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The possible role of ATP and PACAP as mediators of apamin-sensitive NANC inhibitory junction potentials in circular muscle of guinea-pig colon

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1 In the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), nifedipine (1 μM), L-nitroarginine (L-NOARG, 100 μM), and the selective tachykinin NK_1 and NK_2 receptor antagonists, SR 140,333 and GR 94,800, respectively (0.1 μM each), a single pulse of electrical field stimulation (EFS) produced a monophasic non-adrenergic non-cholinergic (NANC) inhibitory junction potential (i.j.p., about 10 mV in amplitude) in the circular muscle of guinea-pig proximal colon, recorded by the modified single sucrose gap technique.

2 The P_2 purinoceptor agonist, α,β methylene ATP (α,β mATP, 100 μM) and the pituitary adenylyl cyclase activating peptide (PACAP, 1 μM) both produced hyperpolarization (11 ± 0.8 mV, $n=14$ and 10.2 ± 0.8 mV, $n=19$, respectively) and relaxation (1.1 ± 0.2 mV, $n=14$ and 1.5 ± 0.2 mN, $n=19$, respectively) of the circular muscle.

3 Apamin (0.1 μM) nearly abolished (about 90% inhibition) the NANC i.j.p. and the α,β mATP-induced hyperpolarization, markedly reduced the α,β mATP-induced relaxation (73% inhibition) and the PACAP-induced hyperpolarization (65% inhibition), while the PACAP-induced relaxation was unaffected.

4 Tetraethylammonium (TEA, 10 mM) increased the EFS-evoked i.j.p. and revealed an excitatory junction potential (e.j.p.). In the presence of TEA, α,β mATP induced a biphasic response: transient depolarization and contraction followed by hyperpolarization and relaxation. The hyperpolarization to PACAP was reduced by TEA (45% inhibition) but the relaxation was unaffected.

5 The combined application of apamin (0.1 μM) and TEA (10 mM) abolished the i.j.p. and single pulse EFS evoked a pure e.j.p. with latency three times longer than that of the i.j.p. In the majority of strips tested, α,β mATP and PACAP elicited a biphasic response : depolarization and small contraction followed by hyperpolarization and relaxation.

6 The P_2 purinoceptor antagonist, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) inhibited the NANC i.j.p. in concentration-dependent manner and inhibited the α,β mATP-induced hyperpolarization and relaxation, without affecting the hyperpolarization and relaxation induced by PACAP. On the other hand, the P_2 purinoceptor antagonist, suramin (100 μM) inhibited to a similar extent (60-80%) the NANC i.j.p. and the hyperpolarization and relaxation induced by α,β mATP or PACAP.

7 PPADS and suramin reduced the NANC e.j.p. evoked by a single pulse EFS in the presence of apamin and TEA (100 μM of PPADS and 300 μM of suramin inhibited the e.j.p. by about 40%).

8 We conclude that ATP, but not PACAP, mediates the apamin-sensitive NANC i.j.p. in the circular muscle of the guinea-pig colon. After blockade of the NANC i.j.p., ATP may act as an excitatory transmitter by activating excitatory P_2 purinoceptors. The subtypes of P_2 purinoceptor involved in the inhibitory and excitatory responses remain to be established. The data suggest that excitatory P_2 purinoceptors may be located extrajunctionally.

Keywords: Non-adrenergic non-cholinergic (NANC); ATP; guinea-pig colon; pituitary adenylyl cyclase activating peptide (PACAP); suramin; apamin

Introduction

The identification of the transmitter(s) responsible for the non-adrenergic non-cholinergic (NANC) inhibitory transmission in smooth muscles (Bennett *et al.*, 1966) has been and still remains a controversial issue. In particular adenosine 5'-triphosphate (ATP) (Burnstock *et al.*, 1970; Burnstock, 1981; Vladimirova & Shuba, 1984; Zagorodnyuk & Shuba, 1986; Crist *et al.*, 1992). Vasoactive intestinal peptide (VIP) (Grider & Rivier, 1990; Crist *et al.*, 1992) and, more recently, nitric oxide (NO, Boeckxstaens *et al.*, 1990; Sanders & Ward, 1992) have been proposed as NANC inhibitory transmitters in the mammalian intestine. It is now held that more than one transmitter is involved in NANC inhibition in the smooth muscle of the gastrointestinal tract, that the relative contribution and importance of different transmitters varies in different regions of the intestine (Costa *et al.*, 1986; Manzini *et al.*, 1986), and that different mechanisms regulate the release of different NANC inhibitory transmitters (Zagorodnyuk & Maggi, 1994; Bridgewater *et al.*, 1995).

In the circular muscle of the guinea-pig colon, three inhibitory NANC mechanisms have been operationally identified as: (1) apamin-sensitive, (2) N^{ω}nitro-arginine (L-NOARG)-sensitive and (3) apamin- and L-NOARG-resistant (Maggi & Giuliani, 1993; Zagorodnyuk *et al.*, 1993; Zagorodnyuk & Maggi, 1994). The L-NOARG-sensitive component of the NANC inhibitory junction potential (i.j.p.) is mediated by NO or NO-generating substance(s) (Zagorodnyuk *et al.*,

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1993; Zagorodnyuk & Maggi, 1994) while the transmitter responsible for the apamin- and NOARG-resistant component of NANC relaxation, is still unknown. With regard to the apamin-sensitive i.j.p., we showed previously that the P_2 purinoceptor antagonist suramin selectively inhibits the apamin-sensitive but not the apamin-resistant component of the NANC i.j.p. evoked by rhythmic (10 Hz) electrical field stimulation (EFS) in the circular muscle of the guinea-pig colon (Zagorodynuk & Maggi, 1994), suggesting a mediator role of ATP for this component.

However, some concern with the role of ATP as mediator of the apamin-sensitive i.j.p. has been raised recently, based on the observations that: (a) a VIP-related peptide, the pituitary adenylyl cyclase activating peptide (PACAP) produces an apamin-sensitive relaxation of guinea-pig taenia caeci smooth muscle (Schworer *et al.*, 1992); (b) suramin but not tetrodotoxin (TTX) inhibits the PACAP-induced relaxation (McConalogue *et al.*, 1995); (c) both PACAP antisera and PACAP receptor antagonist block the apamin-sensitive component of NANC relaxation in the guinea-pig taenia coli (Jin *et al.*, 1994).

