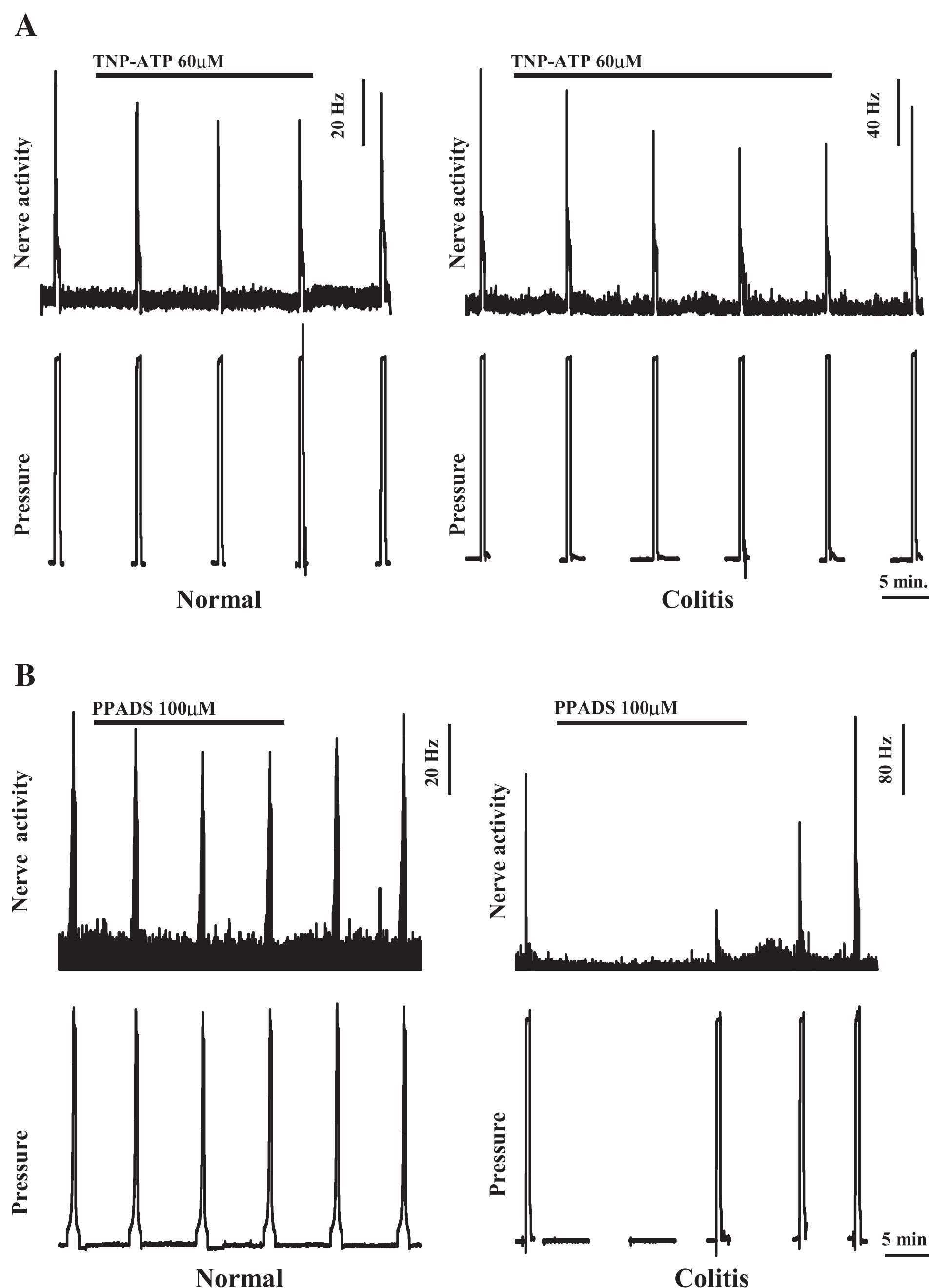


Fig. 3. Comparison of pelvic nerve responses to distension (50 mmHg) in the presence of P2X receptor antagonists 2′,3′-*O*-trinitrophenyl-ATP (TNP-ATP, 60 μM) and pyridoxyl 5-phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS, 100 μM). Colitis models show smaller responses to distension in the presence of the antagonists. Note different scales for recordings.

