Sensitization of visceral afferents to bradykinin in rat jejunum *in vitro*

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1. We have investigated the effects of inflammatory mediators on visceral afferent discharge and afferent responses to bradykinin (BK) in rat jejunum using a novel *in vitro* technique.

2. Prostaglandin E_2 (1 μM) augmented responses to BK without affecting basal firing, while histamine (100 μM) and adenosine (100 μM) activated basal discharge and enhanced BK responses. In contrast, 5-HT (100 μM) increased basal discharge without influencing responses to BK.

3. Afferent discharge induced by histamine was inhibited by both H_1 (pyrilamine) and H_3 (thioperamide) but not H_2 (ranitidine) receptor antagonists at 10 μM. In contrast, sensitization to BK induced by histamine was inhibited by ranitidine (10 μM).

4. Afferent discharge induced by adenosine was blocked by the A_1 receptor antagonist DPCPX (10 μM) but remained unaffected by A_{2A} receptor blockade with ZM241385 (10 μM). In contrast, sensitization of BK responses by adenosine was unaffected by both antagonists. Basal discharge and BK-induced responses were unaffected by the A_3 receptor agonist IB-MECA (1 μM). While involvement of A_{2B} receptors is not excluded, adenosine may activate afferent discharge through A_1 receptors, while sensitization to BK could involve a receptor other than A_1, A_{2A} or A_3, possibly the A_{2B} receptor.

5. Inhibition of cyclo-oxygenase with naproxen (10 μM) prevented sensitization after histamine but not adenosine.

6. Sensitization was mimicked by dibutyryl cAMP. This occurred without changes in basal firing and was unaffected by naproxen.

7. In conclusion, afferent discharge induced by BK is augmented by histamine, adenosine and PGE_2, but not by 5-HT. Evidence suggests that sensitization involves separate mechanisms from afferent activation. Sensitization may be mediated by increases in cAMP following direct activation by mediators at the nerve terminal or through indirect pathways such as the release of prostaglandins.

The gastrointestinal tract has an extensive intrinsic and extrinsic sensory innervation. Despite this, stimuli in the healthy gastrointestinal tract rarely reach the level of conscious perception. In contrast, sensations of abdominal discomfort and pain are common symptoms in patients with gastrointestinal disease. Symptoms include heartburn, chest pain, dyspepsia, bloating, abdominal cramps and feelings of incomplete rectal evacuation, all of which can arise in both organic (e.g. inflammatory) and functional disorders such as irritable bowel syndrome. Visceral afferent hypersensitivity is now a widely accepted mechanism which could explain many of these clinical symptoms associated with functional bowel disease and inflammatory diseases of the gut (Mayer & Raybould, 1990; Bueno *et al.* 1997). However, the mechanisms underlying peripheral sensitization of gastrointestinal afferents is poorly understood.

Much of our understanding of the mechanisms of afferent sensitization and the modulation of painful stimuli has stemmed from studies of cutaneous pain. Sensitization of cutaneous nociceptors is thought to underlie conditions of hyperalgesia (enhanced perception of pain) and allodynia where previously non-noxious stimuli can produce pain (Heller *et al.* 1993). Despite the lack of detailed studies on gastrointestinal afferent sensitivity, it is generally assumed that intestinal afferents behave in a similar way, being activated and/or sensitized by chemical mediators present within an 'inflammatory soup'. Gastrointestinal afferents terminate at different levels within the gut wall (namely

mucosal, muscle and serosal afferents), and reach the CNS via either vagal or spinal pathways. Evidence suggests that the spinal afferent endings in the serosa and mesentery, which have high thresholds for mechanical stimulation, could also serve a nociceptive function (Jaenig & Morrison, 1986; Ness & Gebhart, 1990). Sensitization of these spinal fibres could therefore lead to an altered perception of visceral stimuli.

Bradykinin (BK) is a pain-producing peptide generated in tissues and plasma following tissue damage or inflammation (for review see Regoli & Barabe, 1980) and can be both an algesic and a hyperalgesic agent, stimulating and sensitizing C and Aδ fibres that encode noxious stimuli (Szolscanyi, 1987). In addition, BK stimulates afferents within the gastrointestinal tract (Longhurst *et al.* 1984; Pan *et al.* 1994), is implicated in activation of abdominal visceral afferents during ischaemia (Longhurst & Dittman, 1987) and may play an important role in inflammatory bowel disease where plasma levels appear to correlate well with the onset of gastrointestinal symptoms (Cuschieri & Onabanjo, 1971). Other inflammatory mediators, particularly prostaglandins, have been shown to enhance afferent responses to noxious stimuli such as BK (Handwerker & Reeh, 1991; Nicol & Cui, 1994). We have recently characterized the action of BK on serosal afferents from rat jejunum using a novel *in vitro* model (Maubach & Grundy, 1999) and demonstrated the involvement of prostaglandins in this response. This simple preparation was designed to examine the sensitivity of serosal afferents more directly in the absence of the main body of the jejunum (mucosa, submucosa and muscle layers), thus minimizing the potential for secondary activation of the afferents. The aim of this current study was to investigate the sensitivity of visceral afferents to a variety of potential mediators of inflammation and to examine whether these afferents show sensitization. We have therefore examined the effects of chemical mediators on afferent discharge and explored any possible interaction between these agents and the response to BK.

METHODS

Tissue preparation

Male hooded Lister rats (350–400 g) were overdosed with urethane (1·5 g kg^{-1}) and a mid-line laparotomy performed. A 3 cm-long piece of jejunum complete with mesenteric attachment was then carefully excised. This segment was placed in a Sylgard-lined perfusion chamber and the mesenteric arcade pulled through a small aperture into a separate recording chamber. The mesentery was then dissected free from the main body of the jejunum (which was discarded) and this mesenteric attachment pinned out. Once the aperture had been sealed with vaseline the recording chamber was filled with colourless heavy liquid paraffin (pre-warmed to 34 °C) and the perfusion chamber superfused at 10 ml min^{-1} with bicarbonate buffer (composition (mM): Na^+ 143·5, K^+ 5·9, Cl^- 126, Ca^{2+} 2·5, Mg^{2+} 1·2, H_2PO_4 1·2, SO_4 1·2, HCO^-_3 25, glucose 10 and sodium butyrate 1, pH maintained at 7·4 with 95% O_2–5% CO_2), pre-warmed to yield a chamber temperature of 34 °C. A separate preparation was used for each experiment.

