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Pharmacological evidence for the existence of multiple P2 receptors in the circular muscle of guinea-pig colon

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1 By using the sucrose gap technique, we have investigated the effect of the metabolically stable P2Y receptor agonist, adenosine 5′-O-2-thiodiphosphate (ADPβS), on the membrane potential and tension in the circular muscle of the guinea-pig proximal colon. All experiments were performed in the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), nifedipine (1 μM), L-nitroarginine (L-NOARG, 100 μM) and of the tachykinin NK_1 and NK_2 receptor antagonists, SR 140333 (0.1 μM) and GR 94800 (0.1 μM), respectively.

2 ADPβS (100 μM for 15 s) evoked a tetrodotoxin- (1 μM) resistant hyperpolarization and contraction of the smooth muscle. In the presence of apamin (0.1 μM), the ADPβS-induced hyperpolarization was converted to depolarization and the contraction was potentiated while tetraethylammonium (TEA, 10 mM) did not affect significantly the response to ADPβS. The combined application of apamin and TEA reproduced the effect observed with apamin alone.

3 Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acids (PPADS, 30 μM) slightly but significantly increased the ADPβS-induced hyperpolarization, while the contraction evoked by ADPβS was reduced by about 80%. Suramin (100 μM) did not affect the ADPβS-induced hyperpolarization but totally blocked the ADPβS-induced contraction. In the presence of suramin (100 μM), a small relaxation of the circular muscle was observed upon application of ADPβS.

4 The contraction and hyperpolarization evoked by ADPβS were abolished in Ca^{2+}-free Krebs solution. The blocker of sarcoplasmic reticulum Ca^{2+} pump, cyclopiazonic acid (10 μM) reduced contraction and hyperpolarization induced by ADPβS by about 60 and 50%, respectively.

5 A comparison of our present and previous findings enables to conclude that at least 3 types of P2 receptors are present on the smooth muscle of the guinea-pig colon, as follows: (1) inhibitory P2 receptors, producing an apamin-sensitive hyperpolarization, which are activated by α,β-methylene ATP (α,β-meATP) and by endogenously released purines, sensitive to suramin and PPADS; (2) inhibitory P2 receptors, producing an apamin-sensitive hyperpolarization, which are activated by ADPβS and are resistant to suramin and PPADS; (3) excitatory P2 receptors, producing contraction, which are activated by ADPβS and are sensitive to suramin and PPADS. The data also support the idea of the existence of a restricted pool of specialized junctional P2 receptors producing the apamin-sensitive NANC inhibitory junction potential in response to endogenous ligand(s).

Keywords: Non-adrenergic non-cholinergic (NANC); ATP; ADPβS; PPADS; suramin; guinea-pig colon

Introduction

Adenosine 5′-triphosphate (ATP) has long been suggested to act as a nonadrenergic noncholinergic (NANC) inhibitory neurotransmitter in the mammalian intestine (Burnstock, 1981; Vladimirova & Shuba, 1984; Manzini *et al*., 1986; Zagorodnyuk & Shuba, 1986; Zagorodnyuk *et al*., 1989). More recently, other roles have been proposed for ATP as an enteric neurotransmitter: ATP, or a related P2 receptor ligand, participates in the residual excitatory junction potential (e.j.p.) produced by nerve stimulation in the presence of atropine and tachykinin NK_1 and NK_2 receptor antagonists in the circular muscle of guinea-pig stomach, duodenum and colon (Zagorodnyuk *et al*., 1995; 1996; Zagorodnyuk & Maggi, 1997). Moreover, ATP could act as a synaptic transmitter in the myenteric plexus (Lepard *et al*., 1996), by regulating for e.g. acetylcholine release (Bartho *et al*., 1997).

Following the initial classification based on pharmacological criteria (Burnstock & Kennedy, 1985), the molecular cloning of P2 receptors has now generated structural criteria which are used to distinguish between up to 7 types of ligand-gated cation channels P2X receptors and up to 7 types of metabotropic, G protein-coupled, P2Y receptors (Fredholm *et al*., 1997; Evans, 1996; Webb *et al*., 1996).