*ATP release.* From 4 inflamed colorectal preparations, 100 individual distensions of 6–90 mmHg were performed. These were compared with similar distensions in normal controls, which were consistent with those described by Wynn et al. (58). Figure 5 shows the relationship between rising intraluminal pressure and ATP concentration in the perfusate of each group. Intraluminal fluid was collected before each of the distensions (pressure approximately 0), 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 than in the normal controls: 0.352 ± 0.018 and 0.154 ± 0.004 pmol/ml, respectively. Postdistension samples collected from the inflamed colorectum yielded significantly greater ATP concentrations than those collected from normal colorectal controls, and this was consistently the case over every pressure group ($P \leq 0.0001$ by ANOVA). Compared with the normal colorectum, where the distension-induced rise in ATP release became significant at pressures >11 mmHg, the colitis preparations showed significant increases in ATP release at <10 mmHg ($P = 0.033$).

*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 with two other ganglia ($L_2$ and $S_2$) that are less important in this regard. DRG neurons respond to ATP with three different types of inward current (Fig. 6): transient responses (Fig. 6*A*) correspond to $P2X_3$ receptor activation, sustained responses (Fig. 6*B*) correspond to

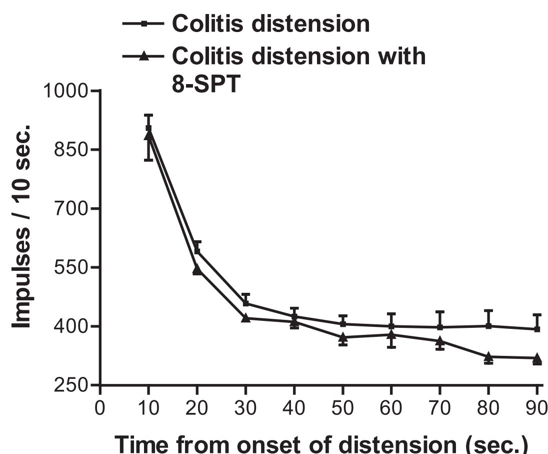


Fig. 4. Ten-second periods of nerve activity plotted for 90-s distensions in 5 colitis preparations with or without the general adenosine antagonist 8-(*p*-sulfophenyl)theophylline (8-SPT). When adenosine receptors are blocked, nerve activity is progressively reduced throughout the period of distension ($P = 0.005$). This may indicate an increasing role for adenosine in the later part of distension-evoked nerve activity.

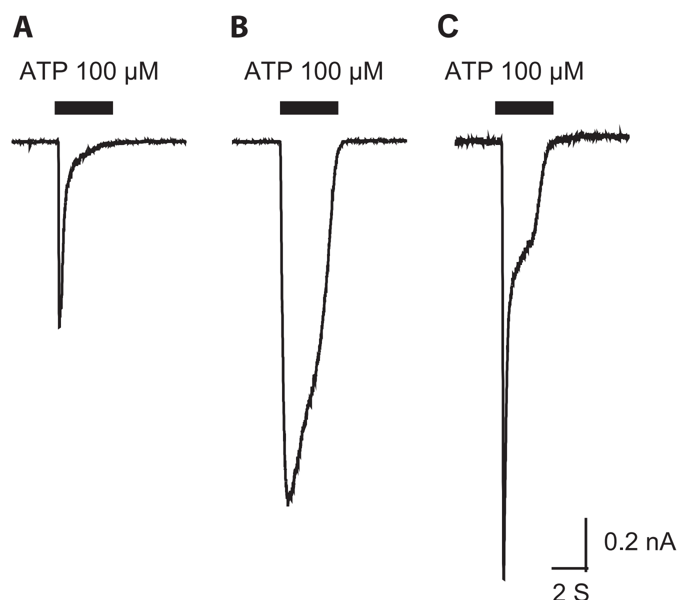


Fig. 6. Typical traces of the 3 different inward currents encountered when ATP is applied to dorsal root ganglia (DRG) neurons: a transient current that corresponds to activation of the $P2X_3$ receptor (*A*), a sustained current that corresponds to activation of the $P2X_2$ receptor (*B*), and a biphasic current corresponding to the $P2X_{2/3}$ heteromultimeric receptor (*C*).

activation of $P2X_2$ receptors, and biphasic responses (Fig. 6*C*) correspond to $P2X_{2/3}$ receptor activation. Table 2 shows the percentage of neurons in each group that were responsive to ATP in $L_1$ and $S_1$ DRG 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 2), there was no difference in the sustained and biphasic responders, but there was 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 four normal rats were studied for immunoreactivity to $P2X_3$ receptors and CGRP. Typical immunostaining of $S_1$ DRG in the normal rat and in the colitis model is compared in Fig. 7. A subpopulation of neurons that are positive for CGRP in the normal rat is shown in Fig. 7*a*, and CGRP-staining cells in the colitis model are shown in Fig. 7*b*. Similarly, a subpopulation of $P2X_3$-immunoreactive neurons in the normal rat DRG is shown in Fig. 7*c*; those staining in the inflammatory model now show staining of axons also (Fig. 7*d*). Colocalization (yellow staining) between CGRP and $P2X_3$ receptors is shown in the normal