Nerve recording

Under a stereomicroscope, a paravascular nerve bundle was teased out from the mesenteric arcade and wrapped around one arm of a bipolar platinum recording electrode with a length of connective tissue attached to the second indifferent electrode. The electrodes were connected to a Neurolog headstage (NL 100), and the signal amplified (NL 104, ×20 000) and filtered (NL 125; band width, 100–1000 Hz) then relayed to a spike processor (Digitimer D130) to allow discrimination of action potentials from noise using a manually set amplitude and polarity window. The whole-nerve recording was displayed on a storage oscilloscope (Tektronix 5111A) and digitized (PCM-2 A/D VCR adapter, Medical Systems Corp.) to allow recording on VHS videotape for future off-line analysis. Whole-nerve activity was continuously monitored as spike discharge (impulses (1·25 s)$^{-1}$) and stored on a PC using Spike2 software (CED).

Experimental protocols

Experiments were performed on preparations in which baseline afferent discharge was maintained and in which robust responses to a priming exposure to a submaximal concentration of BK (1 μM, 2 min; see Maubach & Grundy, 1999, for concentration–reponse data) could be evoked. Once discharge had returned to basal level, the preparation was allowed to stabilize for a further 15 min. Subsequent responses to BK (1 μM, 2 min) were then quantified before and 30 min after a period during which the perfusion fluid was recirculated (reperfusion period) using 60 ml of either buffer alone or buffer containing one of several chemical mediators (see Fig. 1). The first response is referred to as the control response while the second response in the presence of mediator could be compared directly to this control. Reperfusion with buffer alone served as a time control for statistical comparison. This protocol of priming challenge followed after 15 min by a control response and a second challenge after 30 min reperfusion proved reproducible, such that the second response to BK (after reperfusion) was very similar to the control response. Thus, having established this time control, mediators could be tested within the 30 min reperfusion period. The effects of the following mediators were examined: prostaglandin E_2 (1 μM), histamine (100 μM), adenosine (100 μM), 5-HT (100 μM) and dibutyryl cAMP (100 μM). These concentrations were chosen following results from pilot studies and from consideration of sensitization studies in somatic nociceptors *in vitro*, where similar agents are often used as components of an 'inflammatory soup'. In receptor antagonist studies and studies with naproxen, these agents were added after the priming challenge to BK and were present throughout the experiment thereafter. The response in the presence of antagonist alone (first response) could therefore be directly compared to the response after a 30 min reperfusion with antagonist together with the chemical mediator. In addition, this allowed direct comparison between BK responses without the need for extra vehicle controls. In studies with naproxen, naproxen was added after the priming challenge and left for 30 min before the control BK response was obtained. BK was then re-applied after the 30 min reperfusion period during which naproxen was still present together with the chemical mediator concerned (Fig. 4). Thus naproxen was present throughout the experiment following the priming challenge. This acted as a new time control to examine whether mediators act indirectly through the generation of prostanoids. The effects of naproxen on BK-induced discharge *per se* were not under investigation.

Drugs

The following compounds were purchased from Sigma: bradykinin (BK), prostaglandin E_2 (PGE_2), histamine, adenosine, 5-HT

(5-hydroxytryptamine), dibutyryl cAMP (N^6,2′-*O*-dibutyryl-adenosine 3′,5′-cyclic monophosphate), pyrilamine and ranitidine. DPCPX (1,3-dipropyl-8-cyclopentylxanthine) was purchased from Research Biochemicals International. ZM241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol), IB-MECA (1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide) and thioperamide were purchased from Tocris Cookson Ltd. All drug stock solutions were made in distilled water unless otherwise stated. PGE_2 was dissolved in 0·1 ml absolute ethanol + 0·9 ml sodium bicarbonate (2% w/w) giving a stock solution of 1 mg ml^{-1}. ZM241385 was dissolved in DMSO and DPCPX was dissolved in ethanol, giving final concentrations of 0·1 and 0·01% v/v of vehicle, respectively, in perfusion fluids.

Data analysis

BK responses are expressed as total number of impulses calculated as the number of impulses for the entire response (area under response profile) minus the number during an equal period of basal activity. These raw data are given in the text. Peak discharge frequency induced by BK was calculated as the maximum level of discharge after subtraction of the baseline discharge (impulses s^{-1}). Mediator-induced changes in basal discharge were calculated as the mean number of impulses in 3 min following application minus mean basal activity in 3 min prior to application of the mediator, and expressed in impulses per second. Due to differences in the number of fibres in each afferent bundle and the consequent variability in baseline firing and response magnitude, afferent responses to BK were also expressed as a percentage of the control response (i.e. the second response to BK expressed as a percentage of the control response). Some data are also presented as percentage change. The change in afferent discharge upon addition of mediator or vehicle was expressed as a percentage of baseline. The increase in activity induced by BK was also expressed as a percentage of basal discharge prior to addition. Unless otherwise stated data are presented as means ± standard error of the mean (S.E.M.); n, number of experiments. Paired t tests were used to compare control and second BK responses where data were normally distributed, otherwise paired comparisons were made using the Wilcoxon signed rank test. Data involving multiple comparisons to control were analysed using Dunn's multiple comparison method following a Kruskall-Wallis one-way analysis of variance on ranks or Bonferroni's multiple comparison method on data normalized by a simple square root transformation. Values of $P < 0{\cdot}05$ were considered significant.