Previously, we showed that the putative P2X receptor agonist α,β-methyleneATP (α,β-meATP) produces an apamin-sensitive hyperpolarization in the circular muscle of guinea-pig colon (Zagorodnyuk *et al*., 1996). The effect of α,β-meATP is blocked by pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), a P2X receptor antagonist (Lambrecht *et al*., 1992). PPADS also blocks the apamin-sensitive NANC inhibitory junction potential (i.j.p.) produced by electrical field stimulation (EFS) of intramural nerves (Maggi & Giuliani, 1993; Zagorodnyuk & Maggi, 1994; Zagorodnyuk *et al*., 1993; 1996), suggesting that P2X receptors mediate the junctional effects of ATP in this preparation. However, two factors weaken this interpretation: (a) the long latency of the apamin-sensitive NANC i.j.p. in guinea-pig colon can hardly be explained with the involvement of a ligand gated ion channel (Zagorodnyuk *et al*., 1996); (b) the mobilization of intracellular Ca^{2+} and secondary activation of Ca^{2+}-dependent K^+ conductance is responsible for the P2 receptor-activated hyperpolarization and for the genesis of the apamin-sensitive NANC i.j.p. (Den Hertog *et al*., 1985; Zagorodnyuk & Shuba, 1986; Zagorodnyuk *et al*., 1989), a chain of events more consistent with

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the involvement of a metabotropic receptor rather than of a ligand-gated cation channel.

Recently, two relaxation-mediating P2 receptors have been suggested to exist in the smooth muscle of the guinea-pig taenia coli: a prototypic P2Y receptor, for which the most potent agonists are adenosine 5′-O-2-thiodiphosphate (ADPβS) and 2-methylthio ATP and a separate P2 receptor activated by α,β-meATP (Dudeck *et al*., 1995; Windscheif *et al*., 1995; Bultmann *et al*., 1996).

In view of the above, it appeared of interest to investigate, in the same experimental conditions of our previous study (Zagorodnyuk *et al*., 1996), the effect of a preferential P2Y receptor agonist to determine to what extent the response to a ligand of this type overlaps or differs from that observed with α,β-meATP. We therefore studied the electrical and mechanical responses produced in the circular muscle of the guinea-pig colon by the metabolically stable P2Y receptor agonist, ADPβS (Welford *et al*., 1986; Khakh *et al*., 1995).

Methods

A single sucrose-gap method, 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; 1996). The strips were superfused with warmed ($35\pm0.5^{\circ}$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. EFS was produced by means of platinum wire electrodes connected to a Grass S88 stimulator and a SIU5 stimulus isolation unit.

Junction potentials were routinely evoked by single pulse of EFS (30 V, 0.1 ms) in each strip examined to ensure its viability at attainment of steady state conditions. Although the effect of various drugs (apamin, TEA, apamin and TEA, PPADS, suramin) on the NANC i.j.p. was checked in the course of present experiments (Figures 1 and 2), the results were comparable to those obtained previously (Zagorodnyuk *et al*., 1996) and are not systematically presented in this study.

The Krebs solution routinely contained atropine (1 μM) and guanethidine (3 μM) to block the effect of excitatory cholinergic nerves and adrenergic inhibitory nerves of extrinsic origin, respectively. Indomethacin (3 μM) was used to exclude the possible involvement of prostanoids. Nifedipine (1 μM) was used to inhibit spontaneous electrical and mechanical activity and evoked action potentials. The selective NK_1 and NK_2 receptor antagonists, SR 140333 (0.1 μM) (Emonds-Alt *et al*., 1993) and GR 94800 (0.1 μM) (McElroy *et al*., 1992), respectively, were used to prevent the possible effect of endogenous tachykinins on smooth muscle; the nitric oxide synthase inhibitor, L-nitroarginine (L-NOARG, 100 μM) was used to block NO-mediated, apamin-resistant component of the NANC i.j.p.

All drugs, including ADPβS, were added to the Krebs solution and applied by superfusion. The metabolically stable agonist, ADPβS was used to stimulate selectively P2Y receptors. In preliminary experiments, a concentration of 100 μM ADPβS, applied in the superfusate for 15 s, was selected: this concentration produced a hyperpolarization of comparable magnitude to that of the EFS-evoked NANC i.j.p. The effect of ADPβS was reproducible if two consecutive applications of the agonists were made at 10 min or longer intervals.

The concentrations of PPADS (30 μM for 20 min) and suramin (100 μM for 30 min) were selected from previous experiments (Zagorodnyuk *et al*., 1996) in which they were shown to produce about 60 and 70% inhibition of the apamin-sensitive NANC i.j.p. evoked by single pulse of EFS, respectively.

In some experiments the effect of ADPβS was investigated after 10 and 20 min superfusion in a nominally Ca^{2+}-free medium containing 1 mM EDTA and the concentration of $MgSO_4$ was increased up to 3 $mmol^{-1}$. ADPβS was applied twice in Ca^{2+}-free Krebs solution after 10 and 20 min perfusion with the Ca^{2+}-free medium.

Data evaluation and statistical analysis

All data in the text are mean $\pm$ s.e.mean. Statistical analysis was performed by means of Student's t test for paired or unpaired data, or by means of analysis of variance if applicable. A P level <0.05 was considered to be statistically significant.