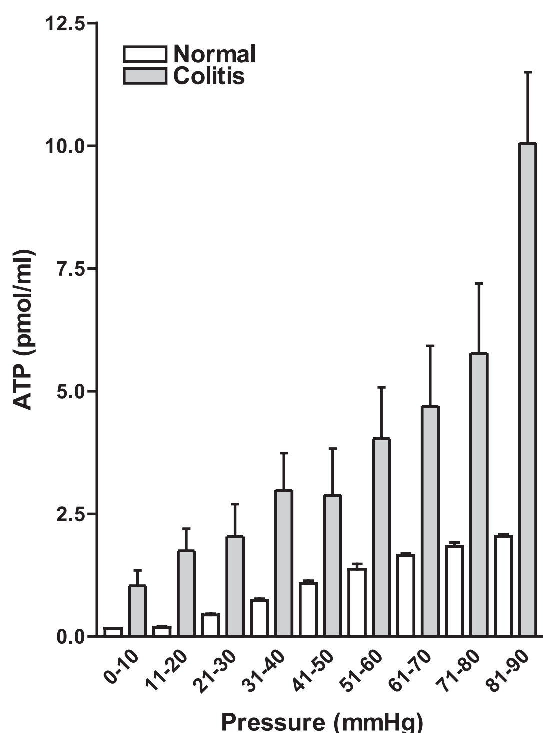


Fig. 5. ATP concentration in luminal fluid samples from normal and inflamed rat colorectum during distension. Values are means ± SE.

Table 2. *DRG neurons that respond to ATP with sustained, transient, or biphasic inward current before and after induction of colitis*

| | *n* | Sustained | Transient | Biphasic | No Response |
|---|---|---|---|---|---|
| | | | *$L_1$ and $S_1$ DRG* | | |
| Normal | 28 | 21 | 46 | 18 | 14 |
| Colitis | 27 | 22 | 56 | 19 | 4 |
| | | | *$L_2$ and $S_2$ DRG* | | |
| Normal | 23 | 22 | 39 | 22 | 17 |
| Colitis | 19 | 21 | 58 | 21 | 0 |

Dorsal respiratory group (DRG) neurons that respond to ATP with a sustained ($P2X_2$ receptor), transient ($P2X_3$ receptor), or biphasic ($P2X_{2/3}$) inward current before and after induction of colitis are shown as percentages. Proportion of $L_2$ and $S_2$ DRG neurons responding to ATP show a similar distribution in the 2 experimental groups.