RESULTS

Extracellular recordings were made from a total of 28 serosal afferent preparations. All multiunit recordings showed a continuous pattern of on-going discharge, the extent of which was highly variable between preparations because of differences in the number of viable units in individual paravascular nerve bundles.

Response to BK

During a 2 min exposure to 1 μM BK there was a marked increase in whole-nerve discharge after a latency of 28·8 ± 1·9 s reaching a peak of 41·7 ± 3·7 impulses s^{-1} from a baseline level of 12·6 ± 1·4 impulses s^{-1} ($n = 28$, $P < 0{\cdot}001$). Discharge returned to baseline levels after the BK challenge and was well maintained for the 30 min reperfusion period (Fig. 1). In time control experiments the baseline discharge before and after 30 min reperfusion was 9·16 ± 3 *vs.* 8·5 ± 3 impulses s^{-1}, respectively ($n = 5$, $P > 0{\cdot}05$). Moreover, the response to BK was also unaffected by the 30 min reperfusion period. The overall effect of BK, expressed as the area under the response curve, was 1962 ± 334 impulses on first exposure compared to 1731 ± 183 impulses during the second challenge ($P > 0{\cdot}05$), such that the second response to BK represented 96 ± 16%

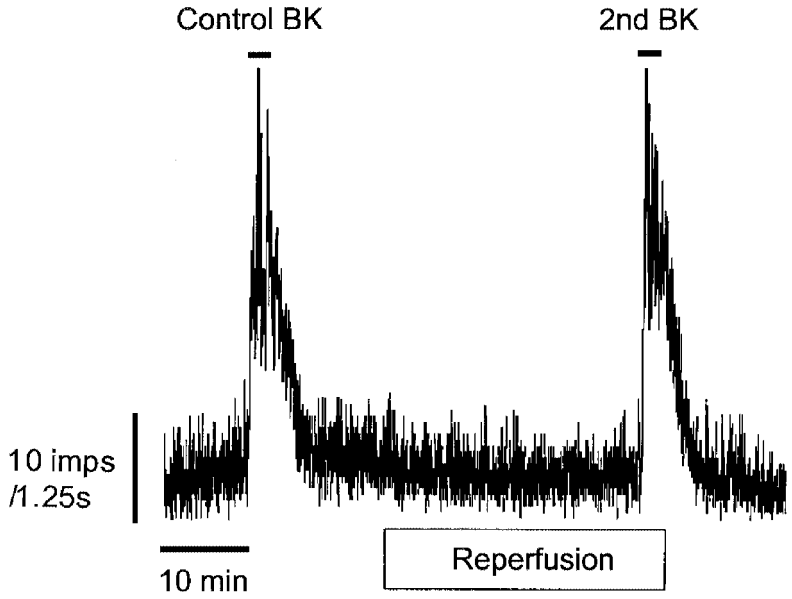

Figure 1. A representative trace of afferent discharge induced by 1 μM BK before and after a 30 min reperfusion period

A 'priming' challenge with 1 μM BK was first added (not shown) and followed after 15 min of stable discharge by a second application (Control BK). BK was added again 30 min later within a fixed volume of the reperfusing solution (2nd BK).

Table 1. Summary of the effects of chemical mediators on activation of afferent discharge and sensitization of responses to BK

	Basal discharge (%)	BK-induced discharge (%)
Time control ($n = 6$)	$-9{\cdot}8 \pm 3{\cdot}9$	$-7{\cdot}7 \pm 12$
PGE_2 ($n = 6$)	$-2{\cdot}4 \pm 4{\cdot}0$	$+117 \pm 48$
Histamine ($n = 4$)	$+29{\cdot}9 \pm 6{\cdot}1$	$+76{\cdot}4 \pm 25$
Adenosine ($n = 4$)	$+39{\cdot}3 \pm 4{\cdot}4$	$+88{\cdot}7 \pm 27$
5-HT ($n = 4$)	$+56{\cdot}2 \pm 18{\cdot}3$	$-13{\cdot}8 \pm 5{\cdot}3$
Dibutyryl cAMP ($n = 4$)	$-5{\cdot}1 \pm 3{\cdot}7$	$+123{\cdot}5 \pm 28$

Data are expressed as the percentage change (means ± S.E.M.) for comparison, while statistical analysis was performed on the raw data presented in the text.

of control ($n = 5$). During subsequent experimental protocols the effect of various mediators on baseline discharge and the magnitude of the response to BK were examined.

Baseline discharge

The effects of PGE_2 (1 μM), histamine (100 μM), adenosine (100 μM) and 5-HT (100 μM) on baseline discharge were examined in separate experiments and representative responses are illustrated in Fig. 2. Discharge was unaffected by PGE_2 ($10{\cdot}6 \pm 2{\cdot}4$ *vs.* $10{\cdot}5 \pm 2{\cdot}5$ impulses s^{-1}, $n = 6$, $P > 0{\cdot}05$). In contrast, histamine ($17{\cdot}7 \pm 2{\cdot}7$ *vs.* $23{\cdot}2 \pm 3{\cdot}9$ impulses s^{-1}, $n = 4$, $P < 0{\cdot}05$) and adenosine ($7{\cdot}1 \pm 1{\cdot}9$ *vs.* $10 \pm 2{\cdot}7$ impulses s^{-1}, $n = 4$, $P < 0{\cdot}05$) caused increases within 3 min, which were sustained throughout the reperfusion. A transient but significant increase in basal discharge was also observed with 5-HT ($10{\cdot}8 \pm 3{\cdot}2$ *vs.* $17{\cdot}4 \pm 1{\cdot}9$ impulses s^{-1}, $n = 4$, $P < 0{\cdot}05$). A summary of the effects of these agents on baseline discharge is shown in Table 1.

