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Inhibition of rat colon contractility by prostacyclin (IP-) receptor agonists: involvement of NANC neurotransmission

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1 The possibility that prostacyclin (IP-) receptor agonists inhibit spontaneous contractions of the rat isolated colon by activating enteric neurones has been investigated. Cicaprost was used as the test agonist because of its high stability, selectivity and potency (IC_{50} = 3.8 nM).

2 The Na^+ channel blockers saxitoxin (STX, 1 nM) and tetrodotoxin (TTX, 1 μM), whilst having little effect on resting spontaneous activity, virtually abolished the inhibitory actions of cicaprost (10 nM) and nicotine (3 μM); inhibitory responses to isoprenaline (20 nM) were not affected. Phentolamine (1 μM), propranolol (1 μM) and atropine (1 μM) had no effect on cicaprost inhibition. These data are compatible with release of inhibitory NANC transmitter(s) by cicaprost.

3 A transmitter role for nitric oxide was investigated. The nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) inhibited the actions of both cicaprost (10 nM) and nicotine (3 μM) by 50–60%, but did not affect responses to isoprenaline (20 nM) or sodium nitroprusside (1–5 μM). The enantiomeric D-NAME (100 μM), which has negligible NOS inhibitory activity, had no effect on the action of cicaprost.

4 The involvement of purinergic transmitters was also investigated. Desensitization to the inhibitory action of ATP did not affect cicaprost responses. The P_{2X}/P_{2Y}-receptor antagonist, suramin, at 300 μM blocked ATP responses, but not those due to adenosine; it did not affect cicaprost inhibition. The selective adenosine A_1-receptor antagonist, DPCPX, used at a sufficiently high concentration (5 μM) to block adenosine A_2-receptors, did not affect cicaprost inhibition. Apamin (25 nM), a blocker of calcium-activated K^+ channels on smooth muscle, abolished or markedly reduced the inhibitory actions of ATP and adenosine, and partially inhibited cicaprost and nicotine responses. The combination of L-NAME (100 μM) and apamin (25 nM) abolished cicaprost and nicotine responses.

5 Investigation of vasoactive intestinal peptide (VIP) as a potential transmitter showed that its inhibitory action on the colon (IC_{50} = 50 nM) was partially inhibited by TTX (1 μM). α-Chymotrypsin abolished the effect of VIP but had no effect on cicaprost inhibition. Attempts to inhibit VIP responses using peptide antagonists and by agonist desensitization were unsuccessful.

6 KCl (40 mM) contracted the colon and abolished spontaneous activity. Under these conditions, isoprenaline, sodium nitroprusside and ATP induced relaxation, whereas cicaprost (10–310 nM) had no effect. Cicaprost inhibited both the tone and the spontaneous activity induced by the EP_1/EP_3-receptor agonist, sulprostone (8.6 nM) but not when either TTX (1 μM) or KCl (40 mM) was also present. On KCl-treated preparations, the prostacyclin analogue, iloprost (10–500 nM), induced contraction, presumably due to activation of EP-receptors.

7 It is concluded that IP-receptor agonists inhibit the contractility of rat colon by stimulating the release of at least two transmitters from NANC enteric neurones. Nitric oxide appears to be one of the transmitters. The second transmitter mechanism is apamin-sensitive; the experimental results do not support ATP, adenosine or VIP as transmitter candidates. However, further studies using more potent and selective receptor antagonists are required.

Keywords: Prostacyclin receptor agonist; cicaprost; colonic smooth muscle; enteric neurones; tetrodotoxin; nitric oxide; ATP; purinoceptor antagonists; adenosine receptor antagonists; vasoactive intestinal peptide

Introduction

Prostacyclin inhibits the spontaneous activity and tone of the rat isolated colon, whereas other products of prostaglandin H_2 (PGH_2) metabolism, such as PGE_2 and $PGF_{2\alpha}$, are contractile; this distinction was particularly useful in the early identification of prostacyclin by superfusion bioassay (Gryglewski *et al.*, 1976). The rat colon was also included in our investigations of the agonist specificity of some of the first stable analogues of prostacyclin (Dong *et al.*, 1986). We showed that iloprost and isocarbacyclin activate both prostacyclin (IP-) and prostaglandin E (EP-) receptors to produce a combination of inhibitory and excitatory actions on the colon (see Watson & Girdlestone, 1994, for nomenclature of prostanoid receptors). On the other hand, cicaprost (Stürzebecher *et al.*, 1986) invariably inhibited the spontaneous activity of the colon and, using data from other smooth muscle preparations, we proposed it as a highly specific IP-receptor agonist (Dong *et al.*, 1986; Lawrence *et al.*, 1992).

IP-receptors on blood platelets can be activated by a range of diphenylalkanoic acids (e.g. EP 157, octimibate), which bear little structural relationship to prostacyclin (see Jones *et al.*, 1993 and Meanwell *et al.*, 1994). In our studies of some of the more lipophilic compounds on the rat colon, inconsistent inhibition of contractility was observed. This appeared to be related to the presence of ethanol, which was essential to the preparation of homogeneous stock solutions. We therefore investigated whether a more robust assay would be obtained if the IP-receptor agonists acted against tone induced by an exogenous agent. To our surprise, we found that cicaprost did not relax colon preparations contracted submaximally with KCl. Further investigations showed that the Na^+ channel blocker, tetrodotoxin (TTX), could abolish the inhibitory action of cicaprost but not that of

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isoprenaline. A neuronal site of action for cicaprost within the colon seemed possible; this paper describes experiments to define the nature of this action.

Methods

Isolated colon preparations

Male Sprague-Dawley rats, weighing 250–300 g, were fasted overnight and killed by stunning and exsanguination. A 4 cm length of the ascending colon was removed and cleaned of mesenteric tissue. Two segments were cut and, without further dissection, were suspended in 10 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.0, KH_2PO_4 1.18, $NaHCO_3$ 25 and glucose 10. The bathing solution was gassed with 95% O_2/5% CO_2, maintained at 37°C, and contained 1 μM indomethacin. Washout of the organ bath was by upward displacement and overflow. Contractions of the longitudinal muscle were measured with Grass FT03 isometric transducers connected to a MacLab data acquisition system (ADInstruments Pty Ltd, Australia). An initial basal tension of 0.5 g was applied; on relaxation further tension was repeatedly applied until the basal tension remained steady at 0.3 g.

Experimental protocols

In all experiments, proximal and distal sections of colon from the same rat were used as matched preparations. After about 60 min equilibration, with frequent washing, several submaximal doses of the appropriate inhibitory agonist were tested on each preparation to ensure that a stable and acceptable level of sensitivity had been reached before the following experimental procedures were begun. Agonist doses were allowed 3–4 min contact with the preparations; blockers/inhibitors were added 10 or 15 min before the agonist dose(s).