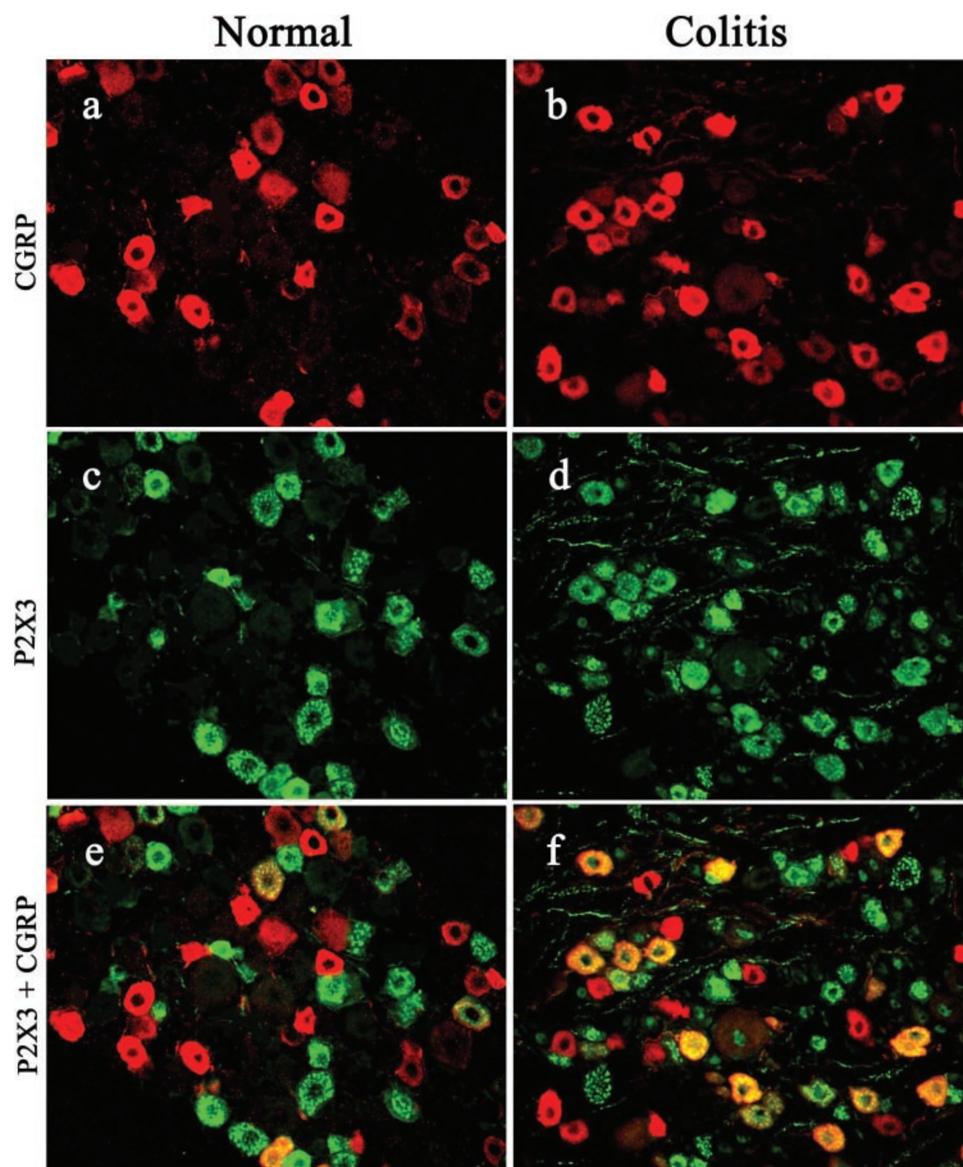


Fig. 7. CGRP (*a* and *b*; red) and $P2X_3$ (*c* and *d*; green) immunoreactivity in $S_1$ DRG in the normal rat and after induction of colitis. Nerve fibers as well as cell bodies stained for $P2X_3$ after induction of colitis (*d*). Note increased number of neurons showing colocalization between CGRP and $P2X_3$ receptors in the colitis models (*e* and *f*; yellow).

state (Fig. 7*e*) and after induction of colitis (Fig. 7*f*). In the normal rat, the percentage of neurons staining for $P2X_3$ receptors (33%) and CGRP (37%) was constant, regardless of the level of the ganglion. In the four 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 $S_2$ (Table 3). Both of these increases were statistically significant ($P \leq 0.0001$ and 0.0001, respectively). More CGRP-positive neurons had also appeared in the inflammatory preparations: increase 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 $S_2$ (Table 3). Again, these increases were highly statistically significant ($P =$ 0.0001 and 0.0008, respectively).

Colocalization between $P2X_3$ receptors and CGRP is quantified in Table 4, which 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 ($P < 0.0001$). $P2X_3$/CGRP colocalization was also increased in $L_2$ and $S_2$ ganglia: from 22.8 ± 0.99 to 29.9 ± 0.84 ($P < 0.0001$). When CGRP neurons were studied, the percentage of $L_1$ and $S_1$ neurons that also stained for $P2X_3$ receptors 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

Table 3. *DRG neurons that stain for the $P2X_3$ receptor and CGRP before and after induction of colitis*

| | *n* | Normal | Colitis | *P* |
|---|---|---|---|---|
| | | *$P2X_3$ receptor* | | |
| $L_1$ 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 |
| | | *CGRP* | | |
| $L_1$ 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 |

Values are percentages.

Table 4. *$P2X_3$-positive DRG neurons that also stain for CGRP and CGRP-positive DRG neurons that also stain for $P2X_3$ before and after induction of colitis*

| | *n* | Normal | Colitis | *P* |
|---|---|---|---|---|
| *$P2X_3$-positive neurons that stain for CGRP* | | | | |
| $L_1$ 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 |
| *CGRP-positive neurons that stain for $P2X_3$* | | | | |
| $L_1$ 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 |

Values are percentages.

in $L_2$ and $S_2$: from 19.5 ± 0.83 to 28.1 ± 0.81% ($P < 0.0001$; Table 4).