Sensitization of BK responses

The response to BK was significantly enhanced by PGE_2 (4179 ± 944 *vs.* 7882 ± 1356 impulses, $n = 6$, $P < 0{\cdot}05$). Similar augmentation of the BK response was observed with histamine (5051 ± 1357 *vs.* 8732 ± 2397 impulses, $n = 4$, $P < 0{\cdot}05$) and adenosine (2604 ± 704 *vs.* 4672 ± 949 impulses, $n = 4$, $P < 0{\cdot}05$). In contrast, 5-HT had no significant effect on BK-induced afferent discharge (5186 ± 933 *vs.* 4315 ± 514 impulses, $n = 4$, $P > 0{\cdot}05$). These data are expressed as percentage changes in Table 1 to emphasize the differential effects of these mediators on baseline discharge and response to BK. Mediators had no effect on the latency of onset of the response to BK (data not shown).

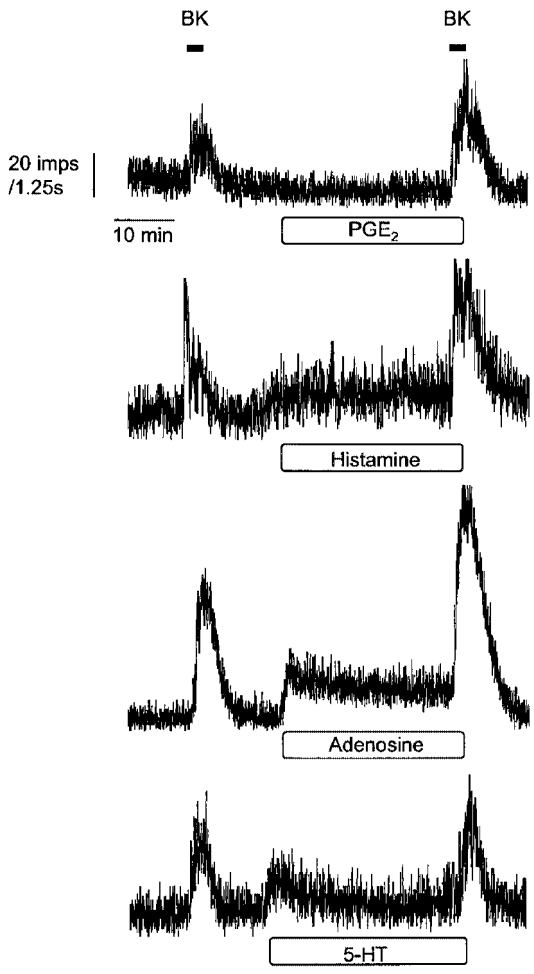

Figure 2. Chemical mediators have differential effects on afferent discharge and discharge induced by BK

Examples of the differential effects of 1 μM PGE_2, 100 μM histamine, 100 μM adenosine and 100 μM 5-HT on basal afferent discharge and afferent activity induced by BK. Mediators were present within the 30 min reperfusion period indicated by the horizontal box and were still present during the second application of BK.

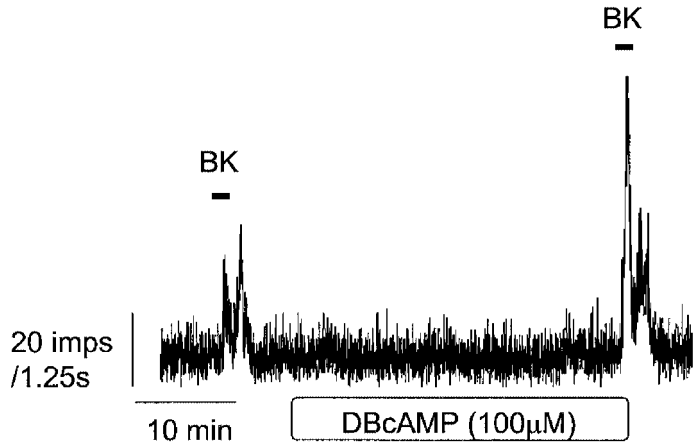

Figure 3. Example of the effects of reperfusion with dibutyryl cAMP (DBcAMP) on basal afferent discharge and afferent activity induced by BK

Since the effect of 5-HT on baseline discharge was only transient, it is possible that the lack of any sensitization of the response to BK could reflect receptor desensitization or reuptake/breakdown of the 5-HT within the 30 min reperfusion period. In a second set of experiments with 5-HT, BK was applied during an on-going response to 5-HT (added during the last 10 min of the reperfusion period). The response to BK was 83·5 ± 14 % of the control response ($n = 4$, $P > 0{\cdot}05$) and still comparable to that seen in the time control group.

Involvement of cAMP in afferent sensitivity

The cell-permeant form of cAMP (dibutyryl cAMP, 100 μM) was used to examine the effects of elevating this intracellular second messenger on the afferent responses to BK. Despite no effect on basal discharge (15·8 ± 4·2 *vs.* 15·1 ± 4·2 impulses s^{-1}, $n = 4$, $P > 0{\cdot}05$), dibutyryl cAMP significantly augmented responses to BK (2650 ± 755 *vs.* 5784 ± 1508 impulses, $n = 4$, $P < 0{\cdot}05$) without influencing the response latency (27·5 ± 2·9 *vs.* 27·5 ± 7 s, $n = 4$, $P > 0{\cdot}05$). The effects of dibutyryl cAMP are also summarized in Table 1 and a representative response is shown in Fig. 3.