Effects of cicaprost and isoprenaline on spontaneous activity and KCl- and sulprostone-induced tone Spontaneous activity: non-cumulative dose-response relationships for cicaprost and isoprenaline were obtained on proximal and distal preparations respectively from 3 rats, and also on distal and proximal preparations respectively from 3 different rats (data combined and quoted as $n = 6$ in Results); this procedure was repeated with cumulative dosing for cicaprost and isoprenaline on KCl-induced tone (6 different rats), and for cicaprost on sulprostone- and KCl/sulprostone-induced tone (6 different rats).

Effects of blockers/inhibitors on responses to single doses of inhibitory agonist Responses to single doses of both cicaprost and isoprenaline were obtained on both proximal and distal preparations from 3 rats, before and after treatment(s) with blocker/inhibitor (sequentially increasing concentrations) ($n = 6$ in Results). In the case of N^{ω}-nitro-L-arginine methyl ester (L-NAME) and/or apamin on nicotine responses, either the distal or proximal preparation was always used as a non-treated preparation to assess the extent of agonist desensitization; during the first treatment period, either L-NAME or apamin was present ($n = 3$), and during the second period, a combination of L–NAME and apamin ($n = 6$).

Effects of blockers/inhibitors on purinoceptor agonists Cumulative dose-response relationships were obtained for a purinoceptor agonist on both proximal and distal preparations from 2 rats. One preparation was used as control and other treated with antagonist/inhibitor/desensitizing agent; second agonist dose-response relationships were then established. This procedure was repeated using 2 different rats with exchange of control and treatment on the proximal and distal preparations ($n = 4$ in Results).

Data analysis and statistical tests

Using the MacLab Chart 3.3 programme, spontaneous activity was measured as the average contractile force over a 2 min period (480 data points). The baseline tension value was set by eye. The response to an inhibitory agonist was taken as the percentage change from the resting spontaneous activity (e.g. −100% corresponds to abolition of spontaneous activity). Using data from a number of experiments, mean agonist responses elicited during control and treatment (receptor antagonist/channel blocker/enzyme inhibitor) periods were compared by Student's unpaired t test; statistical significance was accepted when $P < 0.05$.

Drugs

Cicaprost, iloprost and sulprostone were gifts from Schering AG, Berlin, Germany; ethanolic stock solutions of 5 mg ml^{-1} were kept at −20°C and diluted with 0.9% w/v NaCl (saline) solution for use. Indomethacin, isoprenaline (racemic), phenylephrine, tetrodotoxin (TTX), vasoactive intestinal peptide (VIP), [D-*p*-chloro-Phe^6,Leu^{17}]-VIP, [Lys^1,$Pro^{2,5}$,$Arg^{3,4}$, Tyr^6]-VIP, N^{ω}-nitro-L-arginine methyl ester (L-NAME), D-NAME, ATP, and adenosine were purchased from the Sigma Chemical Company, U.S.A.; saxitoxin (STX) from Calbiochem-Novabiochem Corp., U.S.A.; 2-methylthio ATP, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and apamin from Research Biochemicals International, U.S.A., and suramin from Biomol Research Laboratories, U.S.A. A stock solution of TTX (500 μM) was prepared in saline containing 0.01% acetic acid and diluted with saline. VIP, the VIP analogues, L-NAME and D-NAME were dissolved in distilled water and diluted with saline before use. Stock solutions of purines were prepared in distilled water each day.

Results

In virtually all cases, responses of the proximal and distal sections of the rat colon to the drugs investigated were similar in magnitude, and it was decided to combine the data for graphical and statistical analyses.

Log concentration-response curves for inhibition of the spontaneous activity of the rat colon by cicaprost ($IC_{50} = 3.8$ nM) and isoprenaline (12.5 nM) are shown in Figure 1. In subsequent experiments, the effects of antagonists and inhibitors were assessed against submaximal responses elicited by standard concentrations of 10 nM cicaprost and 20 nM isoprenaline; these responses remained constant or in the case of cicaprost sometimes slightly increased in size (5–10%) over a period of 2–3 h. Nicotine also inhibited the spontaneous activity of the colon ($IC_{50} = 1–2$ μM), but was prone to tachyphylaxis. In subsequent studies, the reduction in response to a standard concentration of 3 μM nicotine applied every 20 min was small enough to allow the testing of inhibitory agents. Deliberate desensitization to nicotine, with three or four 10 μM challenges at 15 min intervals, had no effect on cicaprost inhibition (Figure 3c).

Evidence that cicaprost activates NANC enteric neurones

The Na^+ channel blockers, saxitoxin (STX, 0.1 and 1 nM) and TTX (0.1 and 1 μM), slightly slowed the rate of spontaneous contractions and tended to make the contraction pattern more regular. TTX (1 μM) markedly reduced and in some cases abolished cicaprost responses (Figures 2a and 3a), always abolished nicotine responses (not shown), but had no

effect on isoprenaline responses (Figure 3a). Cicaprost responses (−76.8 ± 2.4%, $n = 5$) were inhibited by STX at 0.1 nM (−57.3 ± 8.9%, $P > 0.05$) and 1 nM (−4.8 ± 1.6%, $P < 0.001$).

Phentolamine at 1 μM did not significantly affect the spontaneous activity of the colon, although in some preparations the basal tension was slightly decreased. Inhibitory responses (−70 to −80%) to phenylephrine (3 μM) were abolished by 1 μM phentolamine, whereas cicaprost and isoprenaline responses were unaffected (Figure 3b). Propranolol at a concentration of 1 μM (which had no significant effect on spontaneous activity or basal tone) markedly inhibited isoprenaline responses, but had no effect on cicaprost responses (Figure 3b). The response of the colon preparation to atropine was complex. Following exposure to 50 nM atropine, there was a small reduction in basal tension and spontaneous activity was reduced by about 20%. Subsequent doses of atropine (0.1–1 μM) produced no further effects. If however a preparation was initially exposed to 1 μM atropine, resting tension was markedly reduced and spontaneous activity abolished for several minutes; these parameters recovered to 60–70% of control values over a period of 20 min. After washout of 1 μM atropine from the organ bath, TTX (1 μM) did not slow the rate, but slightly increased the amplitude of spontaneous contractions. The presence of atropine (1 μM) did not significantly affect responses to either cicaprost or isoprenaline (Figure 3b).

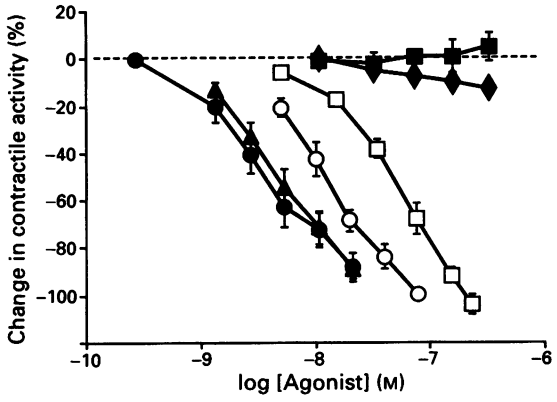

Figure 1 Log concentration-inhibition curves on rat isolated colon. Effects of cicaprost and isoprenaline on spontaneous activity (●, ○) and stable tone induced by 40 mM KCl (■, □), and cicaprost on contractile activity induced by 8.6 nM sulprostone (▲) and 40 mM KCl/8.6 nM sulprostone (◆) are shown. Values are means ± s.e.mean ($n = 6$).