The aim of this study was to address the possible role of ATP and PACAP in mediating the apamin-sensitive NANC i.j.p. in the circular muscle of the guinea-pig colon. All experiments were performed in the presence of atropine and guanethidine, to establish NANC conditions, of L-NOARG to exclude production/release of NO, and of selective tachykinin NK_1 and NK_2 receptor antagonists to exclude the involvement of endogenous tachykinins acting as NANC excitatory transmitters (Zagorodnyuk *et al.*, 1993; Maggi *et al.*, 1994). Under these conditions, single pulse EFS evoked an apamin-sensitive i.j.p.: we have investigated the effect of the new selective P_2 purinoceptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, Lambrecht *et al.*, 1992) and of suramin on the apamin-sensitive i.j.p. as compared to their effect toward the responses evoked by exogenously applied PACAP and the metabolically stable P_2 purinoceptor agonist α,β methylene ATP (α,β mATP) (Kennedy & Leff, 1995). We also found that, in the presence of tetraethylammonium (TEA), and especially of apamin plus TEA, an e.j.p. is produced by single pulse EFS in the presence of atropine and tachykinin receptor antagonists. The effect of PPADS and suramin on this excitatory response was investigated as well.

Methods

A single sucrose-gap, modified as described by Artemenko *et al.* (1982) and Hoyle (1987) was used to investigate simultaneously changes in membrane potential and contractile activity of smooth muscle. Male albino guinea-pigs weighing 250–300 g were stunned and bled. Mucosa-free circular muscle strips of proximal colon approximately 0.5–0.8 mm wide and 10 mm long were excised and prepared for sucrose gap recording of electrical and mechanical activity as described in details previously (Zagorodnyuk *et al.*, 1993; Zagorodynuk & Maggi, 1994). The strips were superfused with warmed (35 ± 0.5°C) and oxygenated (95% O_2 and 5% CO_2, pH 7.4) Krebs solution at a rate of 1 ml min^{-1}. The composition of the Krebs solution was as follows (mM): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11.

Junction potentials were evoked by single pulses of EFS (30 V, 0.1 ms) which were delivered by means of a Grass S88 stimulator. The latency of junctional events was measured as the time interval between stimulus artifact and onset of the change in membrane potential; the time to peak of junctional events was calculated from the onset to the maximal variation in membrane potential; the total duration of the junctional events was calculated from their onset to complete recovery of resting membrane potential.

The Krebs solution routinely contained atropine (1 μM), guanethidine (3 μM), L-NOARG (100 μM), indomethacin (3 μM), nifedipine (1 μM) and the selective NK_1 and NK_2 receptor antagonists, SR 140333 (0.1 μM) (Edmonds-Alt *et al.*, 1993) and GR 94800 (0.1 μM) (McElroy *et al.*, 1992), respectively. Indomethacin was used to exclude the possible involvement of prostanoids. Nifedipine was used to inhibit spontaneous electrical and mechanical activity and evoked action potentials.

The metabolically stable preferential P_2 purinoceptor agonist, α,β mATP was used to avoid the problem of degradation of purinoceptor agonists (Kenney & Leff, 1995). α,β mATP (100 μM) and PACAP (1 μM) were applied in superfusate for 25 s: these concentrations were selected from preliminary experiments consistently to produce a comparable hyperpolarization when tested on the same muscle strips of guinea-pig colon. To study the effect of drugs on the responses to α,β mATP and PACAP, a control response to the agonist was first produced in each strip and a second response to the agonist was then produced 20–25 min later in the presence of the antagonist. In control experiments two consecutive applications of α,β mATP and PACAP were performed at 20–25 min apart from each other.

Data evaluation and statistical analysis

All data in the text are mean ± standard error of the mean (s.e.mean). Statistical analysis was performed by means of Student's test for paired or unpaired data when applicable. A P level <0.05 was considered as statistically significant.

Drugs

Drugs used were: atropine HCl (Serva, Heidelberg, Germany); guanethidine sulphate (ICFI, Milan, Italy); N^{ω}-nitro-L-arginine (L-NOARG), nifedipine, indomethacin, apamin, α,β methylene ATP (α,β mATP), (Sigma); tetraethylammonium chloride (TEA, Merck); pituitary adenylyl cyclase activating peptide (PACAP, Peninsula); pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) and suramin (RBI); GR 94800 (PhCO-Ala-Ala-DTrp-Phe-DPro-Pro-$NleNH_2$) was synthesized by conventional solid phase methods at the Chemistry Department of Menarini Pharmaceuticals. SR 140333 ((S)-1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxy phenylacetyl)piperidin-3-yl]ethyl]-4-phenyl-1-azoniabicyclo[2.2.2.] octane chloride) was a kind gift of Dr X. Edmonds-Alt, Sanofi Research Ctr., Montpellier, France.

Results

General

In the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), nifedipine (1 μM), L-NOARG (100 μM) and of the selective tachykinin NK_1 and NK_2 receptor antagonists SR 140,333 (0.1 μM) and GR 94,800 (0.1 μM), respectively, single pulse EFS (30 V, 0.1 ms) produced a monophasic NANC i.j.p. (latency 110 ± 2 ms, amplitude 9.2 ± 0.3 mV, duration 1.33 ± 0.04 s, time to peak 204 ± 3 ms, $n=51$) which was abolished by tetrodotoxin (TTX, 1 μM). In these conditions the i.j.p. was not accompanied by relaxation of the circular muscle of the guinea-pig colon (Figures 1a and 2a).

Effect of α,β methylene ATP and PACAP

The P_{2x} purinoceptor agonist, α,β mATP (100 μM) and PACAP (1 μM) both produced hyperpolarization (11.0 ± 0.8 and 10.2 ± 0.8 mV, $n=14$ and 19, respectively) and relaxation (1.1 ± 0.2 and 1.5 ± 0.2 mN, $n=14$ and 19, respectively) of the circular muscle of guinea-pig proximal colon (Figures 1b and 2b).

TTX (1 μM) did not affect significantly the α,β mATP (100 μM)-induced hyperpolarization (-2 ± 8%, $n=5$), al-

though it significantly reduced the α,β mATP-induced relaxation (39±6% inhibition, $n=5$, $P<0.05$). TTX (1 μM) did not affect significantly the PACAP (1 μM)-induced hyperpolarization (−6±10%, $n=3$) and relaxation (−8±8%, $n=3$).

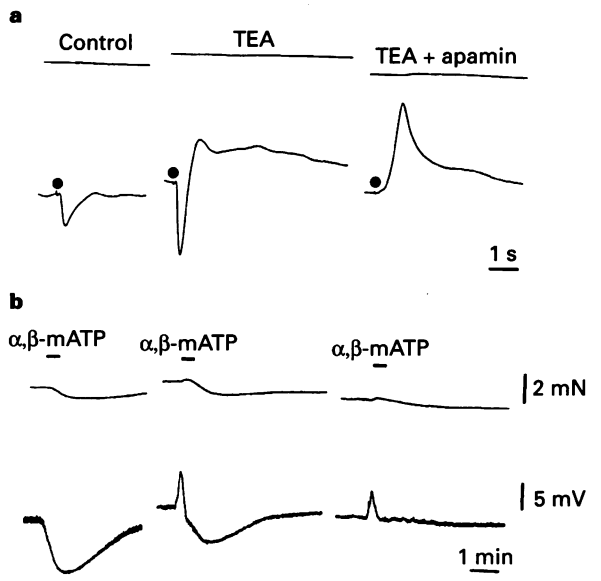

Figure 1 Typical tracings showing the effect of tetraethylammonium (TEA; 10 mM) and TEA plus apamin (0.1 μM) on the i.j.p. evoked by a single pulse of electrical field stimulation (EFS, 30 V, 0.1 ms, applied at dots, a) and on the hyperpolarization and relaxation induced by α,β mATP (100 μM for 25 s, b) in the circular muscle of the guinea-pig proximal colon. Experiments are from different strips obtained from the same animal. In both (a) and (b) upper tracing shows mechanical activity, lower tracing shows changes in membrane potential. The effect of TEA is shown after 20 min of action; the effect of apamin in TEA-containing Krebs solution is shown at 25 min of action.