Effects of naproxen

The involvement of prostanoid synthesis in mediator-induced sensitization of the BK response was investigated using the cyclo-oxygenase (Cox 1 and 2) inhibitor naproxen (10 μM). These studies were confined to the effects of histamine and adenosine since 5-HT did not cause sensitization and PGE_2 has already been shown to restore BK responses after naproxen (Maubach & Grundy, 1999). Naproxen (10 μM) had a negligible effect on baseline discharge (16·9 ± 1·6 *vs.* 15·1 ± 1·3 impulses s^{-1}) but caused a 49 ± 7 % reduction in the BK-induced discharge (4716 ± 661 *vs.* 2189 ± 346 impulses, $n = 12$, $P < 0{\cdot}01$) (Fig. 4). During the subsequent reperfusion period there was no change in basal discharge in time control

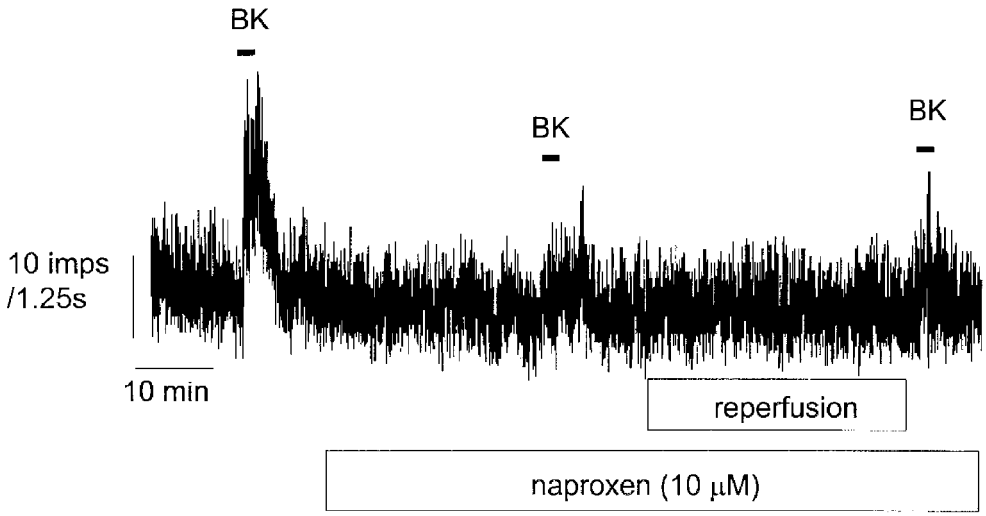

Figure 4. Illustration of the protocol used to investigate the effects of naproxen

Naproxen (10 μM) was added after an initial challenge with BK and was present throughout the experiment. After 30 min, BK was reapplied followed by another addition of BK after a 30 min reperfusion period where mediators could be tested. Note that the two responses to BK in the presence of naproxen before and after reperfusion were similar following this protocol.

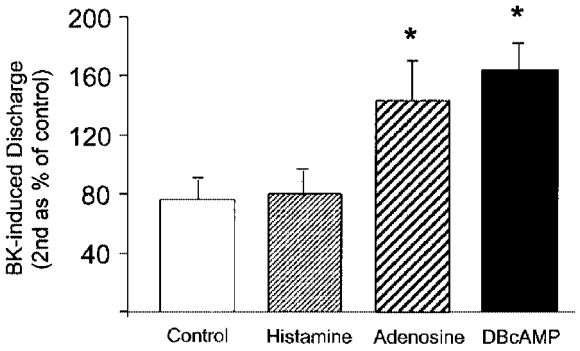

Figure 5. Afferent discharge induced by BK after histamine (100 μM), adenosine (100 μM) and dibutyryl cAMP (100 μM) in the presence of naproxen

Responses to BK are expressed as percentage of control response (means and S.E.M., $n = 4$–5) and were compared against the time control using Bonferroni's multiple comparison test on data normalized following a simple square root transformation (*$P < 0{\cdot}05$).

experiments ($10{\cdot}9 \pm 1{\cdot}2$ *vs.* $10{\cdot}5 \pm 0{\cdot}9$ impulses s^{-1}, $n = 4$, $P > 0{\cdot}05$). The increases in baseline firing by histamine and adenosine were absent after treatment with naproxen ($16{\cdot}9 \pm 3{\cdot}8$ *vs.* $17{\cdot}6 \pm 3{\cdot}9$ impulses s^{-1}, $n = 5$, $P > 0{\cdot}05$ and $13 \pm 2{\cdot}7$ *vs.* $14{\cdot}2 \pm 2{\cdot}6$ impulses s^{-1}, $n = 5$, $P > 0{\cdot}05$, before and after histamine and adenosine, respectively). Similarly there was no change in basal discharge following dibutyryl cAMP after treatment with naproxen ($9{\cdot}5 \pm 1{\cdot}6$ *vs.* $8{\cdot}2 \pm 1{\cdot}0$ impulses s^{-1}, $n = 4$, $P > 0{\cdot}05$).

The histamine-induced augmentation of the BK response was also absent in the presence of naproxen ($81 \pm 17\%$, $n = 5$, $P > 0{\cdot}05$) with a response comparable to that observed in time control experiments ($76 \pm 14\%$, $n = 4$). In contrast, adenosine still caused a significant increase ($156 \pm 25\%$, $n = 5$, $P < 0{\cdot}05$) in the presence of naproxen. In addition, the sensitizing effect of dibutyryl cAMP still occurred in the presence of naproxen ($167 \pm 13\%$ of control, $n = 4$, $P < 0{\cdot}05$). Sensitization induced by adenosine and dibutyryl cAMP, unlike histamine, is therefore independent of prostanoid formation (Fig. 5).

Effects of histamine antagonists

The effects of histamine on baseline discharge and augmentation of the BK response were compared in the presence of selective histamine H_1 (pyrilamine), H_2 (ranitidine) and H_3 (thioperamide) receptor antagonists, all at 10 μM. This concentration was chosen on the basis of effects of histamine antagonists (10–100 μM) on submucosal nerves in guinea-pig colon (Frieling *et al.* 1993). The increase in basal discharge induced by histamine was prevented by pretreatment with either pyrilamine or thioperamide but not ranitidine (Fig. 6*A*). In contrast, the augmentation of the BK response by histamine was unaffected by thioperamide but reduced by pyrilamine and ranitidine although only the latter reached significance (Fig. 6*B*).