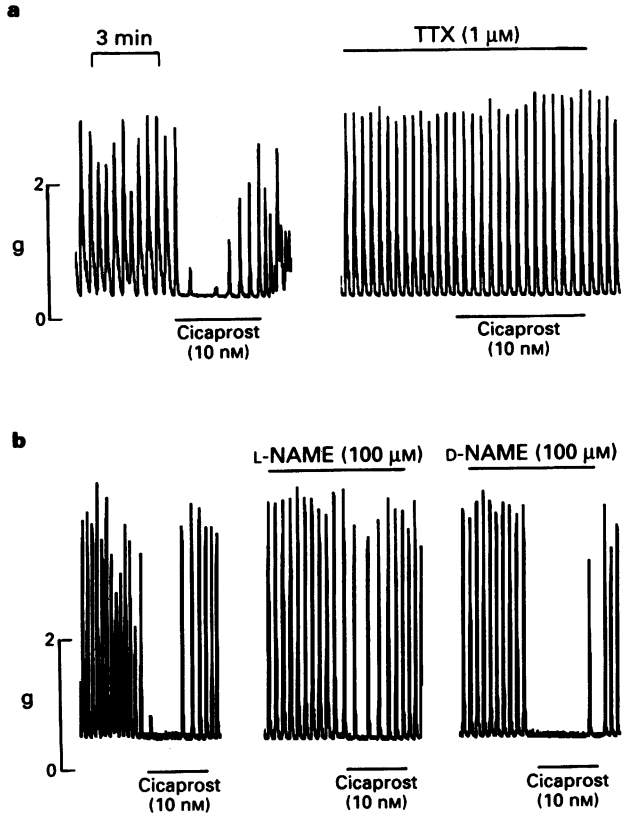

Figure 2 Rat isolated colon: experimental records showing the effect of treatment with (a) tetrodotoxin (TTX, 1 μM) and (b) N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) and D-NAME (100 μM) on inhibitory responses to 10 nM cicaprost.

Effects of nitric oxide synthase inhibition

The nitric oxide synthase (NOS) inhibitor, L-NAME (10–500 μM), produced transient small contractions of the colon preparations. Cicaprost and nicotine responses were partially inhibited by L-NAME, whereas the actions of isoprenaline were unaffected (Figure 2b and 4a,d). The enantiomeric D-NAME, which is inactive as a NOS inhibitor, did not affect the action of cicaprost (control −77.6 ± 2.2%; 100 μM D-NAME treatment −78.7 ± 2.7%, $n = 6$). Sodium nitroprusside, a NO donor, inhibited the spontaneous activity of the colon preparation ($IC_{50} = 0.15$ μM; complete inhibition at 5 μM). Nitroprusside responses were unaffected by either TTX (1 μM) or L-NAME (100 μM).

Interference with postsynaptic purinergic systems

The effects of several treatments intended to inhibit postsynaptic purinergic systems were investigated on inhibitory responses to cicaprost, nicotine, ATP and adenosine.

Log concentration-response curves for ATP derived from two cumulative sequences of doses 80 min apart were identical (Figure 5a); the log concentration-response curve for 2-methylthio-ATP, a specific P_{2Y} agonist, is also shown. Desensitization to ATP was attempted by challenging with three doses of ATP (500 μM) added at 15 min intervals in between the first and second series of ATP doses. Examination of the second ATP curve shows that this procedure was most effective against 10 and 100 μM as opposed to 500 μM ATP (Figure 5a); cicaprost responses were unaffected by this treatment (Figure 5d).

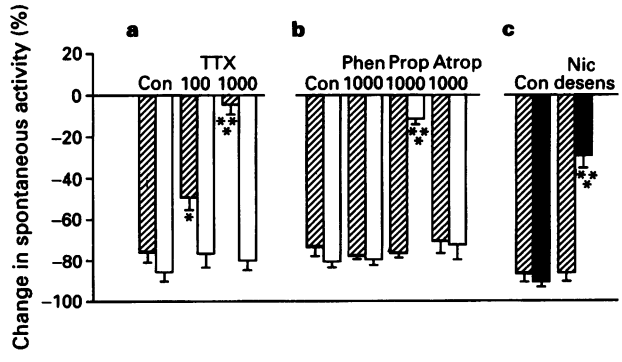

Figure 3 Spontaneous activity of rat isolated colon. Effects of treatment with (a) tetrodotoxin (TTX) and (b) phentolamine (Phen), propranolol (Prop) or atropine (Atrop) on inhibitory responses to cicaprost (10 nM, hatched columns) and isoprenaline (20 nM, open columns). Concentrations are nM. Means ± s.e.mean ($n = 6$). (c) Effects of desensitization with 3–4 applications of 10 μM nicotine (Nic desens) on inhibitory responses to cicaprost (10 nM, hatched columns) and nicotine (10 μM, solid columns) ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to appropriate control (Con).