Drugs

Drugs used were: atropine HCl (Serva, Heidelberg, Germany); guanethidine sulphate (ICF); N^{ω}-nitro-L-arginine (L-NOARG), nifedipine, indomethacin, apamin, adenosine 5′-O-(2-thiodiphosphate) (ADPβS), cyclopiazonic acid (CPA) (Sigma); tetraethylammonium chloride (TEA, Merck); 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. Emonds-Alt, Sanofi Research Ctr., Montpellier, France.

Results

In the presence of atropine (1 μM), guanethidine (3 μM), indomethacin (3 μM), nifedipine (1 μM), L-nitroarginine (L-NOARG, 100 μM) and of the tachykinin NK_1 and NK_2 antagonists SR 140333 (0.1 μM) and GR 94800 (0.1 μM), respectively, a single pulse of EFS (30 V, 0.1 ms) produced a NANC i.j.p. (about 10 mV in amplitude) of the circular muscle of the guinea-pig proximal colon without producing significant changes in tension (Figure 1).

As described in detail previously (Zagorodnyuk *et al*., 1996, Table 1), apamin alone inhibited the NANC i.j.p., whilst TEA alone potentiated the NANC i.j.p.; apamin plus TEA abolished the NANC i.j.p. and, in the majority of cases tested, disclosed a NANC excitatory junction potential (Figure 1a); both suramin (Figure 2a) and PPADS inhibited the NANC i.j.p. induced by single pulse EFS (Table 1).

Effects of ADPβS

The P2Y purinoceptor agonist, ADPβS (100 μM for 15 s, $n=11$) invariably evoked hyperpolarization (11.4 ± 0.7 mV) and a phasic contraction (1.8 ± 0.4 mN) (Figure 1b). In 7 out of 11 strips the hyperpolarization induced by ADPβS

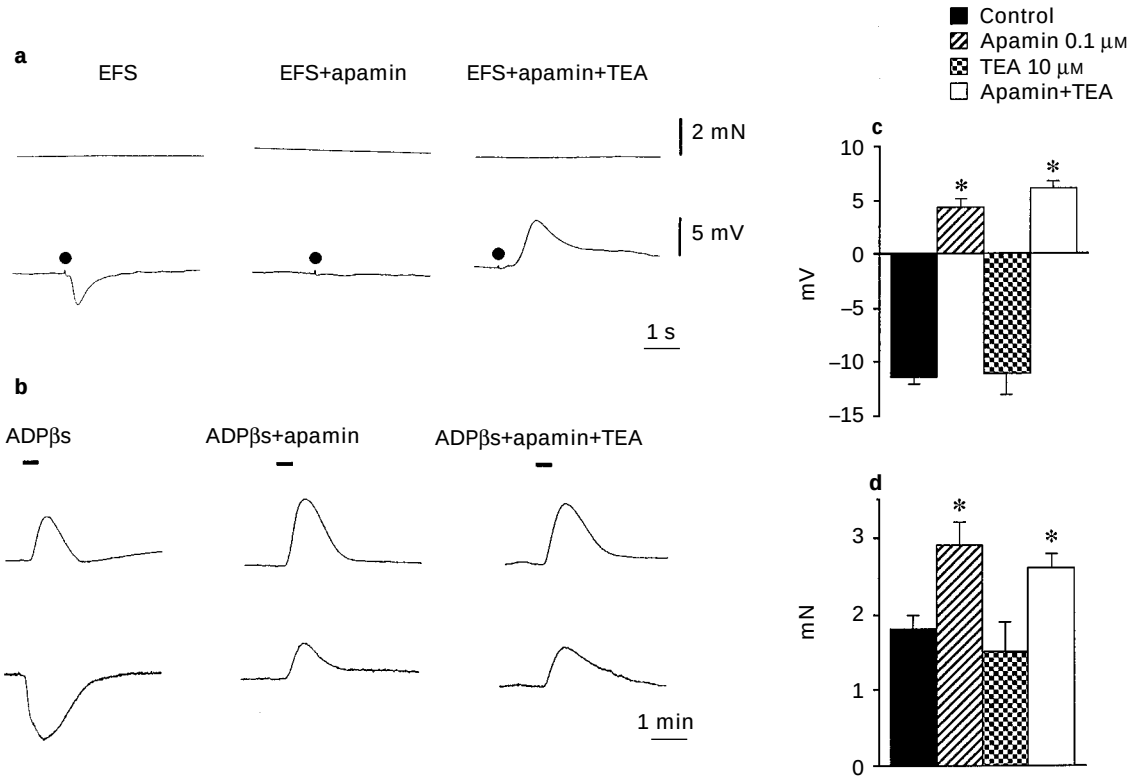

Figure 1 In (a) and (b) typical tracings are shown, representative of the effect of apamin (0.1 μM) and apamin plus TEA (10 mM) on the junction potential evoked by single pulse EFS (30 V, 0.1 ms, applied at dots) and on changes in membrane potential and tension induced by application of ADPβS (100 μM for 15 s) in the circular muscle from guinea-pig proximal colon. The effect of apamin is shown after 25 min superfusion; the effect of TEA in apamin-containing Krebs solution is shown after 20 min superfusion. In both (a) and (b), the upper trace shows mechanical activity and the lower trace is membrane potential. In (c) and (d) quantitative data are shown of the effect of apamin, TEA or apamin plus TEA on changes in membrane potential and tension induced by ADPβS, respectively. Each value is mean$\pm$s.e.mean of 4–10 experiments. *Significantly different from controls, $P<0.05$.