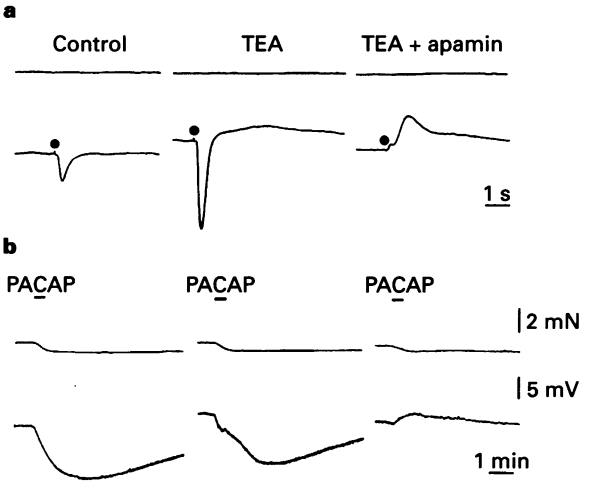

Figure 2 Typical tracings showing the effect of tetraethylammonium (TEA; 10 mM) and TEA plus apamin (0.1 μM) on the i.j.p. evoked by single pulse of electrical field stimulation (EFS, 30 V, 0.1 ms, applied at dots, a) and on the hyperpolarization and relaxation induced by pituitary adenylyl cyclase activating peptide (PACAP) (1 μM for 25 s, b) in the circular muscle of the guinea-pig proximal colon. Experiments are from different strips obtained from the same animal. In both (a) and (b) upper tracing shows mechanical activity, lower tracing shows changes in membrane potential. The effect of TEA is shown after 20 min of action; the effect of apamin in TEA-containing Krebs solution is shown at 25 min of action.

Effects of apamin and TEA on the NANC i.j.p. and on the responses to α,β methylene ATP and PACAP

Apamin (0.1 μM for 20 min) evoked a small depolarization (1.4±0.3 mV, $n=12$) and increased muscle tone (0.5±0.1 mN, $n=12$). Apamin (0.1 μM) inhibited the EFS-induced i.j.p. by about 90% (from 7.63±0.6 to 0.85±0.2 mV, $n=12$) as well as the α,β mATP (100 μM)-induced hyperpolarization and relaxation (by 95 and 73%, respectively, $n=5$; Table 1). Apamin also inhibited significantly the PACAP (1 μM)-induced hyperpolarization by about 65% ($n=7$, $P<0.05$) without significantly affecting the PACAP-induced relaxation (Table 2).

Owing to the uneven and incomplete effect of apamin, we also studied the effect of TEA, alone and in combination with apamin, to check whether that other type(s) of K channels may be involved in the responses under study. TEA (10 mM) produced a sustained depolarization (by 3.3±0.4 mV, $n=14$) of smooth muscle membrane and increased muscle tone (by 0.8±0.2 mN, $n=14$).

In the presence of TEA (10 mM for 20 min), single pulse EFS evoked a biphasic response: the i.j.p. almost doubled in size as compared to the control response (from 8.41±0.6 to 18.1±0.7 mV, $n=16$), was followed by an excitatory junction potential (e.j.p., 6.49±0.7 mV, $n=16$, Figures 1a and 2a). It is worth noting that the time to peak of the i.j.p. evoked in the presence of TEA (206±2 ms, $n=16$) did not significantly change as compared to control. In all strips tested ($n=7$), the monophasic hyperpolarization and relaxation produced by α,β mATP (100 μM) were converted into a biphasic response in the presence of TEA: a transient depolarization and small contraction were followed by hyperpolarization and relaxation (Figure 1b, Table 1). TEA significantly reduced (by about 45%, $P<0.05$) the PACAP (1 μM)-induced hyperpolarization, without affecting the PA-

Table 1 Effect of apamin (0.1 μM) and tetraethylammonium (TEA, 10 mM) on the hyperpolarization and relaxation evoked by α,β mATP(100 μM) in the circular muscle of the guinea-pig colon

Treatment	*Hyperpolarization* (mV)	*Depolarization* (mV)	*Relaxation* (mN)	*Contraction* (mN)
Control	11±0.8 ($n=15$)	–	1.1±0.2 ($n=15$)	–
Apamin	0.5±0.2* ($n=5$)	–	0.3±0.1* ($n=5$)	–
TEA	6.2±0.8* ($n=7$)	5.1±1.4 ($n=7$)	0.8±0.2 ($n=7$)	0.1±0.04 ($n=7$)
Apamin+ TEA	1.3±0.3* ($n=10$)	4.4±0.5 ($n=15$)	0.6±0.1* ($n=13$)	0.2±0.03 ($n=15$)

All values are mean±s.e.mean, *$P<0.05$.

Table 2 Effect of apamin (0.1 μM) and tetraethylammonium (TEA, 10 mM) on the hyperpolarization and relaxation evoked by PACAP (1 μM) in the circular muscle of the guinea-pig colon

Treatment	*Hyperpolarization* (mV)	*Depolarization* (mV)	*Relaxation* (mN)	*Contraction* (mN)
Control	10.2±0.8 ($n=19$)	–	1.5±0.2 ($n=19$)	–
Apamin	3.6±0.6* ($n=7$)	–	1.3±0.3 ($n=7$)	–
TEA	5.3±1.3* ($n=9$)	1.3±0.2 ($n=3$)	1.3±0.2 ($n=9$)	–
Apamin+ TEA	2.4±0.6* ($n=10$)	3.0±0.6 ($n=17$)	0.8±0.1* ($n=17$)	0.5±0.2 ($n=3$)

All values are mean±s.e.mean, *$P<0.05$.

CAP-induced relaxation (Figure 2b, Table 2). In 3 out of 9 strips a small transient depolarization (1.3±0.2 mV, $n=3$, Table 2) was also observed, preceding the development of PACAP-induced hyperpolarization.