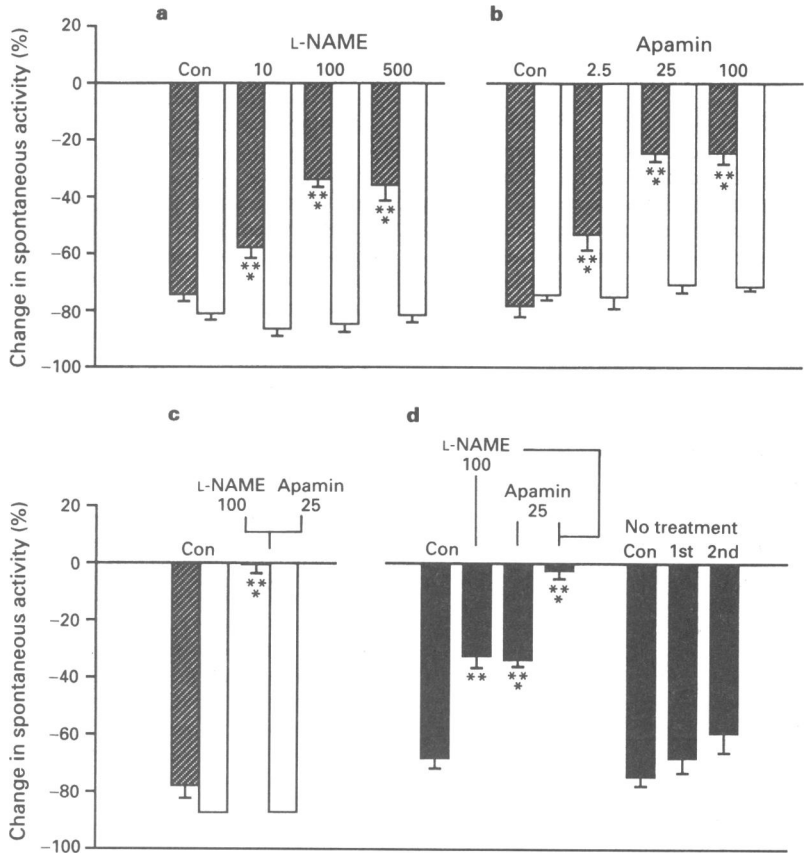

Figure 4 Spontaneous activity of rat isolated colon. Effects of treatment with (a) N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10–500 μM), (b) apamin (2.5–100 nM) and (c) a combination of 100 μM L-NAME and 25 nM apamin on inhibitory responses to cicaprost (10 nM, hatched columns) and isoprenaline (20 nM, open columns). Means ± s.e.mean of 6 experiments, except for isoprenaline in (c), where $n = 2$. (d) Effects of 100 μM L-NAME alone and 25 nM apamin alone (both 1st treatment period, $n = 3$), and in combination (2nd treatment period, $n = 6$) on inhibitory responses to nicotine (3 μM, solid columns); the right hand part of the panel shows nicotine responses on non-treated preparations during control, 1st and 2nd treatment periods ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to appropriate control (Con).

At 300 μM, the P_{2X}/P_{2Y}-receptor antagonist, suramin, produced a rightward shift of the ATP curve for responses within the 0–50% inhibition range (dose ratio = 10–20) (Figure 5b). However, no inhibition was seen when the ATP concentration was raised to 500 μM. Suramin did not inhibit adenosine responses (Figure 5c). DPCPX, a potent antagonist at adenosine A_1-receptors, was used at high concentrations of 5 μM in an attempt to block adenosine A_2-receptors in the colon. The success of this strategy is indicated by marked inhibition of adenosine responses without significant inhibition of ATP responses (Figure 5b,c). Neither suramin (300 μM) nor DPCPX (5 μM) had any effect on cicaprost responses (Figure 5d).

Apamin (25 nM) markedly inhibited responses to ATP and adenosine (Figure 5b,c), and partially inhibited responses to both cicaprost and nicotine, while not affecting those to isoprenaline (Figure 4b,d). Cicaprost and nicotine induced no obvious change in spontaneous activity ($< \pm 5\%$ and $< \pm 10\%$ respectively) or basal tension in the presence of 100 μM L-NAME and 25 nM apamin; isoprenaline inhibition was unaffected by this combination (Figure 4c,d).

Interference with the action of vasoactive intestinal peptide

VIP inhibited the spontaneous activity of the colon with an IC_{50} value of about 50 nM. On five preparations, 100 nM VIP produced 92 ± 2% inhibition, which was reduced to 33 ± 4% in the presence of 1 μM TTX ($P < 0.01$).

Two VIP analogues, [Lys^1,$Pro^{2,5}$,$Arg^{3,4}$,Tyr^6]-VIP and [D-*p*-chloro-Phe^6,Leu^{17}]-VIP were examined as potential antagonists of VIP action. The former at 1 μM transiently contracted the colon; subsequent submaximal responses to either VIP (100 nM) or cicaprost were unaffected. The latter analogue at 1 and 5 μM also had no effect on VIP responses; it was not tested against cicaprost.

Prolonged exposure to VIP as a means of desensitization was attempted on three colon preparations (which turned out to be somewhat more sensitive to VIP than normal). VIP at 100 nM completely inhibited spontaneous activity of the colon, but after about 5 min contact, spontaneous activity resumed and had returned to the control level after 20 min. Although this appeared to indicate substantial desensitization, a second 100 nM VIP challenge (without washout of the first dose) resulted in total inhibition, which returned to control level slightly quicker than the first response. Similarly, a third 100 nM VIP challenge produced about 95% inhibition, followed by complete recovery; at this time, addition of cicaprost induced a response identical to the pre-VIP control.

Inhibitory responses to 100 nM VIP were almost abolished when α-chymotrypsin (2 u ml^{-1}) was present in the organ bath (control −74.7 ± 5.0%; treatment −1.7 ± 3.8%, $n = 5$). In contrast, cicaprost responses were not inhibited (control −66.5 ± 3.9%; treatment −76.5 ± 4.6%, $n = 5$).

Relaxation of KCl-induced tone

Addition of 40 mM KCl to the organ bath resulted in the generation of a stable level of tone, some 30 to 50% of that obtained with a near maximal KCl concentration of 120 mM; spontaneous activity was completely or almost completely

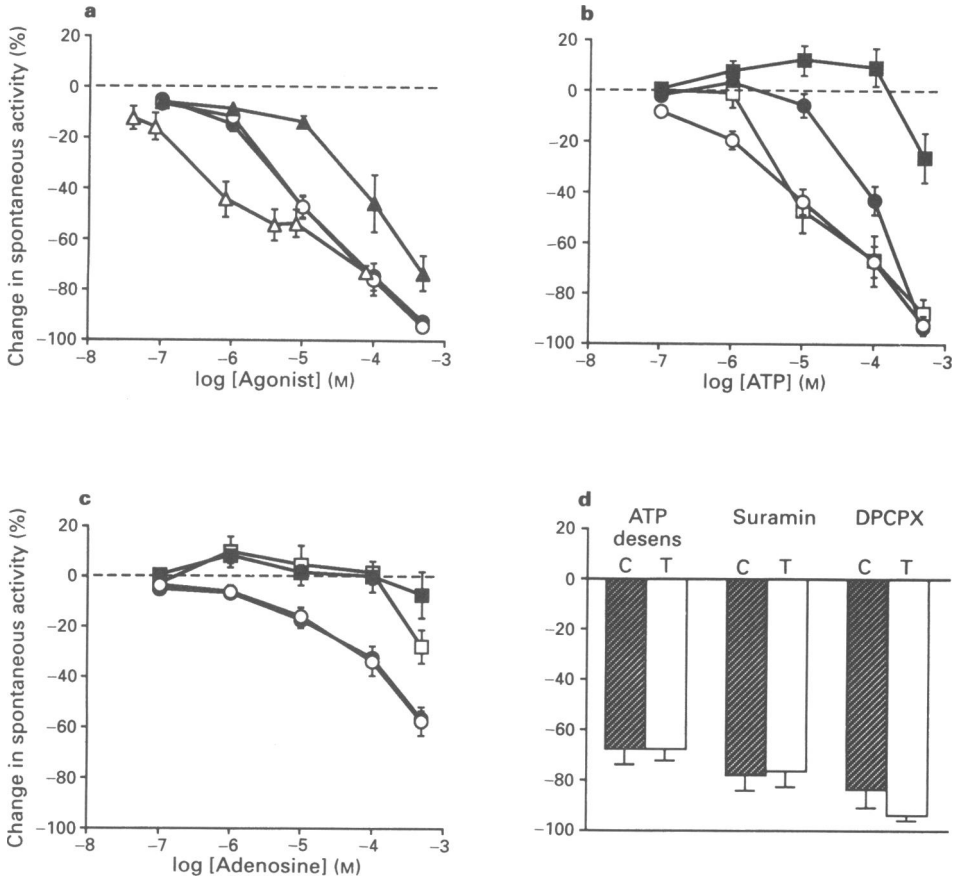

Figure 5 Inhibition of spontaneous activity of rat isolated colon; log concentration-response curves for second cumulative sequences (all $n = 4$). (a) ATP acting alone (○) and corresponding ATP first sequence (●); ATP after desensitization by 3 × 500 μM ATP (▲); 2-methylthio-ATP (△). (b) ATP acting alone (control curve for suramin) (○) and in the presence of suramin (300 μM, ●), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 5 μM, □) and apamin (25 nM, ■). ATP control curves for DPCPX and apamin are not shown, but are similar to the suramin control curve. (c). Adenosine, same conditions as (b). (d) Effects of desensitization to ATP (as in a), suramin (300 μM) and DPCPX (5 μM) on inhibitory responses to cicaprost (10 nM); C = control, T = treatment ($n = 4$). Means ± s.e.mean.