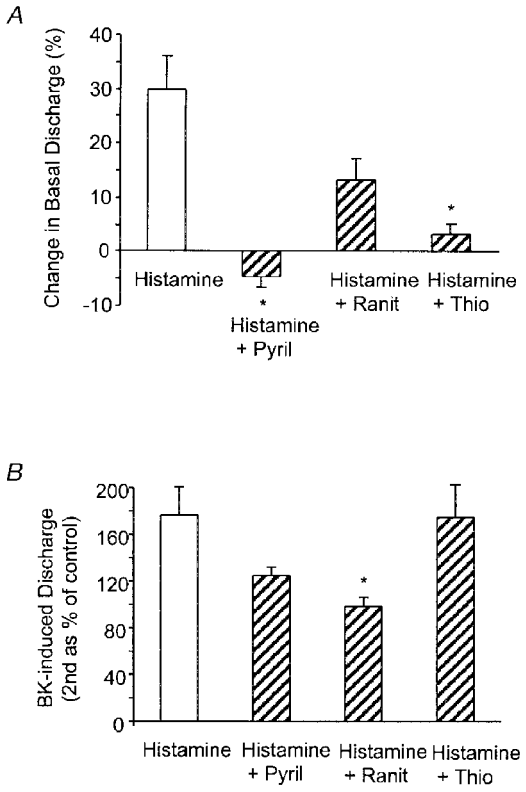

Figure 6. Effects of histamine antagonists

Effects of the histamine antagonists pyrilamine (Pyril), ranitidine (Ranit) and thioperamide (Thio) at 10 μM on the afferent responses to histamine (*A*) and BK-induced discharge following reperfusion with histamine (100 μM; *B*). Data are presented as means and S.E.M. ($n = 4$–6) and were compared statistically against control (Histamine) using Dunn's method for multiple comparison (*$P < 0{\cdot}05$).

Effects of adenosine antagonists

The effect of adenosine on baseline discharge and the augmented BK response was examined in the presence of antagonists of adenosine A_1 and A_{2A} receptors, namely DPCPX and ZM241385, respectively, at 10 μM and the A_3 receptor agonist IB-MECA at 1 μM. The increase in basal discharge induced by adenosine was clearly abolished by DPCPX but not by ZM241385 (Fig. 7*A*). In contrast, neither antagonist influenced the adenosine-induced augmentation of afferent responses to BK (Fig. 7*B*). The A_3 receptor agonist IB-MECA had no effect on basal discharge ($11{\cdot}8 \pm 3{\cdot}4$ *vs.* $12{\cdot}1 \pm 3{\cdot}3$ impulses s^{-1}, $n = 3$, $P > 0{\cdot}05$, paired *t* test) and while BK-induced discharge was augmented (174 ± 4%), this was similar to vehicle controls (DMSO 0·1% v/v, 162 ± 24%).

DISCUSSION

This study extends our earlier observations that the algesic peptide BK activates mesenteric afferents from rat jejunum *in vitro* via B_2 receptors and interacts with prostanoids at the level of the afferent nerve terminal (Maubach & Grundy, 1999). Thus the response to BK was attenuated by cyclo-oxygenase inhibitors such as naproxen, an effect that could be reversed upon addition of PGE_2 (Maubach & Grundy, 1999). The present study demonstrates that mesenteric afferents are also influenced by other inflammatory mediators, including histamine, adenosine and 5-HT. However, unlike PGE_2, which augments the response to BK without affecting baseline afferent discharge, these three agents activated afferent activity in the absence of BK. Moreover, with the exception of 5-HT, they also enhanced the afferent response to BK. Thus inflammatory agents can be divided into those which stimulate afferents, those which sensitize the response to BK and those which can both stimulate and sensitize. Clearly, excitation and sensitization of sensory nerves may be mediated by separate mechanisms. In addition, mediators could enhance the BK response by increasing the sensitivity of active fibres or possibly by causing the recruitment of previously 'silent' afferents. Clearly, single unit analysis is required to investigate this further. Given that these mesenteric afferents could be nociceptive (spinal origin) and that these chemical mediators can be released in pathological states, the afferent responses described in the present study could provide the basis for peripheral sensitization leading to the production of pain in inflamed, damaged or ischaemic intestine.

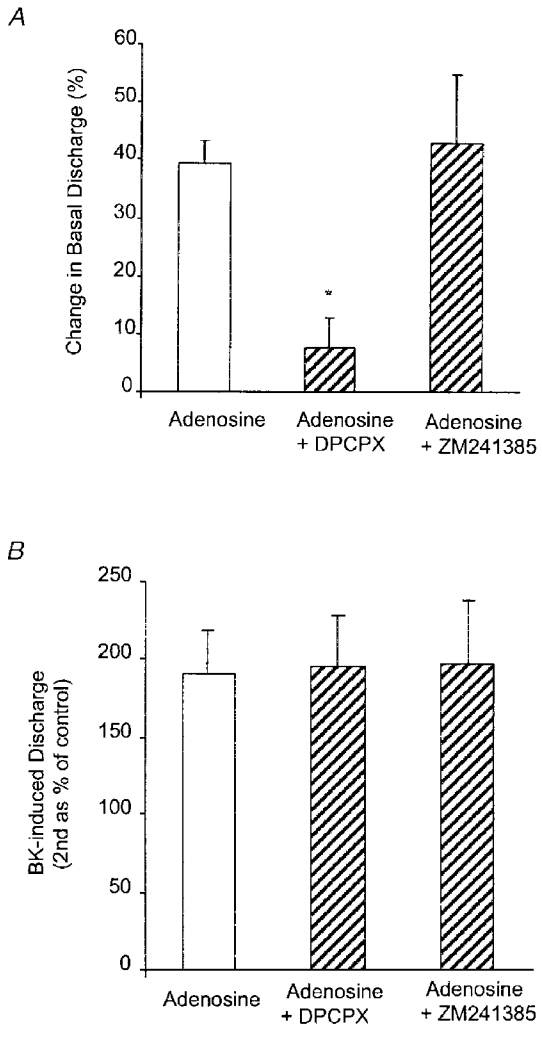

Figure 7. Effects of adenosine antagonists

Effects of the adenosine A_1 antagonist DPCPX and the A_{2A} antagonist ZM241385 at 10 μM on the afferent discharge induced by adenosine (*A*) and BK-induced discharge following reperfusion with adenosine (100 μM; *B*). Data are presented as means and S.E.M. ($n = 4$–6) and were statistically compared against control (Adenosine) using Dunn's method for multiple comparison (*$P < 0{\cdot}05$).