The combined application of apamin (0.1 μM) and TEA (10 mM) evoked a depolarization (4.7±0.7 mV, $n=9$) and increased muscle tone (1.0±0.2 mN, $n=9$). In the presence of apamin and TEA the NANC i.j.p. was totally abolished in 28 out of 33 strips tested (Figures 1a and 2a); only in 5 cases was a small i.j.p. (1.5±0.5 mV) observed. More importantly, single pulse EFS invariably evoked a large NANC e.j.p. (Figures 1a and 2a; latency 314±8 ms, amplitude 15.5±0.8 mV, duration 14.7±0.6 s, time to peak 541±14 ms, $n=33$), often accompanied by a small (<0.1 mN) increase in tension. In 25 out of 33 strips tested, the e.j.p. was followed by a small long-lasting hyperpolarization (mean amplitude 0.7±0.1 mV, duration 22±1.0 s, not shown). TTX (1 μM, $n=3$) blocked all the responses to single pulse EFS in the presence of apamin and TEA.

In the presence of apamin and TEA, α,β mATP (100 μM) (Figure 1b, Table 1) induced a transient depolarization and contraction in all strips tested ($n=15$): these were followed by hyperpolarization in 10 out of 15 strips and by relaxation in 13 out of 15 strips.

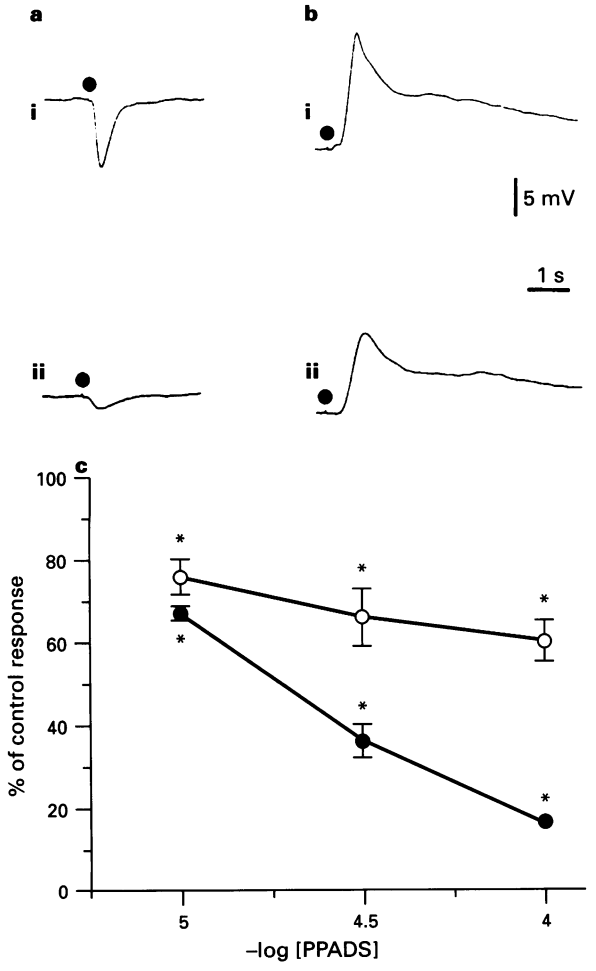

Figure 3 Effect of pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on the NANC i.j.p. and e.j.p. evoked by electrical field stimulation (EFS) in the circular muscle of the guinea-pig colon. (a) EFS (applied at dots) evoked a monophasic NANC i.j.p. (a(i)) which was markedly inhibited by PPADS (100 μM for 20 min, a(ii)). (b) In the presence of apamin (0.1 μM) and tetraethylammonium (TEA; 10 mM), EFS (applied at dots) evoked a NANC e.j.p. which was partly inhibited by PPADS (100 μM for 20 min). (c) Concentration-dependent inhibition by PPADS of EFS-evoked NANC i.j.p. (●) and e.j.p. (○), in the presence of apamin and TEA; $n=3-12$ experiments, $^*P<0.05$.

In the same conditions (Figure 2b, Table 2) PACAP (1 μM) induced a transient depolarization in all cases tested ($n=17$) along with a small contraction in 3 out of 17 strips; these responses were followed by hyperpolarization and relaxation in 7 out of 17 strips.

Effect of PPADS and suramin on the NANC i.j.p. and e.j.p. and on responses to α,β mATP and PACAP

The P_2 purinoceptor antagonist PPADS (30 μM) produced a slight relaxation (0.3±0.1 mN, $n=11$) without affecting the membrane potential of the smooth muscle. PPADS produced a concentration-dependent inhibition of the NANC i.j.p. (Figure 3a,c): at the highest concentration tested, 100 μM, PPADS inhibited the i.j.p. by 84±1%. A lower concentration of PPADS (30 μM for 20 min) inhibited the NANC i.j.p. by 64±4% and the α,β mATP (100 μM)-induced hyperpolarization and relaxation by 83±2 and 54±4%, respectively ($n=5$) (Figure 4). In sharp contrast, PPADS (30 μM, $n=5$) did not affect significantly by PACAP(1 μM)-induced hyperpolarization and relaxation (Figure 4d).

Suramin (100 μM for 30 min) produced relaxation (0.7±0.3 mN, $n=8$) without affecting the membrane potential of the circular muscle. Suramin concentration-dependently inhibited the NANC i.j.p. (Figure 5d): at the highest concentration tested (300 μM), suramin inhibited the i.j.p. by 86±1% ($n=3$). A lower concentration of suramin (100 μM) inhibited the i.j.p. by 66±3% ($n=9$) and the hyperpolarization induced by α,β mATP and PACAP by 84±2 and 77±10%, respectively, ($n=4$ for each agonist) as well as the accompanying relaxation (by 70±7 and 66±6%, respectively, $n=4$) (Figure 5).

Both PPADS (Figure 3b and c) and suramin (Figure 5d) partly antagonized the NANC e.j.p. evoked by a single pulse of EFS in the presence of apamin and TEA. As shown in Figures 3 and 5, the inhibitory effect was concentration-dependent for both PPADS and suramin; although the effective concentrations of both agents were similar, both PPADS and suramin were distinctly less effective in inhibiting the NANC e.j.p. recorded in the presence of apamin and TEA than the i.j.p. in the absence of apamin and TEA. At the highest concentrations tested, the maximal effect of PPADS (100 μM) and suramin (300 μM) on the NANC e.j.p. averaged 40±5 and 40±10% inhibition ($n=3$ and 4, respectively).

In a separate series of experiments, we investigated the effect of PPADS on the excitatory effect produced by α,β mATP in the presence of apamin (0.1 μM) and TEA (10 mM). In this series of experiments, α,β mATP evoked depolarization (3.5±0.1 mV, $n=3$) and a very small transient contraction (0.05±0.03 mN, $n=3$) followed by relaxation (0.4±0.1 mN, $n=3$) of the circular muscle. PPADS (30 μM) abolished the α,β mATP-induced depolarization and contraction while relaxation was reduced by 57±5% ($n=3$).

Discussion

ATP but not PACAP mediates the apamin-sensitive NANC i.j.p.