**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 than in 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 the existence of an important purinergic component in sensory nerve signaling 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 (22). ATP and α,β-MeATP produce dose-dependent nocifensive behavior when injected into the rat hindpaw, and the effect of these agonists is greatly augmented after ultraviolet irradiation, before injection with carageenan, and immediately after prostaglandin $E_2$ treatment (23). The formalin rat paw model has been used to demonstrate the antinociceptive effects of intrathecally administered P2X antagonists (19, 54). In addition to bladder hyporeflexia, $P2X_3$-null mice have reduced inflammatory pain-related behavior (14). When $P2X_3$ and $P2X_{2/3}$ receptors are expressed by *Xenopus* oocytes, their currents are potentiated by SP and BK (43). Mechanosensory function in a model of esophagitis was sensitized by α,β-MeATP (41), and another study suggested that $P2X_3$ receptors on intrinsic enteric neurons are increased in human IBD (60).

Recordings from the pelvic nerve in the present study showed that background activity was significantly higher in the colitis models than in normal colorectal preparations. This finding was paralleled during distension, where individual units from inflamed colon fired at a higher frequency than those from controls at a given intraluminal pressure. Other studies have shown that intrinsic neurons in the guinea pig jejunum (42) and dorsal horn neurons receiving input from the colon in the rat (40) also exhibit enhanced excitability after 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 (58). Briefly, passive permeation of hydrophilic molecules and ions across the gastrointestinal epithelium is conducted for the most part by tight junctions that allow selective absorption. The colon has a very high transepithelial electrical resistance ($10^6$ $\Omega \cdot cm^2$), and hydrophilic molecules with a Stokes radius greater than ~11.5 Å are excluded (35). 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 with 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 metabolized 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 ~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 signaling available, even if there was no effect on enzyme activity. We have not specifically investigated whether ATPases are up- or downregulated 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 signaling molecule itself. ATP is released from endothelial cells subjected to shear stress (6) and from urothelial cells during bladder (20, 56) and ureteric distension (33). There is good evidence that the mechanism of release is vesicular exocytosis (6, 33). Endothelial cells increase their release in acute inflammation (5), and in the bladder, stretch-activated ATP release is increased in interstitial cystitis (52) and coincides with an augmented component of purinergic neurotransmission in this condition (for review, see Ref. 12). The present study demonstrates that, in the normal rat colorectum, there is a strong relation 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 in normal controls. ATP is released in other painful pathological conditions also. Tumor cells are known to contain exceptionally high levels of ATP, and in sympathetic reflex dystrophy, surgical sympathectomy, sympathetic ganglion blockade, and guanethidine are more

effective at relieving pain than adrenoceptor antagonists, suggesting a role for the release of the cotransmitter ATP (25). ATP exists within cells in millimolar concentrations; therefore, any significant cellular damage is also likely to increase local release. In a model of postoperative 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 (53). ATP activates visceral sensory nerves in a dose-dependent way (32, 46), so it follows that in situations where release of ATP is greater, there is augmented activation of purinergic nerves. 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 (61). 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 threefold increase in ATP-activated currents and altered the voltage dependence of P2X receptors of neurons in the DRG (59).

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 $P2X_3$ upregulation 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 (28). In other experimental conditions where sensory nerves detect injury, there are also changes to expression of $P2X_3$ receptors. After 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 with sham-operated animals (39). Studies using tight ligation of a spinal nerve (30) or axotomy (7) report a reduction in $P2X_3$ expression in the relevant DRG. Tsuzuki et al. (55) demonstrated that axotomized neurons reduced the expression of $P2X_3$ mRNA, whereas adjacent neurons that were spared increased their expression. Together with the knowledge that $P2X_3$ receptors accumulate proximal to the site of nerve ligation, indicating receptor transport to the periphery (57), this gives indirect evidence that $P2X_3$ receptors located on the peripheral terminals of colorectal afferents are upregulated during colitis. $P2X_3$ receptors may be increased also in the intrinsic nervous system of the colorectum during inflammation (60).