lost. Subsequent addition of cicaprost (10-310 nM) was without effect, whereas isoprenaline could still elicit complete relaxation (IC_{50} = 50 nM) (Figures 1 and 6a,b). Both SNP (0.1–5 μM) and ATP (100 μM) also inhibited KCl tone (not shown).

The PGE analogue, sulprostone, at 8.6 nM induced contractions of the colon preparation equivalent to the 40 mM KCl response; there was some enhancement of spontaneous activity. The contractile responses, which were more pronounced in distal segments, were not blocked by pretreatment with phentolamine, propranolol, atropine or TTX (all at 1 μM). Cicaprost reduced both the tone and the rhythmic contractions in sulprostone-treated preparations; its EC_{50} value was 4.5 nM and 90% inhibition was achieved at 20 nM (Figure 1). TTX at 1 μM abolished these cicaprost responses. In the presence of 40 mM KCl, sulprostone (8.6 nM) elicited a further increase in tension but spontaneous activity remained absent; cicaprost (10–310 nM) produced only minimal relaxation (Figures 1 and 6c).

In the presence of tone elicited by 40 mM KCl, iloprost (10–500 nM) contracted the colon preparation (Figure 6d).

Discussion

There are a number of reports in the literature on the neuronal stimulant actions of IP-receptor agonists. For example, in relation to sensory events, prostacyclin activates 'cardiopulmonary receptors' in the large pulmonary vessels of the dog to elicit vagal bradycardia (Nganele & Hintze, 1987), and cicaprost potentiates sensory discharge from the arthritic ankle joint of the rat following depression with either acetylsalicylic acid or paracetamol (McQueen *et al.*, 1991). In a spinal cord/functionally-attached tail preparation from the neonate rat, cicaprost also enhanced responses of peripheral nociceptors to thermal and chemical stimuli (Rueff & Dray, 1993). In terms of motor actions, cicaprost elicits contraction of the longitudinal muscle of the guinea-pig ileum by releasing both acetylcholine (Gaion & Trento, 1983) and a substance P-like transmitter (Jones & Lawrence, 1993) from enteric nerves. In addition, preliminary experiments indicate that cicaprost increases the twitch response of the guinea-pig vas deferens to electrical field stimulation (EFS) by enhancing ATP release (Jones, 1993).

As a result of the present studies, activation of IP-receptors on enteric neurones in the rat colon to release inhibitory transmitters can now be added to the above list. Thus the neuronal Na^+ channel blockers, STX and TTX, abolish the inhibitory action of cicaprost on the spontaneous activity of the colon. Their selectivity at the concentrations used is evidenced by a lack of effect on the pacemaker function of the colon: anatomical and electrophysiological investigations of the contractility of rat and dog colon preparations have shown that three tissue components are involved: smooth muscle cells, a network of pacemaker cells (interstitial cells of Cajal, ICC) linked to each other and to smooth muscle cells by gap junctions, and a largely inhibitory innervation which is concentrated on the ICC (Huizinga *et al.*, 1990; Faussone-Pellegrini, 1992; references therein). The inhibitory action of nicotine on the colon is

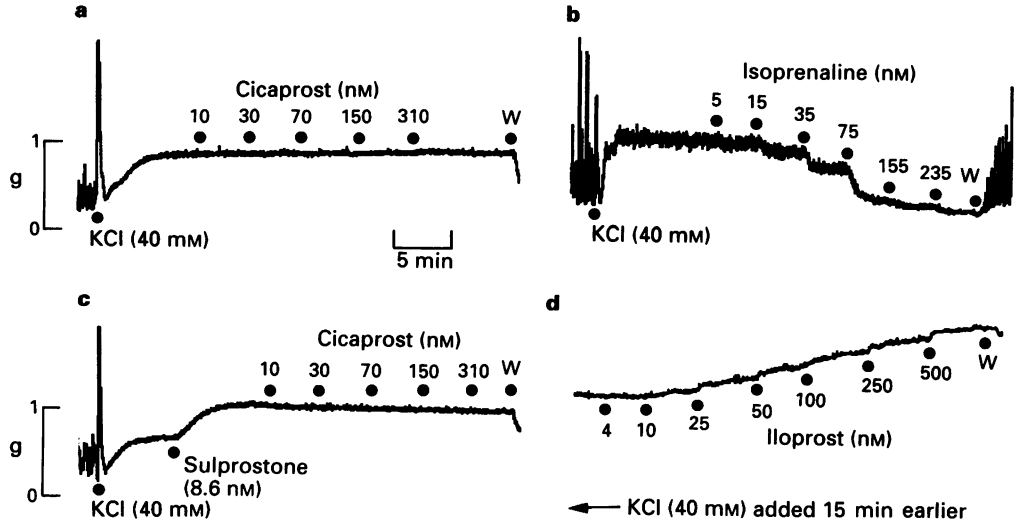

Figure 6 Rat isolated colon: experimental records showing the effects of (a) cicaprost, (b) isoprenaline and (d) iloprost in the presence of tone generated by 40 mM KCl, and (c) cicaprost on tone generated by a combination of 40 mM KCl and 8.6 nM sulprostone. Cumulative concentrations. W = wash.

also abolished by TTX. However, nicotine inhibition is more difficult to study because of its tendency to desensitization. Deliberate desensitization with nicotine did not affect cicaprost inhibition, implying that the action of cicaprost is not dependent on the integrity of nicotinic cholinergic synapses. In contrast to cicaprost and nicotine, the inhibitory action of isoprenaline is unaffected by TTX and appears to be non-neuronal in nature. Inhibitory β_3-adrenoceptors, with a low affinity for propranolol (pA_2 ~6.5), are present in the rat colon (Bianchetti & Manara, 1990; McLaughlin & MacDonald, 1990); this would explain the incomplete block of isoprenaline action by 1 μM propranolol in our studies. The inability of phentolamine, propranolol and atropine to inhibit the action of cicaprost suggests that this agent releases a non-adrenergic, non-cholinergic (NANC) inhibitory transmitter. NANC neurotransmission (Burnstock, 1986) to gut structures appears to be common and the main transmitter candidates are ATP (Burnstock *et al.*, 1970), VIP (Goyal & Rattan, 1980) and nitric oxide (Bult *et al.*, 1990). In general, two or more NANC transmitters may operate simultaneously to elicit a relaxant effect (Manzini *et al.*, 1986; Maggi & Giuliani, 1993), although one of them may play a dominant role in particular segments of the intestine of some species.