ATP was the first candidate proposed as a transmitter for NANC inhibitory transmission in the intestine (Burnstock *et al.*, 1970; Burnstock, 1981; Vladimirova & Shuba, 1984). In the absence of potent and selective antagonists, the parallel blockade of the hyperpolarization induced by purinoceptor agonists and the NANC i.j.p. exerted by the bee venom polypeptide, apamin, has provided support for a transmitter role of ATP. In recent years, blockade of the apamin-sensitive i.j.p. by the proposed selective P_2 purinoceptor antagonist, suramin, has been taken as further evidence for the transmitter role of ATP (Hoyle *et al.*, 1990; Zagorodnyuk & Maggi, 1994).

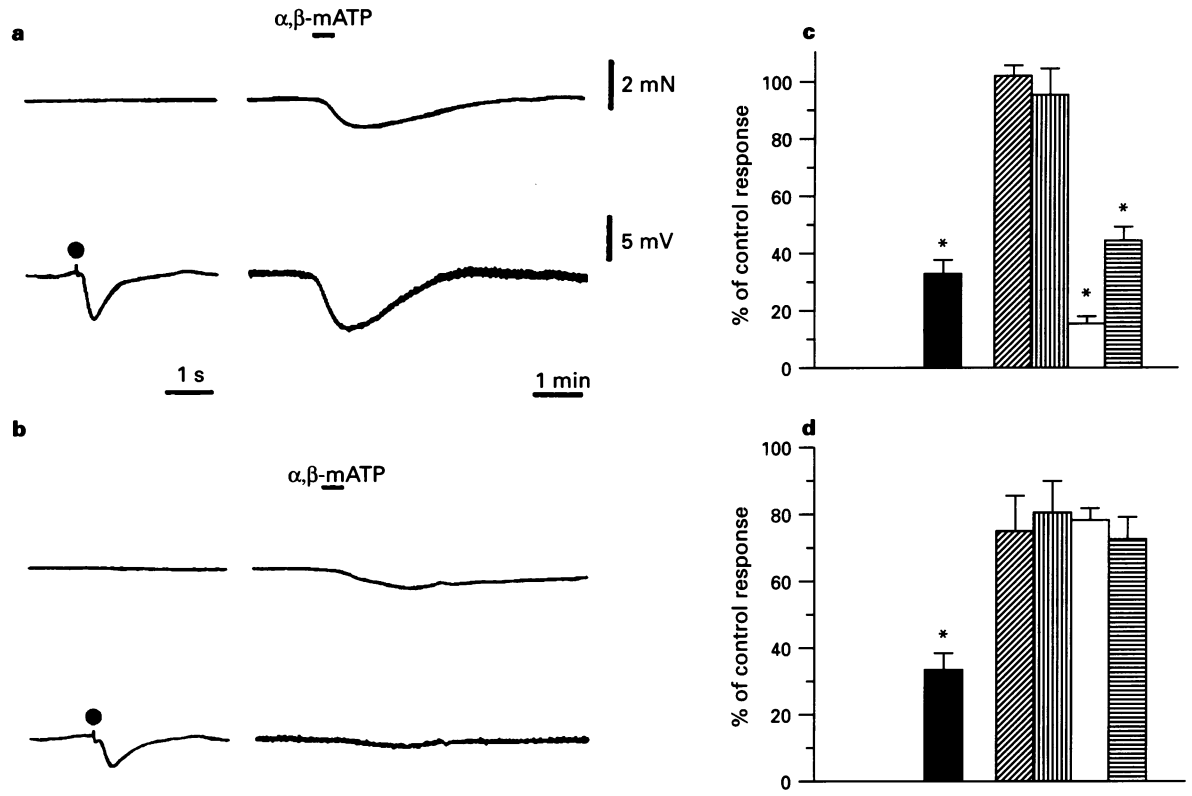

Figure 4 Effect of pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on the EFS-evoked apamin-sensitive i.j.p. and hyperpolarization and relaxation produced by α,β mATP (100 μM for 25 s) or pituitary adenylyl cyclase activating peptide (PACAP; 1 μM for 25 s) in the circular muscle of the guinea-pig colon. In (a) and (b), the upper tracings show mechanical activity and lower tracings show changes in membrane potential in response to EFS (applied at dots) and α,β mATP, in the absence (a) and presence (b) of PPADS (30 μM for 20 min). (c) The effect of PPADS (30 μM for 20 min) on the amplitude of EFS-evoked i.j.p. (solid column) and the hyperpolarization (open column) and relaxation (horizontally-hatched column) evoked by α,β mATP. Control experiments indicate that the second application of α,β mATP induced hyperpolarization (diagonally cross-hatched column) and relaxation (squared column) not significantly different from the first response; $n = 5-12$ experiments, $^*P < 0.05$. (d) The effect of PPADS (30 μM for 20 min) on the amplitude of the EFS-evoked i.j.p. (solid column) and the hyperpolariziation (open column) and relaxation (horizontally-hatched column) evoked by PACAP. The hyperpolarization (diagonally-hatched column) and relaxation (squared column) evoked by a second application of PACAP in control muscle strips did not likewise show significant variations from control; $n = 4-12$ experiments. $^*P < 0.05$.

Under the conditions of the present study, single pulse EFS produced an apamin-sensitive monophasic i.j.p. which is unable to evoke a significant relaxation of the muscle strips (cf. Zagorodynuk & Maggi, 1994; Maggi *et al.*, 1994) but is suitable for discerning the relative roles of ATP and PACAP in its genesis.

Our findings indicate that both apamin and suramin inhibit the TTX-resistant hyperpolarization and relaxation induced by PACAP in the circular muscle of guinea-pig colon, in keeping with results obtained in the guinea-pig taenia coli (Schworer *et al.*, 1992; McConalogue *et al.*, 1995); therefore, suramin cannot be considered a selective purinoceptor antagonist here. On the other hand, the data obtained with the P_2 purinoceptor antagonist PPADS (Lambrecht *et al.*, 1992) strongly indicate that ATP, but not PACAP, is involved in generating the apamin-sensitive i.j.p. in guinea-pig colon. The evidence supporting this conclusion can be summarized as follows: (1) the metabolically stable P_2-purinoceptor agonist, α,β mATP, evoked a TTX-resistant hyperpolarization; (2) apamin blocked to a similar extent the EFS-induced i.j.p. and the α,β mATP-induced hyperpolarization; (3) PPADS likewise blocked to a similar extent the EFS-evoked i.j.p. and the α,β mATP-induced hyperpolarization; (4) PPADS was without effect on the hyperpolarization induced by PACAP.