The $P2X_3$ receptor is normally found largely in nonpeptidergic sensory neurons that bind the lectin IB4; however, a minority of $P2X_3$-positive neurons also contain the neuropeptide CGRP (7). 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 (47). CGRP released in response to inflammation is thought to provide tissue protection by increasing blood flow to damaged areas, and rats treated with CGRP antagonists develop more severe colitis after TNBS enema (44). These data suggest that purinergic signaling 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 the number of $P2X_3$ receptors per neuron and in the number and type of neurons expressing $P2X_3$ receptors suggest 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 upregulation 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 while significantly reducing pelvic nerve afferent activity in response to distension (58). ATP released during noxious colorectal distension 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 upregulation and/or sensitization 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, e.g., BK, prostaglandins, histamine, and cytokines such as IL-1β and IL-6 (49). Many of the properties of ATP suggest that it may play a role similar to that of some of these mediators. ATP causes pain when injected into the base of blisters (6), and this pain is increased in states of inflammation (24). 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 understand the etiology 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, have an inflammatory episode as the trigger for sensory neuron dysfunction (3, 45). ATP may act as one of the signaling 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 remodeling and plasticity in response to their local environment and the ongoing need for the mucosa to renew itself (51). If abnormal or prolonged sensitization 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.

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## REFERENCES

1. **Ardizzone S, Bollani S, Manzionna G, and Bianchi Porro G.** Inflammatory bowel disease approaching the 3rd millennium: pathogenesis and therapeutic implications? *Eur J Gastroenterol Hepatol* 11: 27–32, 1999.
2. **Ardizzone S and Porro GB.** Inflammatory bowel disease: new insights into pathogenesis and treatment. *J Intern Med* 252: 475–496, 2002.
3. **Barbara G, De Giorgio R, Stanghellini V, Cremon C, and Corinaldesi R.** A role for inflammation in irritable bowel syndrome? *Gut* 51 *Suppl* I: I41–I44, 2002.
4. **Bjorck S, Dahlstrom A, Johansson L, and Ahlman H.** Treatment of the mucosa with local anaesthetics in ulcerative colitis. *Agents Actions* C60–C72, 1992.
5. **Bodin P and Burnstock G.** Increased release of ATP from endothelial cells during acute inflammation. *Inflamm Res* 47: 351–354, 1998.
6. **Bodin P and Burnstock G.** Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. *J Cardiovasc Pharmacol* 38: 900–908, 2001.
7. **Bradbury EJ, Burnstock G, and McMahon SB.** The expression of $P2X_3$ purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Mol Cell Neurosci* 12: 256–268, 1998.
8. **Bueno L and Fioramonti J.** Visceral perception: inflammatory and non-inflammatory mediators. *Gut* 51, *Suppl* I: I19–I23, 2002.
9. **Burnstock G.** Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J Anat* 194: 335–342, 1999.
10. **Burnstock G.** P2X receptors in sensory neurones. *Br J Anaesth* 84: 476–488, 2000.
11. **Burnstock G.** Purine-mediated signalling in pain and visceral perception. *Trends Pharmacol Sci* 22: 182–188, 2001.
12. **Burnstock G.** Potential therapeutic targets in the rapidly expanding field of purinergic signalling. *Clin Med* 2: 45–53, 2002.
13. **Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, and Wood JN.** A P2X purinoceptor expressed by a subset of sensory neurons. *Nature* 377: 428–431, 1995.
14. **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, and Ford AP.** Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* 407: 1011–1015, 2000.
15. **Cohen RD and Hanauer SB.** Nicotine in ulcerative colitis. How does it work and how can we use it? *Clin Immunother* 5: 169–174, 1996.
16. **Collins SM.** The immunomodulation of enteric neuromuscular function: implications for motility and inflammatory disorders. *Gastroenterology* 111: 1683–1699, 1996.
17. **De Giorgio R, Barbara G, Blennerhassett P, Wang L, Stanghellini V, Corinaldesi R, Collins SM, and Tougas G.** Intestinal inflammation and activation of sensory nerve pathways: a functional and morphological study in the nematode-infected rat. *Gut* 49: 822–827, 2001.
18. **Dray A.** Inflammatory mediators of pain. *Br J Anaesth* 75: 125–131, 1995.