Involvement of NO generation in the action of cicaprost

There is good agreement that NO makes a major contribution to inhibition of colonic activity elicited by activation of enteric neurones. Hata *et al.* (1990) showed that relaxation of the circular muscle of the rat proximal colon due to local distension was abolished by N^G-nitro-L-arginine (L-NOARG), a NOS inhibitor; L-arginine, the precursor of NO (Palmer *et al.*, 1988a), but not D-arginine, largely reversed the effect of L-NOARG. Similarly in rat colon, relaxations of circular muscle to distension and longitudinal muscle to EFS were essentially abolished by L-NOARG (Suthamnatpong *et al.*, 1993; 1994). In the guinea-pig colon, L-NOARG almost abolished the relaxation induced by EFS (Zagorodnyuk & Maggi, 1994). In our experiments, inhibitory responses to both cicaprost and nicotine were partially (50–60%) blocked by L-NAME at appropriate concentrations (see Rees *et al.*, 1990). D-NAME however did not affect cicaprost action, compatible with stereospecific inhibition of NOS by arginine analogues (see Palmer *et al.*, 1988b). Thus it appears that cicaprost- and nicotine-induced inhibitions of spontaneous activity in the rat colon are mediated in part by NO. Constitutive NOS has been found in enteric nerves (Bredt *et al.*, 1990), but there has been some discussion in the literature (see Zagorodnyuk & Maggi, 1994) as to whether colonic relaxation is produced by NO generated within nerves or by another transmitter, for example VIP (Grider *et al.*, 1992), inducing NO generation in smooth muscle. Our experiments do not distinguish between these two possibilities.

The involvement of purinergic transmission in the action of cicaprost

Bailey & Hourani (1992) have shown that ATP and adenosine relax the carbachol-contracted rat colon (IC_{25} = 35 and 80 μM) through activation of P_{2Y}-purinoceptors and adenosine A_2-receptors respectively. From our experiments, these two receptors also appear to mediate inhibition of spontaneous activity, although both ATP and adenosine show somewhat greater potency (IC_{25} = 2.5 and 35 μM respectively). 2-Methylthio-ATP was more potent than ATP, consistent with the known properties of this specific P_{2Y} agonist (Gough *et al.*, 1973; O'Connor *et al.*, 1991).

Our studies provide no real evidence for a purinergic contribution to the inhibitory action of cicaprost. Firstly, desensitization to ATP had no effect on cicaprost responses. Secondly, suramin, a P_{2X}/P_{2Y}-receptor antagonist (Dunn & Blakely, 1988; Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990; Leff *et al.*, 1990), did not affect cicaprost-induced inhibition. Although suramin is a weak antagonist (300 μM was used), it did act specifically, blocking the inhibitory action of ATP but not that of adenosine. Higher concentrations of suramin could not be used because of their depressant effect on spontaneous activity. Thirdly, the potent adenosine A_1-receptor antagonist, DPCPX (Collis *et al.*, 1989; Bruns, 1990), used at high concentration to block adenosine A_2-receptors, did not inhibit cicaprost responses.

The bee venom peptide apamin has been shown to block inhibitory responses to ATP in guinea-pig taenia coli (Den Hertog *et al.*, 1985), guinea-pig internal anal sphincter (Lim & Muir, 1986) and rat gastric fundus (LeFebvre *et al.*, 1991). In our experiments, apamin also markedly inhibited or abolished the inhibitory effects of ATP and adenosine. These observations indicate that ATP and adenosine probably inhibit spontaneous activity by opening low conductance calcium-activated K^+-channels in smooth muscle, since apamin is known to block these channels specifically (Banks *et al.*, 1979; Maas & Den Hertog, 1979). Apamin partially inhibited and in combination with L-NAME abolished the

inhibitory actions of both cicaprost and nicotine on the colon. This suggests that the second transmitter released from enteric nerves opens calcium-activated K^+-channels. Although the value of this observation in terms of transmitter identification is limited (α_1-adrenoceptor-mediated inhibition is also apamin-sensitive), it does provide further support against a β_3-adrenoceptor contribution, since isoprenaline inhibition was apamin-insensitive. Since cicaprost and nicotine produced no excitatory effects in the presence of the L-NAME/apamin combination, it would appear that their stimulant actions are limited to inhibitory enteric neurones (at least at the concentrations employed).

Involvement of VIP in the action of cicaprost

The inhibitory effects of VIP on the rat colon were partially blocked by TTX, suggesting that both prejunctional and postjunctional sites of action are involved. A prejunctional action of VIP has also been reported in the dog and cat trachea (Hakoda & Ito, 1990); however, in these tissues VIP inhibits the release of an excitatory transmitter.

In the studies of Suthamnatpong *et al.* (1993), the VIP antagonist, VIP 10–28, inhibited the EFS response of the distal rat colon to a maximum of 35%; it had no effect on EFS responses of the proximal and middle colon. In our experiments, two VIP antagonists, [Lys^1,Pro^{2,5},Arg^{3,4},Tyr^6]-VIP (Gozes *et al.*, 1989) and [D-*p*-chloro-Phe^6,Leu^{17}]-VIP (Pandol *et al.*, 1986), did not inhibit responses to either VIP or cicaprost. In this context, [D-*p*-chloro-Phe^6,Leu^{17}]-VIP and another antagonist, [Ac-Tyr^1,D-Phe^2]-GRF(1-29)-NH_2, were without effect on responses to exogenous VIP in the guinea-pig isolated trachea (Ellis & Farmer, 1989). However in the cat trachea, these two antagonists abolished the pre-juctional action of VIP to suppress the excitatory junction potential (Xie *et al.*, 1991). Clearly we are in the position of having useful antagonists for one subtype of VIP receptor and not for the other(s).

Information was then sought from VIP desensitization experiments. However, as in the studies of Suthamnatpong *et al.* (1993), it proved difficult to achieve significant desensitization to VIP. Although inhibitory responses to VIP faded with time, subsequent addition of VIP still elicited good inhibitory responses. The mechanism underlying this profile of action is not clear.

As a last resort, evidence of the involvement of VIP as a transmitter was sought from the use of α-chymotrypsin, an enzyme that cleaves peptide bonds adjacent to tyrosine, lysine and arginine residues; all three amino acids are present in VIP. In previous studies, evidence has been obtained both for (rat gastric fundus, De Beurme & Lefebvre, 1987) and against (guinea-pig taenia coli, Mackenzie & Burnstock, 1980; rat duodenum, Manzini *et al.*, 1986) a role for VIP in NANC relaxation. The finding that α-chymotrypsin treatment did not inhibit the relaxation induced by cicaprost appears to exclude a transmitter role for not only VIP but also other susceptible peptides such as calcitonin gene-related peptide (CGRP). We emphasise 'appears to exclude', since, as suggested by others, there is no evidence that VIP released from neurones within the tissue is subject to the same degree of inactivation as VIP in the bathing fluid.