Effects of PGE_2

The effects of PGE_2 were tested since this is the predominant prostanoid found in inflamed tissues. Prostaglandins are known to increase the sensitivity of cutaneous sensory nerves to chemical mediators of inflammation and pain including BK (see Handwerker & Reeh, 1991). The mechanism of sensitization involved in these present studies was independent of activation of basal discharge. This may be similar to that seen in embryonic rat sensory neurones in culture where PGE_2 brought about a 3-fold increase in the number of action potentials evoked by BK without altering resting membrane potential (Nicol & Cui, 1994). In contrast, however, prostaglandins are themselves powerful stimuli of mesenteric afferents *in vivo* (Mense, 1981; Longhurst & Dittman, 1987), although *in vivo* there is considerable potential for multiple mechanisms, both direct and indirect, to influence afferent firing. Indeed, recent evidence (Haupt *et al.* 1999) suggests that PGE_2 activates mesenteric afferents *in vivo* by a direct action on mucosal afferents and indirectly following an increase in intraluminal pressure (ILP). This may explain the lack of effect of PGE_2 on serosal afferent discharge *in vitro*.

PGE_2 produces many of its biological effects through the generation of cAMP (e.g. Wellman & Schwabe, 1973). Raised intracellular cAMP, following stimulation by PGE_2, may be involved in sensitizing these mesenteric nerves to BK since the effect of PGE_2 was mimicked by the addition of dibutyryl cAMP. cAMP has also been implicated in a mechanism for hyperalgesia produced by prostaglandins in rat paw (Ferreira & Nakamura, 1979) and enhanced responses to BK (Cui & Nicol, 1995) and capsaicin (Pitchford & Levine, 1991) in sensory nerve cultures. The lack of effect of PGE_2 on baseline afferent activation suggests that baseline firing is independent of cAMP since directly raising cAMP with dibutyryl cAMP also failed to affect afferent discharge.

Effects of histamine

Histamine is an important mediator in gastrointestinal hypersensitivity reactions (Perdue *et al.* 1984), and can be released from mast cells which are in turn closely associated with afferent nerves in the gastrointestinal tract (Williams *et al.* 1995). In the present study histamine both increased basal discharge and sensitized the afferent response to BK. Inhibition of histamine-induced discharge by pyrilamine and thioperamide, but not by ranitidine, would implicate H_1 and H_3 receptors in the response. While we are cautious about this interpretation in the absence of concentration–response data it appears that H_2 receptors are unlikely to mediate afferent activation by histamine. This contrasts with effects of histamine on intrinsic submucosal nerves in guinea-pig colon where both postsynaptic H_2 and presynaptic H_3 receptors are involved (Frieling *et al.* 1993). This may reflect the differential expression of receptors on intrinsic and extrinsic nerves. However, in the small intestine of the cat *in vivo*, histamine-induced afferent discharge was attenuated by the H_2 antagonist cimetidine (Akoev *et al.* 1996), although this response may have been secondary to motor responses triggered by high concentrations of histamine. Other studies have implicated the involvement of H_1 receptors in mediating the actions of endogenous histamine on ischaemically sensitive visceral afferents in the cat (Fu *et al.* 1997) and the activation of mesenteric afferents in the rat (Kreis *et al.* 1998). In contrast to the effect on baseline discharge, the H_2 antagonist ranitidine significantly inhibited the ability of histamine to sensitize afferents to BK. However, the actions of histamine may involve indirect effects on afferents through the release of other mediators of sensitization. For example, histamine can generate prostaglandins in the gastrointestinal tract and these in turn mediate histamine-induced Cl^- secretion in rat small intestine (Hardcastle & Hardcastle, 1987). Indeed, the sensitization of BK-induced afferent discharge by histamine seen in the present study was inhibited by naproxen. Thus, sensitization of mesenteric afferents by histamine may occur indirectly following prostaglandin release which in turn raises intracellular cAMP.

Effects of adenosine

Adenosine can be generated in large amounts in hypoxic and ischaemic tissue (Edlund & Sollevi, 1993), activates unmyelinated afferents (Cherniak *et al.* 1987) and causes pain in human blister base studies (Bleehen & Keele, 1977). Adenosine is also a potential sensitizing mediator since it is implicated in mucosal inflammation (Pratt *et al.* 1986). Recent work has shown clear activation by adenosine of mesenteric afferents in rat intestine *in vivo* via an action at both A_1 and A_{2B}-like receptors with no apparent contribution from A_{2A} and A_3 receptors (Kirkup *et al.* 1998). In the present *in vitro* study adenosine was seen to both stimulate and sensitize serosal afferents. The stimulation was abolished by the A_1 receptor antagonist DPCPX but not by an equal concentration of the A_{2A} antagonist ZM241385. Since these antagonists have very similar potencies at A_1 and A_{2A} receptors (DPCPX, −log of antagonist concentration producing a 2-fold rightward shift of the dose–response curve (pA_2) = 8·5; and ZM241385, $pA_2 = 9$, respectively), and ZM241385 can maintain selectivity even at 10 μM (Poucher *et al.* 1995), these results suggest the involvement of A_1 receptors in the stimulation of mesenteric afferents. While these studies cannot exclude the involvement of A_{2B} receptors, A_3 receptors are unlikely to mediate the actions of adenosine since the A_3 receptor agonist IB-MECA had no effect on basal discharge. The concentration of IB-MECA used (1 μM) is not excessive and should have been adequate to activate any A_3 receptors in our preparation since this compound relaxes mesenteric artery with an EC_{50} of approximately 4 μM (Prentice *et al.* 1997).