Table 1 A comparison of the effect of apamin (0.1 μM), tetraethylammonium (TEA, 10 mM), apamin plus TEA, PPADS (30 μM) and suramin (100 μM) on the amplitude of the NANC i.j.p. evoked by EFS and on the hyperpolarization evoked by α,β-meATP (100 μM) and ADPβS (100 μM) in the circular muscle of the guinea-pig colon

	NANC i.j.p.	*α,β-meATP*	*ADPβS*
Apamin	−90%*	−95%*	block[d]
	($n=12$)	($n=5$)	($n=5$)
TEA	+115%[a,]*	−44%*	0%
	($n=16$)	($n=7$)	($n=4$)
Apamin	−84%[b,]*	−88%[c,]*	100%[d]
+TEA	($n=5$)	($n=10$)	($n=13$)
PPADS	−84%*	−83%*	+25%*
	($n=5$)	($n=5$)	($n=4$)
Suramin	−66%*	−84%*	+19%
	($n=9$)	($n=4$)	($n=4$)

Data on the NANC i.j.p. and hyperpolarization induced by α,β-meATP are from Zagorodnyuk *et al.* (1996). *$P<0.05$. [a]i.j.p. was followed by e.j.p. [b]In an additional 28 preparations only e.j.p. was recorded. [c]In all strips depolarization was observed before hyperpolarization. [d]Only depolarization was recorded.

was followed by a long-lasting depolarization (1.2$\pm$0.3 mV) and a tonic contraction (1.0$\pm$0.2 mN) (Figures 1, 2 and 3). A late small relaxation (0.5$\pm$0.1 mN) was observed in 4 strips.

Tetrodotoxin (1 μM, $n=4$) did not significantly affect the hyperpolarization (+14$\pm$6%) and contraction (+2$\pm$2%) induced by ADPβS.

Effect of apamin and TEA on the responses to ADPβS

Apamin (0.1 μM, $n=5$) totally blocked the hyperpolarization induced by ADPβS (100 μM), unmasking a depolarization (4.3$\pm$0.8 mV) and potentiated the contraction induced by ADPβS (2.9$\pm$0.3 mN, $n=5$) (Figure 1b).

TEA (10 mM, $n=4$), had no significant effect on the hyperpolarization and contraction induced by ADPβS (100 μM, $n=4$) (Table 1).

The combined application of apamin and TEA ($n=10$) totally blocked the hyperpolarization induced by ADPβS (100 μM): in the presence of apamin and TEA a depolarization (6.1$\pm$0.6 mV) and contraction (2.6$\pm$0.2 mN) were observed, similar to the effect produced by apamin alone (Figure 1b).

Effects of PPADS and suramin on the responses to ADPβS

PPADS (30 μM for 20 min, $n=4$) slightly but significantly increased the ADPβS-induced hyperpolarization (11.5$\pm$0.4 and 14.4$\pm$0.6 mV, in the absence and presence of PPADS, respectively, $P<0.05$), while the contraction evoked by ADPβS was markedly inhibited (1.4$\pm$0.2 and 0.25$\pm$0.2 mN in the absence and presence of PPADS, respectively, $P<0.05$) (Figure 2, Table 1).

Suramin (100 μM for 30 min, $n=4$) did not affect the ADPβS-induced hyperpolarization (12.4$\pm$1.6 and 14.7$\pm$0.9 mV in the absence and presence of suramin, respectively), but completely blocked the ADPβS-induced contraction (Figure 2, Table 1). Indeed, a small relaxation (0.3$\pm$0.4 mN,