The P_2 purinoceptor subtype involved in generating the NANC i.j.p. and e.j.p. cannot be established at present. Formerly, it was held that P_{2X}- and P_{2Y}-purinoceptors mediate contraction and relaxation, respectively (Burnstock & Kennedy, 1985); thus, P_{2Y}-purinoceptors were considered to mediate the NANC i.j.p. in guinea-pig taenia coli (Kennedy, 1990). PPADS is considered a preferential P_{2X} antagonist (Ziganshin *et al.*, 1994) but it also inhibits competitively ($pK_B = 5.9$) the P_{2Y}-purinoceptor-stimulated phospholipase C activity in turkey erythrocytes (Boyer *et al.*, 1994). With regard to agonists, α,β mATP is a preferential P_{2X} ligand, yet in preliminary experiments we found that P_{2Y} agonists such as ADPβS and 2-methylthio ATP also produced hyperpolarizations of the circular muscle of the guinea-pig colon (Zagorodnyuk & Maggi, unpublished data). Thus multiple P_2 purinoceptor subtypes may be present in this preparation. Moreover, there is a rapid evolution of the literature in this field, with the identification of several novel subtypes of P_2 purinoceptors (Kennedy & Leff, 1995; Buell *et al.*, 1996). It is important to note, however, that all P_{2X}-purinoceptors are ligand-gated ion channels whilst P_{2Y}-purinoceptors are G-protein coupled. The latency of the PPADS-sensitive i.j.p. in guinea-pig colon is 10 fold longer than that of the P_{2X}-purinoceptor-mediated e.j.p. in guinea-pig vas deferens (Reilly & Hirst, 1996). Moreover, the ATP-induced hyperpolarization of intestinal smooth muscle is produced via increase of intracellular Ca^{2+} and activation of Ca^{2+}-dependent K^+ channels (Zagorodnyuk & Shuba, 1986), as is also the case for the apamin-sensitive i.j.p. described in this study. These observations suggest the involvement of G-protein-coupled P_2 purinoceptors in the PPADS-sensitive i.j.p. of the guinea-pig colon.

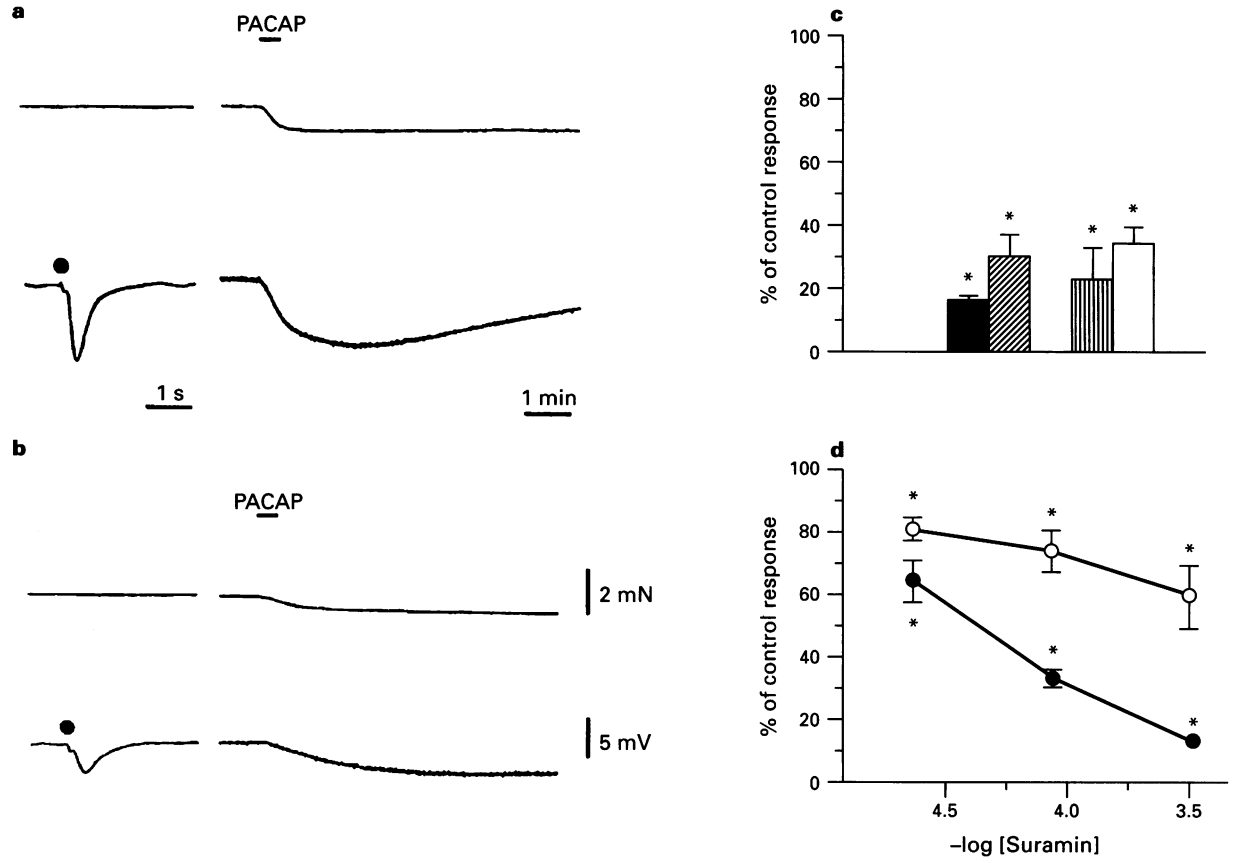

Figure 5 Effect of suramin on EFS evoked NANC i.j.p. and e.j.p. and on hyperpolarization and relaxation produced by α,β mATP or pituitary adenylyl cyclase activating peptide (PACAP) in the circular muscle of the guinea-pig colon (a) and (b). The EFS-evoked NANC i.j.p. (applied at dots) and PACAP-induced (1 μM for 25 s) hyperpolarization and relaxation, in the absence (a) and presence (b) of suramin (100 μM for 30 min). In both (a) and (b), the upper tracings show mechanical activity and lower tracings show changes in membrane potential. (c) The effect of suramin (100 μM for 30 min) on the amplitude of hyperpolarization (solid column) and relaxation (cross-hatched column) evoked by α,β mATP (100 μM for 25 s) and on hyperpolarization (squared column) and relaxation (open column) evoked by PACAP (1 μM for 25 s); $n=4-8$ experiments, $^*P<0.05$. (d) The concentration-dependent inhibition the EFS-evoked NANC i.j.p. (●) and e.j.p. (○) by suramin; $n=3-9$ experiments, $^*P<0.05$.

Similarities and differences in the responses to α,β mATP and PACAP

The present findings indicate both similarities and differences in the mechanism of action of α,β mATP and PACAP on the circular muscle of the guinea-pig colon: both agonists evoked a TTX-resistant hyperpolarization sensitive to potassium channel blockers, apamin and TEA, although important differences appear to exist in mechanisms involved in the coupling between changes in membrane potential and mechanical activity. The hyperpolarization produced by α,β mATP was TTX-resistant and, as expected, apamin alone produced a large inhibitory effect on this response; in parallel, a large fraction of the relaxation was inhibited by apamin. Part of the relaxation induced by α,β mATP is TTX-sensitive: since the experiments were performed in the presence of L-NOARG, this suggests the existence of neuronal P_{2X}-purinoceptors mediating the release of other relaxant transmitters, possibly PACAP or VIP.