19. **Driessen B, Reimann W, Selve N, Friderichs E, and Bultmann R.** Antinociceptive effect of intrathecally administered P2-purinoceptor antagonists in rats. *Brain Res* 666: 182–188, 1994.
20. **Ferguson DR, Kennedy I, and Burton TJ.** ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes—a possible sensory mechanism? *J Physiol* 505: 503–511, 1997.
21. **Grundy D.** Neuroanatomy of visceral nociception: vagal and splanchnic afferents. *Gut* 51, *Suppl* I: I2–I5, 2002.
22. **Hamilton SG, McMahon SB, and Lewin GR.** Selective activation of nociceptors by P2X receptor agonists in normal and inflamed rat skin. *J Physiol* 534: 437–445, 2001.
23. **Hamilton SG, Wade A, and McMahon SB.** The effects of inflammation and inflammatory mediators on nociceptive behaviour induced by ATP analogues in the rat. *Br J Pharmacol* 126: 326–332, 1999.
24. **Hamilton SG, Warburton J, Bhattacharjee A, Ward J, and McMahon SB.** ATP in human skin elicits a dose-related pain response which is potentiated under conditions of hyperalgesia. *Brain* 123: 1238–1246, 2000.
25. **Hannington-Kiff JG.** Intravenous regional sympathetic block with guanethidine. *Lancet* 1: 1019–1020, 1974.
26. **Hicks GA, Coldwell JR, Schindler M, Bland-Ward PA, Jenkins D, Lynn PA, Humphrey PPA, and Blackshaw LA.** Excitation of rat colorectal afferent fibres by $5\text{-}HT_3$ receptors. *J Physiol* 544: 861–869, 2002.
27. **Jacobson K, McHugh K, and Collins SM.** Experimental colitis alters myenteric nerve function at inflamed and noninflamed sites in the rat. *Gastroenterology* 109: 718–722, 1995.
28. **Jacobson K, McHugh K, and Collins SM.** The mechanism of altered neural function in a rat model of acute colitis. *Gastroenterology* 112: 156–162, 1997.
29. **Jarvis MF, Wismer CT, Schweitzer E, Yu H, van Biesen T, Lynch KJ, Burgard EC, and 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* 132: 259–269, 2001.
30. **Kage K, Niforatos W, Zhu CZ, Lynch KJ, Honore P, and Jarvis MF.** Alteration of dorsal root ganglion $P2X_3$ receptor expression and function following spinal nerve ligation in the rat. *Exp Brain Res* 147: 511–519, 2002.
31. **King BF, Ziganshina LE, Pintor J, and Burnstock G.** Full sensitivity of $P2X_2$ purinoceptor to ATP revealed by changing extracellular pH. *Br J Pharmacol* 117: 1371–1373, 1996.
32. **Kirkup AJ, Booth CE, Chessell IP, Humphrey PP, and Grundy D.** Excitatory effect of P2X receptor activation on mesenteric afferent nerves in the anaesthetised rat. *J Physiol* 520: 551–563, 1999.
33. **Knight GE, Bodin P, De Groat WC, and Burnstock G.** ATP is released from guinea pig ureter epithelium on distension. *Am J Physiol Renal Physiol* 282: F281–F288, 2002.
34. **Lewis C, Neidhart S, Holy C, North RA, Buell G, and Surprenant A.** Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. *Nature* 377: 432–435, 1995.
35. **Madara JL.** Loosening tight junctions. Lessons from the intestine. *J Clin Invest* 83: 1089–1094, 1989.
36. **Mazelin L, Theodorou V, More J, Fioramonti J, and Bueno L.** Protective role of vagal afferents in experimentally-induced colitis in rats. *J Auton Nerv Syst* 73: 38–45, 1998.
37. **McCafferty DM, Wallace JL, and Sharkey KA.** Effects of chemical sympathectomy and sensory nerve ablation on experimental colitis in the rat. *Am J Physiol Gastrointest Liver Physiol* 272: G272–G280, 1997.
38. **Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL.** Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 96: 795–803, 1989.
39. **Novakovic SD, Kassotakis LC, Oglesby IB, Smith JA, Eglen RM, Ford AP, and Hunter JC.** Immunocytochemical localization of $P2X_3$ purinoceptors in sensory neurons in naive rats and following neuropathic injury. *Pain* 80: 273–282, 1999.
40. **Olivar T, Cervero F, and Laird JM.** Responses of rat spinal neurones to natural and electrical stimulation of colonic afferents: effect of inflammation. *Brain Res* 866: 168–177, 2000.
41. **Page AJ, O'Donnell TA, and Blackshaw LA.** P2X purinoceptor-induced sensitisation of ferret vagal mechanoreceptors in oesophageal inflammation. *J Physiol* 523: 403–411, 2000.
42. **Palmer JM, Wong-Riley M, and Sharkey KA.** Functional alterations in jejunal myenteric neurons during inflammation in nematode-infected guinea pigs. *Am J Physiol Gastrointest Liver Physiol* 275: G922–G935, 1998.
43. **Paukert M, Osteroth R, Geisler HS, Brandle U, Glowatzki E, Ruppersberg JP, and Grunder S.** Inflammatory mediators potentiate ATP-gated channels through the $P2X_3$ subunit. *J Biol Chem* 276: 21077–21082, 2001.
44. **Reinshagen M, Flamig G, Ernst S, Geerling I, Wong H, Walsh JH, Eysselein VE, and Adler G.** Calcitonin gene-related peptide mediates the protective effect of sensory nerves in a model of colonic injury. *J Pharmacol Exp Ther* 286: 657–661, 1998.