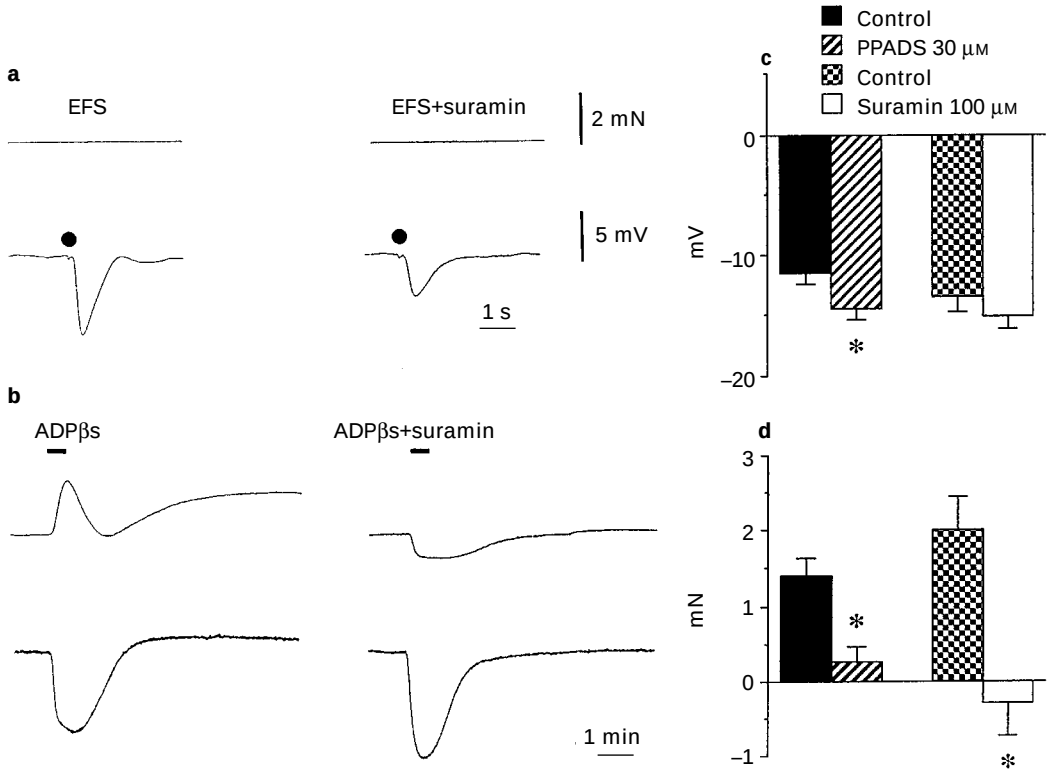

Figure 2 In (a) and (b) typical tracings are shown, representative of the effect of suramin (100 μM) on the i.j.p. evoked by EFS (30 V, 0.1 ms, applied at dots) and on changes in membrane potential and tension induced by application of ADPβS (100 μM for 15 s) in the circular muscle from guinea-pig proximal colon. The effect of suramin is shown after 30 min of superfusion. In both (a) and (b), the upper trace shows mechanical activity and the lower trace is membrane potential. In (c) and (d) quantitative data are shown of the effect of PPADS (30 μM) or suramin (100 μM) on the ADPβS-induced changes in membrane potential and tension, respectively. Each value is mean$\pm$s.e.mean of 4–10 experiments. *Significantly different from controls, $P<0.05$.

$n=4$) of the circular muscle was observed upon application of ADPβS in the presence of suramin (100 μM) (Figure 2).

We observed previously that a PPADS-sensitive NANC excitatory junction potential (e.j.p.) was unmasked by apamin and TEA in response to single pulse EFS in the presence of tachykinin receptor antagonists (Zagorodnyuk *et al*., 1996). Therefore, we studied the effect of PPADS on the excitatory effect produced by ADPβS in the presence of apamin (0.1 μM) and TEA (10 mM). In these conditions, the ADPβS-induced depolarization (6.3$\pm$0.7 mV) was not significantly affected (19$\pm$9% inhibition, $n=8$, NS) by PPADS. On the other hand, the ADPβS-induced contraction (2.0$\pm$0.3 mN) was inhibited by 60$\pm$10% ($n=8$, $P<0.05$).

Effects of Ca^{2+}-free Krebs solution and cyclopiazonic acid on the ADPβS-induced response

We next investigated the effects of Ca^{2+}-free Krebs solution and of the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, cyclopiazonic acid (CPA) on the ADPβS-induced contraction to clarify which source of Ca^{2+} (intra- or extracellular) is involved in the action of this drug.

The first application of ADPβS (100 μM for 15 s) in Ca^{2+}-free EDTA (1 mM)-containing Krebs solution evoked a transient hyperpolarization which was about 3 fold smaller than in control (10.9$\pm$0.7 and 2.8$\pm$0.5 mV, in normal and Ca^{2+}-free Krebs solution, respectively, $n=4$, $P<0.05$, Figure 3a). On the other hand, the long-lasting depolarization induced by ADPβS was not significantly affected (2.5$\pm$0.8 and 3.7$\pm$0.4 mV, in normal and Ca^{2+}-free Krebs solution, respectively, $n=4$). The contraction (2.7$\pm$0.5 mN, $n=4$) induced by ADPβS was strongly inhibited (by 92$\pm$2%, $n=4$) in Ca^{2+}-free Krebs solution. The second application of ADPβS in Ca^{2+}-free EDTA (1 mM)-containing Krebs solution produced only depolarization (2.8$\pm$0.3 mV, $n=4$, Figure 3a).