The inhibitory responses to PACAP were totally TTX-resistant, indicating a direct action on smooth muscle cells, yet we found some discrepancies between changes in membrane potential and mechanical activity under the action of this neuropeptide. Both apamin and TEA significantly inhibited the PACAP-induced hyperpolarization (by 65 and 50%, respectively) but neither agent alone significantly affected the accompanying relaxation; the combined application of apamin and TEA inhibited the PACAP-induced hyperpolarization by about 80% and relaxation was reduced by about 45% only. It appears therefore that the relaxant action of PACAP involves multiple mechanisms, including some which produce relaxation independently from changes of membrane potential. A similar dissociation was shown recently to occur for relaxation induced by calcitonin gene-related peptide in guinea-pig colon (Maggi *et al.*, 1996). Furthermore, in most cases, the relaxation induced by PACAP in the presence of apamin and TEA was accompanied by a transient depolarization. The latter observation also suggests the existence of multiple PACAP receptors in guinea-pig colon.

The NANC e.j.p. produced in the presence of atropine, tachykinin receptor antagonists and nifedipine

In previous studies we characterized acetylcholine and tachykinins (the latter acting via both NK_1 and NK_2 receptors) as transmitters responsible for e.j.p.s and contraction in the circular muscle of guinea-pig colon (Zagorodnyuk *et al.*, 1993; Maggi *et al.*, 1994). To eliminate such excitatory events, the present experiments were performed in the presence of atropine and of high concentrations of selective and potent tachykinin receptor antagonists. Surprisingly, in the presence of TEA and, even more clearly, in the presence of apamin and TEA, a NANC e.j.p. was revealed in response to single pulse EFS. This finding recalls, to some extent, the observation that a suramin-sensitive e.j.p. is elicited by single pulse EFS in the circular muscle of guinea-pig duodenum in the presence of apamin, L-

NOARG, atropine and tachykinin receptor antagonists, suggesting that, under certain circumstances, ATP may also act as NANC excitatory transmitter in the intestine (Zagorodnyuk *et al.*, 1995).

The NANC e.j.p. detected in this study has several properties which deserve comment: the observation that both PPADS and suramin inhibit the e.j.p. suggest that ATP could be involved, although further studies are needed to make a firm conclusion on this point. In particular, the observation that PPADS completely blocks the depolarization induced by α,β mATP in the presence of apamin and TEA, whilst it inhibits the e.j.p. by about 40% only deserves further investigation. Alternatively, the long latency and time to peak of the e.j.p., and the weak sensitivity to the P_2 antagonist, PPADS and suramin, may argue against the role of ATP in generation of NANC e.j.p. in the guinea-pig colon.

It is interesting to note that apamin alone, while largely inhibiting the i.j.p., did not reveal an e.j.p. in response to single pulse EFS; whilst TEA alone which actually increased the i.j.p., revealed the e.j.p.; and that the PPADS-sensitive e.j.p. evoked in the presence of apamin and TEA had a latency and time to peak about three times longer than the preceding i.j.p. Assuming that ATP may be involved in generating the e.j.p., as suggested by the action of PPADS, it appears that the addition of TEA is essential to demonstrate that ATP-mediated e.j.p. in the colon. This effect of TEA could involve both pre- and postjunctional mechanisms, as indicated by (a) the increased amplitude of the NANC i.j.p. and (b) the appearance of an early depolarization to α,β mATP in presence of TEA alone. We speculate that TEA increases the amount of ATP released per nerve impulse and also makes the postjunctional membrane more responsive to the excitatory action(s) of ATP. The influence of apamin would be that of blocking the effector K^+ channel producing the ATP-induced hyperpolarization, thus enabling the expression of a pure e.j.p. in the presence of TEA. Since apamin alone failed to reveal an e.j.p. and owing to the longer latency and slower time to peak of the e.j.p. as compared to the i.j.p., we suggest that in addition to junctional receptors which mediate the i.j.p., a subset of extrajunctional P_2 purinoceptors may exist which mediate membrane depolarization and the e.j.p. Similarly, the existence of specialized junctional and extrajunctional receptors has been proposed to account for differences between the excitatory action of endogenous and exogenous acetylcholine or ATP in the guinea-pig ileum or vas deferens, respectively (Cousins *et al.*, 1993; Reilly & Hirst, 1996).

Conclusions

We conclude that ATP, but not PACAP, mediates the apamin-sensitive NANC i.j.p. in the circular muscle of the guinea-pig colon. The hyperpolarization produced by the P_2 agonist α,β mATP is sensitive to blockade by the P_2 purinoceptor antagonist, PPADS, and is mainly due to the activation of apamin-sensitive K^+ channels but TEA-sensitive K^+ channels are also partly involved. After blockade of the NANC i.j.p., ATP may act as a fast excitatory transmitter by activating excitatory P_2 purinoceptors. The subtypes of P_2 purinoceptors involved in the generation of the NANC i.j.p. and e.j.p. remain to be established. The data suggest that the excitatory P_2 purinoceptors may be located extrajunctionally.

This work is dedicated to Prof. Ernst Mutschler, J.W. Goethe Universitat, Frankfurt/Main, Germany, on his 65th birthday.

References

ARTEMENKO, D.P., BURY, V.A., VLADIMIROVA, I.A. & SHUBA, M.F. (1982). Modification of the single sucrose-gap. *Physiol. Zhur.*, **28,** 374–380.

BENNETT, M.R., BURNSTOCK, G. & HOLMAN, M.E. (1966). Transmission from perivascular inhibitory nerves to the smooth muscle of the guinea-pig taenia coli. *J. Physiol.*, **182,** 527–540.

BOECKXTAENS, PELCKMANS, P.A., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990). Non-adrenergic non-cholinergic relaxations mediated by nitric oxide in the canine ileocolonic junction. *Eur. J. Pharmacol.*, **190,** 239–246.

BOYER, J.L., ZOHN, I.E., JACOBSON, K.A. & HARDEN, T.K. (1994). Differential effects of P_2-purinoreceptor antagonists on phospholipase C- and adenylyl cyclase-coupled P_{2y}-purinoceptors. *Br. J. Pharmacol.*, **113,** 614–620.

BRIDGEWATER, M., CUNNANE, T.C. & BRADING A.F. (1995). Characteristic features of inhibitory junction potentials evoked by single stimuli in the guinea-pig isolated taenia caeci. *J. Physiol.*, **485,** 145–155.

BUELL, G., LEWIS, C., COLLO, G., NORTH, R.A. & SURPRENANT, A. (1996). An antagonist-insensitive P_{2x} receptor expressed in epithelia and brain. *EMBO J.*, **15,** 55–62.