45. **Rodriguez LA and Ruigomez A.** Increased risk of irritable bowel syndrome after bacterial gastroenteritis: cohort study. *Br Med J* 318: 565–566, 1999.
46. **Rong W, Spyer KM, and Burnstock G.** Activation and sensitisation of low- and high-threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder. *J Physiol* 541: 591–600, 2002.
47. **Roza C and Reeh PW.** Substance P, calcitonin gene-related peptide and $PGE_2$ co-released from the mouse colon: a new model to study nociceptive and inflammatory responses in viscera, in vitro. *Pain* 93: 213–219, 2001.
48. **Ruan HZ and Burnstock G.** Localisation of $P2Y_1$ and $P2Y_4$ receptors in dorsal root, nodose and trigeminal ganglia of the rat. *Histochem Cell Biol* 120: 415–426, 2003.
49. **Sharkey KA and Kroese AB.** Consequences of intestinal inflammation on the enteric nervous system: neuronal activation induced by inflammatory mediators. *Anat Rec* 262: 79–90, 2001.
50. **Souslova V, Cesare P, Ding Y, Akopian AN, Stanfa L, Suzuki R, Carpenter K, Dickenson A, Boyce S, Hill R, Nebenuis-Oosthuizen D, Smith AJ, Kidd EJ, and Wood JN.** Warm-coding deficits and aberrant inflammatory pain in mice lacking $P2X_3$ receptors. *Nature* 407: 1015–1017, 2000.
51. **Stead RH, Kosecka-Janiszewska U, Oestreicher AB, Dixon MF, and Bienenstock J.** Remodeling of B-50 (GAP-43)- and NSE-immunoreactive mucosal nerves in the intestines of rats infected with *Nippostrongylus brasiliensis*. *J Neurosci* 11: 3809–3821, 1991.
52. **Sun Y, Keay S, DeDeyne PG, and Chai TC.** Augmented stretch activated adenosine triphosphate release from bladder urothelial cells in patients with interstitial cystitis. *J Urol* 166: 1951–1956, 2001.
53. **Tsuda M, Koizumi S, and Inoue K.** Role of endogenous ATP at the incision area in a rat model of postoperative pain. *Neuroreport* 12: 1701–1704, 2001.
54. **Tsuda M, Ueno S, and Inoue K.** Evidence for the involvement of spinal endogenous ATP and P2X receptors in nociceptive responses caused by formalin and capsaicin in mice. *Br J Pharmacol* 128: 1497–1504, 1999.
55. **Tsuzuki K, Kondo E, Fukuoka T, Yi D, Tsujino H, Sakagami M, and Noguchi K.** Differential regulation of $P2X_3$ mRNA expression by peripheral nerve injury in intact and injured neurons in the rat sensory ganglia. *Pain* 91: 351–360, 2001.
56. **Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne DA, Ford AP, and Burnstock G.** $P2X_3$ knock-out mice reveal a major sensory role for urothelially released ATP. *J Neurosci* 21: 5670–5677, 2001.
57. **Vulchanova L, Riedl MS, Shuster SJ, Stone LS, Hargreaves KM, Buell G, Surprenant A, North RA, and Elde R.** $P2X_3$ is expressed by DRG neurons that terminate in inner lamina. II. *Eur J Neurosci* 10: 3470–3478, 1998.
58. **Wynn G, Rong W, Xiang Z, and Burnstock G.** Purinergic mechanisms contribute to mechanosensory transduction in the rat colorectum. *Gastroenterology* 125: 1398–1409, 2003.
59. **Xu GY and Huang LY.** Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons. *J Neurosci* 22: 93–102, 2002.
60. **Yiangou Y, Facer P, Baecker PA, Ford AP, Knowles CH, Chan CL, Williams NS, and Anand P.** ATP-gated ion channel $P2X_3$ is increased in human inflammatory bowel disease. *Neurogastroenterol Motil* 13: 365–369, 2001.
61. **Zhong Y, Dunn PM, Bardini M, Ford AP, Cockayne DA, and Burnstock G.** Changes in P2X receptor responses of sensory neurons from $P2X_3$-deficient mice. *Eur J Neurosci* 14: 1784–1792, 2001.