The depolarization (7.6$\pm$0.4 mV, $n=4$) induced by ADPβS in the presence of apamin (0.1 μM) and TEA (10 mM) was reduced (by 38$\pm$2%, $n=4$, $P<0.05$) if elicited in Ca^{2+}-free EDTA (1 mM)-containing Krebs solution. The concomitant contraction (3.2$\pm$1.4 mN, $n=3$) was strongly inhibited (92$\pm$1%, $n=4$, $P<0.05$).

CPA (10 μM, $n=4$) evoked transient depolarization (2.2$\pm$0.3 mV, in 3 out of 7 strips) followed by a small hyperpolarization (1.9$\pm$0.5 mV, in 5 out of 7 strips) and sustained increase of muscle tone (1.8$\pm$0.2 mN, $n=7$) which persisted through the whole period of application of CPA (30 min). The ADPβS-induced hyperpolarization and contraction were reduced by CPA (10 μM) by 52$\pm$6% ($n=4$, $P<0.05$) and 62$\pm$2% ($n=4$, $P<0.05$), respectively (Figure 3b). CPA (10 μM, $n=4$) also reduced the ADPβS-induced long-lasting depolarization and tonic contraction by 55$\pm$11% and 68$\pm$8%, respectively ($P<0.05$). At the same time the NANC i.j.p. was increased by CPA (10 μM for 30 min) by 53$\pm$6% ($P<0.05$, $n=7$) (Figure 3b).

Discussion

At the present time, the information on the structure of P2X and P2Y receptors largely outscores the availability of the selective ligands (agonists and antagonists) which would be needed for a proper pharmacological analysis of the role of