Effects of agonists when tone is raised with K^+

Raising the external K^+ concentration to produce submaximal contraction of the colon preparation was accompanied by loss of spontaneous activity. Under these conditions, isoprenaline, sodium nitroprusside and ATP induced relaxation, whereas cicaprost was completely inactive. Use of the EP_1/EP_3-receptor agonist, sulprostone, to raise the tone of the preparation did not result in loss of spontaneous activity; in this case cicaprost still induced a TTX-sensitive relaxation. However if sulprostone was combined with high K^+, spontaneous activity was lost and cicaprost was again inactive. Two mechanisms may be relevant to the lack of effect of cicaprost in the presence of high K^+. Firstly, cicaprost may still release inhibitory transmitters, but they are ineffective since their action on the pacemaker is abrogated by high K^+. Secondly, the high K^+ may prevent the transmitter-releasing action of cicaprost. This is an interesting concept, since it raises the possibility that activation of IP-receptors on enteric neurones leads to closing of K^+ channels and membrane depolarization. This action may not proceed through the G_S-adenylate cyclase pathway typical of the inhibitory actions of prostacyclin on platelet function. An analogous situation is the presynaptic inhibitory actions of opioid agonists, where mediation through cyclic AMP is being abandoned in favour of direct G-protein links with K^+ and/or Ca^{2+} channels in the plasma membrane (see Di Chiara & North, 1992).

The loss of IP-receptor-mediated inhibition in the presence of high K^+ exposes the true contractile action of iloprost on the rat colon. Iloprost is known to be a potent agonist at EP_1-receptors (Dong *et al.*, 1986). However, the nature of the EP-receptor mediating contraction of the rat colon has not been reported; preliminary observations in our laboratory suggest that it is more likely to be an EP_3- than an EP_1-receptor.

In conclusion, the present study demonstrates that the inhibitory actions of IP-receptor agonists on the contractility of the rat colon are neuronally-mediated and that at least two NANC transmitter mechanisms appear to be involved. NO is likely to be one of the inhibitory transmitters, whereas some evidence has been obtained against a role for ATP, adenosine and VIP. Further studies are warranted to characterize the 'neuronal' IP-receptors involved, in terms of molecular structure, G-protein coupling and structure-activity relationships.

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References

BAILEY, S.J. & HOURANI, S.M.O. (1992). Effects of purines on the longitudinal muscle of the rat colon. *Br. J. Pharmacol.*, **105**, 885–892.

BANKS, B.E., BROWN, C., BURGESS, G.M., BURNSTOCK, G., CLARET, M., COCKS, T.M. & JENKINSON, D.H. (1979). Apamin blocks certain neurotransmitter-induced increases in potassium permeability. *Nature*, **282**, 415–417.

BIANCHETTI, A. & MANARA, L. (1990). *In vitro* inhibition of intestinal motility by phenylethanolaminotetralines: evidence of atypical β-adrenoceptors in rat colon. *Br. J. Pharmacol.*, **100**, 831–839.

BREDT, D.S., HWANG, P.M. & SNYDER, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **347**, 768–770.

BRUNS, R.F. (1990). Adenosine receptors: role and pharmacology. *Ann. N.Y. Acad. Sci.*, **603**, 211–226.

BULT, H., BOECKXSTAENS, G.E., PELKMANS, P.A., JORDAENS, F.H., VAN MAERCHE, Y.M. & HERMAN, A.G. (1990). Nitric oxide as an inhibitory non-adrenergic, non-cholinergic neurotransmitter. *Nature*, **345**, 346–347.

BURNSTOCK, G. (1986). The non-adrenergic, non-cholinergic nervous system. *Arch. Int. Pharmacodyn. Ther.*, **280**, 1–15.

BURNSTOCK, G., CAMPBELL, G., SATCHELL, D. & SMYTHE, A. (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br. J. Pharmacol.*, **40**, 668–688.

COLLIS, M.G., STOGGALL, S.M. & MARTIN, F.M. (1989). Apparent affinity of 1,3-dipropyl-8-cyclopentylxanthine for adenosine A_1 and A_2 receptors in isolated tissues from guinea-pigs. *Br. J. Pharmacol.*, **97**, 1274–1278.

DE BEURME, F.A. & LEFEBVRE, R.A. (1987). Influence of α-chymotrypsin and trypsin on the non-adrenergic non-cholinergic relaxation in the gastric fundus. *Br. J. Pharmacol.*, **91**, 171–177.

DEN HERTOG, A., NELEMANS, A. & VAN DEN AKKER, J. (1989). The inhibitory action of suramin on the P_2-purinoceptor response in smooth muscle cells of guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **166**, 531–534.

DEN HERTOG, A., PIELKENROOD, J. & VAN DEN AKKER, J. (1985). Effector mechanisms for α,β-methylene ATP derivatives in guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **110**, 95–101.

DI CHIARA, G. & NORTH, R.A. (1992). Neurobiology of opiate abuse. *Trends Pharmacol. Sci.*, **13**, 185–193.

DONG, Y.J., JONES, R.L. & WILSON, N.H. (1986). Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *Br. J. Pharmacol.*, **87**, 97–107.

DUNN, P.M. & BLAKELY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

ELLIS, J.L. & FARMER, S.G. (1989). The effects of vasoactive intestinal peptide (VIP) antagonists, and VIP and peptide histidine isoleucine antisera on non-adrenergic, non-cholinergic relaxations of tracheal smooth muscle. *Br. J. Pharmacol.*, **96**, 513–520.

FAUSSONE-PELLEGRINI, M.S. (1992). Histogenesis, structure and relationships of interstitial cells of Cajal (ICC): from morphology to functional interpretation. *Eur. J. Morphol.*, **30**, 137–148.

GAION, R.M. & TRENTO, M. (1983). The role of prostacyclin in modulating cholinergic neurotransmission in guinea-pig ileum. *Br. J. Pharmacol.*, **80**, 279–286.

GOUGH, G.R., MAGUIRE, M.H. & SATCHELL, D.G. (1973). Three new adenosine-triphosphate analogs. Synthesis and effects on isolated gut. *J. Med. Chem.*, **16**, 1188–1190.

GOYAL, R.K. & RATTAN, S. (1980). VIP as a possible neurotransmitter of non-cholinergic, non-adrenergic inhibitory neurones. *Nature*, **288**, 378–380.

GOZES, I., MELTZER, E., RUBINROUT, S., BRENNEMAN, D.E. & FRIDKIN, M. (1989). Vasoactive intestinal peptide potentiates sexual behavior: inhibition by novel antagonist. *Endocrinology*, **125**, 2945–2949.