BURNSTOCK, G. (1981). Neurotransmitter and trophic factors in the autonomic nervous system. *J. Physiol.*, **313,** 1–35.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P2-purinoceptor? *Gen. Pharmacol.*, **16,** 433–440.

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

COSTA, M., FURNESS, J.B. & HUMPHREYS, C.M.S. (1986). Apamin distinguishes two types of relaxation mediated by enteric nerves in the guinea-pig gastrointestinal tract. *Naunyn-Schmeiderberg's Arch. Pharmacol.*, **332,** 79–88.

COUSINS, H.M., EDWARDS, F.R., HRST, G.D.S. & WENDT, I.R. (1993). Cholinergic neuromuscular transmission in the longitudinal muscle of the guinea-pig ileum. *J. Physiol.*, **471,** 61–86.

CRIST, J.R., HE, X.D. & GOYAL, R.K. (1992). Both ATP and the peptide VIP are inhibitory neurotransmitters in the guinea-pig ileum circular muscle. *J. Physiol.*, **447,** 119–131.

EMONDS-ALT, X., DOUTREMEPUICH, J.D., HEAULME, M., NELIAT, G., SANTUCCI, V., STEINBERG, R., VILAIN, P., BICHON, D., DUCOUX, J.P., PROIETTO, V., VAN BROECK, D., SOUBRIE, P., LE FUR, G. & BRELIERE, J.C. (1993). In vitro and in vivo biological activities of SR 140,333 a novel potent nonpeptide tachykinin NK_1 receptor antagonist. *Eur. J. Pharmacol.*, **250,** 403–413.

GRIDER, J.R. & RIVIER, J.R. (1990). Vasoactive intestinal peptide (VIP) as transmitter of inhibitory motor neurons of the gut: evidence from use selective VIP antagonists and VIP antiserum. *J. Pharmacol. Exp. Ther.*, **253,** 738–742.

HOYLE, C.H.V. (1987). A modified single sucrose gap-junction potentials and electronic potentials in gastro-intestinal smooth muscle. *J. Pharmacol. Methods*, **18,** 219–226.

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

JIN, J.G., KATSOULIS, S., SCHMIDT, W.E. & GRIDER, J.R. (1994). Inhibitory transmission in taenia coli mediated by distinct vasoactive intestinal peptide and apamin-sensitive pituitary adenylate cyclase-activating peptide receptors. *J. Pharmacol. Exp. Ther.*, **270,** 433–439.

KENNEDY, C. (1990). P_1- and P_2-purinoceptor subtypes - an update. *Arch. Int. Pharmacodyn. Ther.*, **303,** 30–50.

KENNEDY, C. & LEFF, P. (1995). How should P_{2x}-purinoceptors be classified pharmacologically? *Trends Pharmacol. Sci.*, **16,** 168–174.

LAMBRECHT, G., FRIEBE, T., GRIMM, U., WINDSCHEIF, U., BUNGARDT, E., HILDEBRANNDT, C., BAUMERT, H.G., SPATZ-KUMBEL, G. & MUTSCHLER, E. (1992). PPADS, a novel functionally selective antagonist of P2-purinoreceptor mediated responses. *Eur. J. Pharmacol.*, **217,** 217–219.

MAGGI, C.A. & GUILIANI, S. (1993). Multiple inhibitory mechanisms mediate NANC relaxation in the circular muscle of the guinea-pig colon. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **347,** 630–634.

MAGGI, C.A., GIULIANI, S. & ZAGORODNYUK, V.P. (1996). Calcitonin gene-related peptide (CGRP) in the circular muscle of guinea-pig colon: role as inhibitory transmitter and mechanisms of relaxation. *Regul. Pept.*, **61,** 27–36.

MAGGI, C.A., ZAGORODNYUK, V. & GIULIANI, S. (1994). Specialization of tachykinin NK_1 and NK_2 receptors in producing fast and slow atropine-resistant neurotransmission to the circular muscle of the guinea-pig colon. *Neuroscience*, **63**, 1137–1152.

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

MCCONALOGUE, K., FURNESS, B., VREMEC, M.A., HOLST, J.J., TORNOE, K. & MARLEY, P.D. (1995). Histochemical, pharmacological, biochemical and chromatographic evidence that pituitary adenylyl cyclase activating peptide is involved in inhibitory neurotransmission in the taenia of the guinea-pig caecum. *J. Autonom. Nerv. System*, **50**, 311–322.

MCELROY, A.B., CLEGG, S.P., DEAL, M.J., EWAN, G.B., HAGAN, R.M., IRELAND, S.J., JORDAN, C.C., PORTER, B., ROSS, B.C., WARD, P. & WHITTINGTON, A.R. (1992). Highly potent and selective heptapeptide antagonists of the neurokinin NK_2 receptor. *J. Med. Chem.*, **35**, 2582–2591.

REILLY, M.J. & HIRST, G.D.S. (1996). Differences in the response to purinergic nerve stimulation and applied ATP in the guinea-pig vas deferens. *J. Autonom. Nerv. System*, **57**, 93–100.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.*, **262**, G379–G392.

SCHWORER, H., KATSOULIS, S., CREUTZFELDT, W. & SCHMIDT, W.E. (1992). Pituitary adenylate cyclase activating peptide, a novel VIP-like gut-brain peptide, relaxes the guinea-pig taenia caeci via apamin-sensitive potassium channels. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **346**, 511–514.

VLADIMIROVA, I.A. & SHUBA, M.F. (1984). Synaptic processes in smooth muscle. *Neurophysiology*, **16**, 307–319.

ZAGORODNYUK, V.P. & 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.

ZAGORODNYUK, V.P., SANTICIOLI, P. & MAGGI, C.A. (1993). Tachykinin NK_1 but not NK_2 receptor mediate non-cholinergic excitatory junction potentials in the circular muscle of guinea-pig colon. *Br. J. Pharmacol.*, **110**, 195–803.

ZAGORODNYUK, V.P., SANTICIOLI, P., MAGGI, C.A. & GIACHIETTI, A. (1995). Evidence that tachykinin NK_1 and NK_2 receptors mediate non-adrenergic non-cholinergic excitation and contraction in the circular muscle of guinea-pig duodenum. *Br. J. Pharmacol.*, **115**, 237–246.

ZAGORODNYUK, V.P. & SHUBA, M.F. (1986). The nature of nonadrenergic inhibition in smooth muscle of human intestine. *Neurophysiology*, **18**, 373–381.

ZIGANSHIN, A.U., HOYLE, C.H.V., LAMBRECHT, G., MUTSCHLER, E., BAUMERT, H.G. & BURNSTOCK, G. (1994). Selective antagonism by PPADS at P_{2X} purinoceptors in rabbit isolated blood vessels. *Br. J. Pharmacol.*, **111**, 923–929.

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