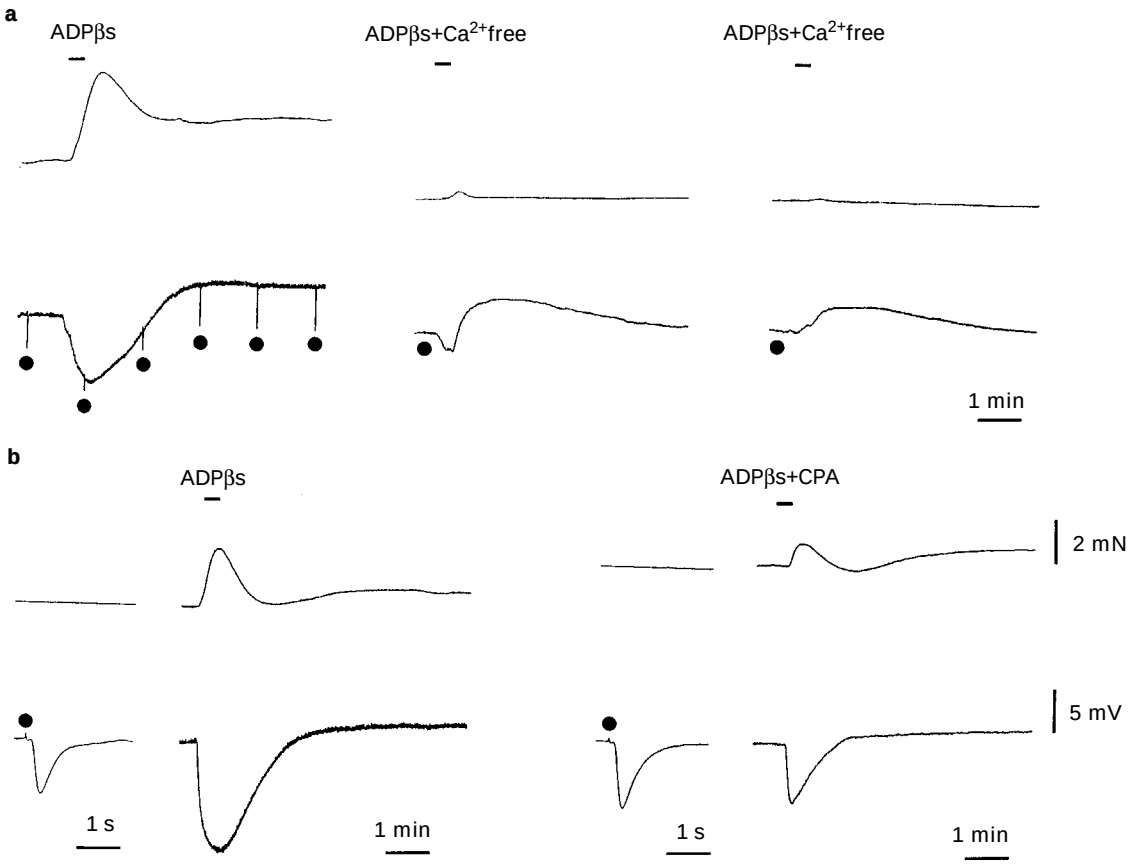

Figure 3 In (a) and (b) typical tracings are shown, representative of superfusion with Ca^{2+}-free Krebs solution or CPA (10 μM), respectively, on the NANC i.j.p. evoked by a single pulse of EFS (30 V, 0.1 ms, applied at dots) and changes in membrane potential and tension induced by ADPβS (100 μM for 15 s)-induced response in the circular muscle of the guinea-pig colon. In (a) the responses to EFS and ADPβS are shown in normal Krebs and after 10 and 20 min superfusion in Ca^{2+}-free solution. In (b), the responses to EFS and ADPβS are shown before and 30 min after the beginning of superfusion with CPA. In both (a) and (b) the upper trace is mechanical activity and the lower trace shows membrane potential.

different P2 receptors in purinergic transmission. In particular, no antagonists are available which, in absolute terms, discriminate between P2X and P2Y receptors. Although PPADS was originally claimed to be a preferential P2X receptor antagonist (Lambrecht *et al*., 1992; Ziganshin *et al*., 1994) this compound, like suramin, effectively blocks some P2Y receptors (Boyer *et al*., 1994; Charlton *et al*., 1996; Bultmann *et al*., 1996). Likewise agonist selectivity is poor: α,β-meATP is usually regarded as a selective P2X ligand but it efficiently acts as an agonist in preparations expressing prototypical P2Y receptors, such as the guinea-pig taenia caeci and rat duodenum (Windscheif *et al*., 1995; Bultmann *et al*., 1996). Also, some cloned P2X receptors do not respond to α,β-meATP (Evans, 1996 for review). At the same time, the preferential P2Y receptor agonist, ADPβS, is known to contract the rat urinary bladder not only via 'ADPβS-sensitive' receptors but also via P2X receptors (Suzuki & Kokubun, 1994).

Redundancy in the expression of different receptor types could be a rule rather than an exception for P2 receptors: up to 5 different P2Y receptors have been shown to be expressed on rat neonatal cardiac myocytes (Webb *et al*., 1996). A comparison of our present and previous (Zagorodnyuk *et al*., 1996) results (Table 1) indicates the existence of multiple types of P2 receptor modulating membrane potential and tension in the circular muscle of the guinea-pig colon. A conclusive statement about their P2X/P2Y nature could only be made following a molecular analysis of the receptors expressed at this level.

A striking aspect of the action of ADPβS in the guinea-pig colon was its ability to induce concomitantly hyperpolarization and contraction. Both effects are tetrodotoxin-resistant, implying a direct action of ADPβS on the colonic smooth muscle. Since the experiments were performed in the presence of indomethacin and L-NOARG, we can exclude a tetrodotoxin-resistant generation of prostanoids/nitric oxide in their genesis. It is worth noting that the effect of another preferential P2Y receptor agonists, 2-methylthio ATP is similar to that of ADPβS:2-methylthio ATP (100 μM) evoked a suramin-resistant hyperpolarization accompanied by a suramin-sensitive contraction of the circular muscle of the guinea-pig colon (Zagorodnyuk & Maggi, unpublished observation).

The present findings indicate that ADPβS acts on at least two different receptors in the circular muscle of the guinea-pig colon:(1) a class of PPADS/suramin-resistant P2 receptors which mediates hyperpolarization; (2) a class of PPADS/suramin-sensitive P2 receptors which mediate a nifedipine-resistant contraction of the smooth muscle. A comparison (Table 1) of our previous results with EFS and α,β-meATP and present results with ADPβS indicates that the receptor mediating hyperpolatization in response to α,β-me-ATP is sensitive to blockade by PPADS/suramin and is therefore pharmacologically distinct from the one which is activated by ADPβS. Importantly, the pharmacology of the hyperpolarization activated by α,β-meATP matches that of the apamin-sensitive NANC i.j.p. evoked by single pulse

EFS (Table 1). Therefore, the same receptor activated by α,β-meATP appears also to be junctionally activated by an endogenous ligand for P2 receptors, whilst the two receptors activated by ADPβS do not appear to have a role in junctional transmission activated by a single pulse EFS. It is possible that the inhibitory and excitatory receptors activated by ADPβS are located extrajunctionally: in other words, only the PPADS-sensitive inhibitory receptor activated by α,β-meATP would possess a specialized junctional localization (Hirst *et al*., 1992) suitable for its activation in response to the release of minute amounts of transmitter (Zagorodnyuk *et al*., 1996).