GRIDER, J.R., MURTHY, K.S., JIN, J.G. & MAKHLOUF, G.M. (1992). Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am. J. Physiol.*, **262**, G774–G778.

GRYGLEWSKI, R.J., BUNTING, S., MONCADA, S., FLOWER, R.J. & VANE, J.R. (1976). Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. *Prostaglandins*, **12**, 685–713.

HAKODA, H. & ITO, Y. (1990). Modulation of cholinergic neurotransmission by the peptide VIP, VIP antiserum and VIP antagonists in dog and cat trachea. *J. Physiol.*, **428**, 133–154.

HATA, F., ISHII, T., KANADA, A., YAMANO, N., KATAOKA, T., TAKEUCHI, T. & YAGASAKI, O. (1990). Essential role of nitric oxide in descending inhibition in the rat proximal colon. *Biochem. Biophys. Res. Commun.*, **172**, 1400–1406.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

HUIZINGA, J.D., BEREZIN, I., DANIEL, E.E. & CHOW, E. (1990). Inhibitory innervation of colonic smooth muscle cells and interstitial cells of Cajal. *Can. J. Physiol. Pharmacol.*, **68**, 447–454.

JONES, R.L. (1993). Prostacyclin analogues potentiate contraction of the guinea-pig vas deferens to electrical field stimulation. *Br. J. Pharmacol.*, **108**, 16P.

JONES, R.L. & LAWRENCE, R.A. (1993). The NK_1-receptor antagonist CP-96,345 partially inhibits cicaprost-induced contractions of the guinea-pig ileum. *Pharmacol. Commun.*, **3**, 147–155.

JONES, R.L., WILSON, N.H., MARR, C.G., MUIR, G. & ARMSTRONG, R.A. (1993). Diphenylmethylazine prostanoids with prostacyclin-like actions on human platelets. *J. Lipid Mediators*, **6**, 405–410.

LAWRENCE, R.A., JONES, R.L. & WILSON, N.H. (1992). Characterization of receptors involved in the direct and indirect actions of prostaglandins E and I on the guinea-pig ileum. *Br. J. Pharmacol.*, **105**, 271–278.

LEFEBVRE, R.A., DE BEURME, F.A. & SAS, S. (1991). Effect of apamin on the responses to VIP, ATP and NANC neurone stimulation in the rat and cat gastric fundus. *J. Auton. Pharmacol.*, **11**, 73–83.

LEFF, P., WOOD, B.E. & O'CONNOR, S.E. (1990). Suramin is a slowly-equilibrating but competitive antagonist at P_{2X}-receptors in the rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 645–649.

LIM, S.P. & MUIR, T.C. (1986). Neuroeffector transmission in the guinea-pig internal anal sphincter: an electrical and mechanical study. *Eur. J. Pharmacol.*, **128**, 17–24.

MAAS, A.J.J. & DEN HERTOG, A. (1979). The effect of apamin on the smooth muscle cells of the guinea-pig taenia coli. *Eur. J. Pharmacol.*, **58**, 151–156.

MACKENZIE, I. & BURNSTOCK, G. (1980). Evidence against vasoactive intestinal polypeptide being the non-adrenergic, non-cholinergic inhibitory transmitter released from nerves supplying the smooth muscle of the guinea-pig taenia coli. *Eur. J. Pharmacol.*, **67**, 255–264.

MAGGI, C.A. & GIULANI, S. (1993). Multiple inhibitory mechanisms mediate non-adrenergic non-cholinergic relaxation in the circular muscle of the guinea-pig colon. *Naunyn-Schmied. Arch. Pharmacol.*, **347**, 630–634.

MANZINI, S., MAGGI, C.A. & MELI, A. (1986). Pharmacological evidence that at least two different non-adrenergic non-cholinergic inhibitory systems are present in the rat small intestine. *Eur. J. Pharmacol.*, **123**, 229–236.

MCLAUGHLIN, D.P. & MACDONALD, A. (1990). Evidence for the existence of 'atypical' β-adrenoceptors (β_3-adrenoceptors) mediating relaxation in the rat distal colon *in vitro*. *Br. J. Pharmacol.*, **101**, 569–574.

MCQUEEN, D.S., IGGO, A., BIRRELL, G.J. & GRUBB, B.D. (1991). Effects of paracetamol and aspirin on neural activity of joint mechanonociceptors in adjuvant arthritis. *Br. J. Pharmacol.*, **104**, 178–182.

MEANWELL, N.A., ROMINE, J.L & SEILER, S.M. (1994). Non-prostanoid prostacyclin mimetics. *Drugs Future*, **19**, 361–385.

NGANELE, D.M. & HINTZE, T.H. (1987). Prostacyclin reduces preload in conscious dogs via a vagal reflex mechanism. *Am. J. Physiol.*, **253**, H1477–H1483.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PALMER, R.M., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

PANDOL, S.J., DHARMSATHAPHORN, K., SCHOEFFIELD, M.S., VALE, W. & RIVIER, J. (1986). Vasoactive intestinal peptide receptor antagonist [4Cl-D-Phe^6,Leu^{17}]VIP. *Am. J. Physiol.*, **250**, G553–G557.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

RUEFF, A. & DRAY, A. (1993). Pharmacological characterization of the effects of 5-hydroxytryptamine and different prostaglandins on peripheral sensory neurons *in vitro*. *Agents Actions*, **38**, C13–C15.

STURZEBECHER, S., HABEREY, M., MUELLER, B., SCHILLINGER, E., SCHRODER, G., SKUBALLA, W., STOCK, G., VORBRUGGEN, H. & WITT, W. (1986). Pharmacological profile of a novel carbacyclin derivative with high metabolic stability and oral activity in the rat. *Prostaglandins*, **31**, 95–109.

SUTHAMNATPONG, N., HATA, F., KANADA, A., TAKEUCHI, T. & YAGASAKI, O. (1993). Mediators of nonadrenergic, noncholinergic inhibition in the proximal, middle and distal regions of rat colon. *Br. J. Pharmacol.*, **108**, 348–355.

SUTHAMNATPONG, N., HOSOKAWA, M., TAKEUCHI, T., HATA, F. & TAKEWAKI, T. (1994). Nitric oxide-mediated inhibitory response of rat proximal colon: independence from changes in membrane potential. *Br. J. Pharmacol.*, **112**, 676–682.

WATSON, S. & GIRDLESTONE, D. (ed.). (1994). Prostanoid receptors. In *Receptor and Ion Channel Nomenclature Supplement. Trends Pharmacol. Sci.* pp. 36–37. Cambridge: Elsevier Trends Journals.

XIE, Z., HIROSE, T. & ITO, Y. (1991). Effects of vasointestinal polypeptide antagonists on cholinergic neurotransmission in dog and cat trachea. *Br. J. Pharmacol.*, **104**, 938–944.

ZAGORODNYUK, V. & MAGGI, C.A. (1994). Electrophysiological evidence for different release mechanism of ATP and NO as inhibitory NANC transmitters in guinea-pig colon. *Br. J. Pharmacol.*, **112**, 1077–1082.

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