In contrast to the clear inhibition of adenosine-induced afferent discharge by DPCPX, this A_1 receptor antagonist had no effect on the ability of adenosine to sensitize afferents to BK. Sensitization was also unaffected by the A_{2A} antagonist (ZM241385). It seems unlikely therefore that

A_1 receptors mediate adenosine-induced sensitization of mesenteric afferents to BK. Again, this supports the idea that afferent sensitization and stimulation are mediated by separate mechanisms. The A_3 receptor agonist IB-MECA did not sensitize afferents to BK and while we must be cautious in excluding the involvement of A_3 receptors on this basis, we speculate that the A_{2B} receptor remains a likely candidate for mediating afferent sensitization by adenosine. Furthermore, adenosine A_{2B} receptors, unlike A_1 and A_3 receptors, are positively coupled to adenylate cyclase (Snyder, 1985). In addition, adenosine stimulates Cl^- secretion in the T84 colonic cell line by an A_{2B}-mediated rise in intracellular cAMP (Strohmeier *et al.* 1995). Thus, adenosine-induced sensitization of BK responses may be mediated by a rise in intracellular cAMP, possibly generated following activation of adenosine A_{2B} receptors. Adenosine may therefore act 'directly' at the afferent terminals. However, while sensitization by adenosine is independent of prostanoids, we cannot exclude possible interactions of adenosine with non-neural cells such as mast cells or even blood vessels within the mesentery.

5-HT

Serotonin (5-HT), while involved in many gastrointestinal functions, is also implicated in the regulation of nociception. Activation of 5-HT_1 (Sufka *et al.* 1992), 5-HT_2 (Grubb *et al.* 1988; Abbott *et al.* 1996) and 5-HT_3 receptors (Eschalier *et al.* 1989) has been associated with pain and hyperalgesia. The novel 5-HT_3 receptor antagonist alosetron attenuates c-fos expression in the spinal cord following noxious levels of colonic distension in the rat (Kozlowski *et al.* 1999). In the present study, 5-HT caused transient increases in basal afferent discharge but did not sensitize these afferents to BK. This contrasts with sensitization of BK responses by 5-HT in cutaneous preparations *in vitro* (Lang *et al.* 1990) and the ability of 5-HT to reduce mechanical nociceptive thresholds in rat hindpaw (Taiwo & Levine, 1992). This discrepancy could be due to other secondary effects of 5-HT or simply to differences in receptor subtypes present on cutaneous and mesenteric afferents. We have yet to identify the 5-HT receptor responsible for activation of these serosal afferents. While 5-HT_3 receptors mediate a direct action of 5-HT on vagal mucosal afferents in rat jejunum (Hillsley & Grundy, 1998), spinal fibres could also possess 5-HT_3 receptors (Fu & Longhurst, 1998). The 5-HT_3 receptor is linked to a ligand-gated cation channel (Hoyer *et al.* 1993) and as such is unlikely to sensitize the nerve ending to BK, which appears to rely on adenylate cyclase.

Role for cAMP in a mechanism of afferent sensitization to BK

Our present findings implicate a role for cAMP in a mechanism of afferent sensitization to BK. While there is a great deal of evidence already implicating cAMP/cAMP-dependent protein kinase in afferent sensitization and hyperalgesia in cutaneous systems (see Heller *et al.* 1993), our results now support a similar role for cAMP in visceral afferents from the gastrointestinal tract. In general, neuronal excitability can be influenced by changes in intracellular cAMP following the phosphorylation of membrane ion channels by cAMP-dependent protein kinase. Indeed, hyperalgesia induced by forskolin (an activator of adenylate cyclase) in rat hindpaw is antagonized by an inhibitor of cAMP-dependent protein kinase (Taiwo & Levine, 1991). However, the precise actions further downstream are not fully understood. cAMP has a variety of actions on sensory nerves depending on the preparation used. In rat embryonic dorsal root ganglion (DRG) cells, PGE_2 (which acts through cAMP) increased the number of action potentials induced by BK without altering resting membrane potential (Nicol & Cui, 1994). Thus, cAMP may act on ion channels that do not normally contribute to resting potential. Indeed, cAMP has also been implicated in prolonging Ca^{2+}-dependent action potentials in cultured mouse DRG cells (Grega & Macdonald, 1987) through inhibition of voltage-dependent K^+ channels. Changes in repolarization could therefore affect sensitivity. Other studies where prostaglandins influence Ca^{2+} currents suggest that cAMP could alter excitability through changes in Ca^{2+} conductance. However, augmentation of BK-induced peptide release by PGE_2 is unaffected by L-, N- or P-type Ca^{2+} channel blockade in rat DRG cells (Evans *et al.* 1996). Finally, cAMP could influence receptor expression, as demonstrated in rat mesangial cells where BK receptors are upregulated by cAMP (Castano *et al.* 1998).

Conclusions

The present investigation demonstrates that certain potential inflammatory mediators can sensitize serosal afferents to BK in rat jejunum *in vitro*. Sensitization was independent of activation of basal discharge and could be mediated by cAMP. Agents such as PGE_2 and adenosine may act 'directly' while histamine acts indirectly through the generation of prostanoids which in turn can generate cAMP. Since these afferents are spinal in origin and readily activated by BK, they may be implicated in signalling nociceptive stimuli from the gastrointestinal tract. While afferent hypersensitivity is known to underlie conditions of hyperalgesia in many cutaneous models, analogous mechanisms could also operate in visceral organs. Such sensitization, which could occur in the presence of elevated levels of inflammatory mediators or products of ischaemia, may eventually lead to an altered perception of physiological stimuli and possibly the type of symptom commonly associated with both organic and functional bowel disorders.

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