Recently two different relaxation-mediating P2 receptors have been suggested to exist in the smooth muscle of the guinea-pig taenia coli (Dudeck *et al*., 1995; Windscheif *et al*., 1995; Bultmann *et al*., 1996). Our findings demonstrate that there are two types of inhibitory P2 receptors in the circular muscle of the guinea-pig colon, a PPADS/suramin-sensitive receptor activated by α,β-meATP and a PPADS/suramin-resistant receptor activated by ADPβS (Table 1). We suggest that they could both represent different types of the P2Y receptors family. The data supporting this suggestion are as follows: (i) the hyperpolarization evoked by ATP, α,β-meATP and ADPβS in the intestinal smooth muscle is due to the mobilization of intracellular Ca^{2+} and the secondary activation of Ca^{2+}-dependent K^+ channels (Den Hertog *et al*., 1985; Zagorodnyuk & Shuba, 1986; Zagorodnyuk *et al*., 1989; present study); (ii) the latency of the apamin-sensitive NANC i.j.p. which has an ionic mechanism similar to that of the ATP- and α,β-meATP-mediated hyperpolarization, is about 10 fold longer than that of the P2X receptor-mediated NANC e.j.p. in the guinea-pig vas deferens (Zagorodnyuk *et al*., 1989; Reilly & Hirst, 1996; Zagorodnyuk *et al*., 1996). Therefore, the events which occur following the activation of the inhibitory P2 receptors are likely to occur through the involvement of G protein-mediated receptors rather than ligand-gated cation channels.

A class of suramin-sensitive P2 receptor activated by ADPβS, defined as 'ADPβS-sensitive' receptors, has been found in the rat urinary bladder and anococcygeus muscle in addition to classical P2X receptors antagonized by desensitization of α,β-me-ATP (Suzuki & Kokubun, 1994; Najbar *et al*., 1996). The ADPβS-induced depolarization observed in the circular muscle of the guinea-pig colon in the presence of apamin is PPADS-resistant while the contraction induced by ADPβS is PPADS-sensitive. At this stage we cannot determine whether the depolarization is due to activation of a fourth type of P2 purinoceptor by ADPβS or whether the same receptor mediating the ADPβS-induced hyperpolarization also determines the depolarization to ADPβS in the presence of apamin.

The activation of G protein-coupled P2 receptors usually evokes an increase in intracellular Ca^{2+} concentration which is due to both Ca^{2+} mobilization from intracellular stores and Ca^{2+} entry (Boarder *et al*., 1995). In the circular muscle of guinea-pig colon, the contraction evoked by ADPβS involves the influx of extracellular Ca^{2+} through nifedipine-resistant Ca^{2+} channels, since the contraction evoked by ADPβS was almost totally abolished in Ca^{2+}-free Krebs solution. At the same time, cyclopiazonic acid (CPA), an inhibitor of Ca^{2+}-ATPase in sarcoplasmic reticulum (Seidler *et al*., 1989), which can be used as a selective tool to estimate intracellular Ca^{2+} stores (Uyama *et al*., 1993) significantly reduced the ADPβS-induced contraction. The discrepancy in evaluating of the ratio between intra- and extracellular Ca^{2+} sources involved in the ADPβS-induced contraction may be explained by the fact that CPA evoked a sustained increase in muscle tone on its own. This elevation in tone may lead to an overestimation of the inhibitory action of CPA on the ADPβS-induced contraction. Alternatively, the CPA-sensitive Ca^{2+} stores could have been depleted during the incubation of the circular muscle strips in Ca^{2+}-free Krebs solution containing EDTA (1 mM).

CPA increased the amplitude of the apamin-sensitive NANC i.j.p. evoked by EFS. Under the same experimental conditions, the α,β-me-ATP-induced hyperpolarization was inhibited by CPA (10 μM) by about 70% (Zagorodnyuk & Maggi, unpublished observation). Qualitatively, the effect of CPA resembles that exerted by TEA:CPA reduced the hyperpolarization evoked by α,β-meATP and increased the amplitude of i.j.p. (Zagorodnyuk *et al*., 1996). We suggest that an increase in the amplitude of i.j.p. may be due to an increase in the amount of ATP released from nerve terminals. CPA could decrease the rate of fall of the action potential in prejunctional nerve terminals, as that has been shown to occur in several smooth muscle cells (Imaizumi *et al*., 1996), and/or it may induce a sustained increase of free Ca^{2+} concentration in the nerve terminals by blocking Ca^{2+}- ATPase of endoplasmic reticulum.

In conclusion, the present findings indicate that application of the metabolically stable P2 receptor agonist ADPβS induces complex changes in membrane potential and tension of the circular muscle of guinea-pig colon which appear to involve at least two different types of P2 receptors. The receptors activated by ADPβS do not appear to be involved in PPADS/suramin-sensitive neuromuscular transmission to single pulse of EFS (Zagorodnyuk *et al*., 1996), supporting the idea of the existence of a restricted pool of junctional P2 receptors in the circular muscle of the guinea-pig colon.

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