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Dependence of P2-nucleotide receptor agonist-mediated endothelium-independent relaxation on ectonucleotidase activity and A_{2A}-receptors in rat portal vein

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1 The mechanism of action of P2 nucleotide receptor agonists that produce endothelium-independent relaxation and the influence of ecto-ATPase activity on this relaxing effect have been investigated in rat portal vein smooth muscle.

2 At 25°C, ATP, 2-methylthioATP (2-MeSATP) and 2-chloroATP (2-ClATP), dose-dependently inhibited spontaneous contractile activity of endothelium-denuded muscular strips from rat portal vein. The rank order of agonist potency defined from the half-inhibitory concentrations was 2-ClATP (2.7 ± 0.5 μM, $n=7$) > ATP (12.9 ± 1.1 μM, $n=9$) $\geqslant$ 2-MeSATP (21.9 ± 4.8 μM, $n=4$). In the presence of $\alpha\beta$-methylene ATP ($\alpha\beta$-MeATP, 200 μM) which itself produced a transient contractile effect, the relaxing action of ATP and 2-MeSATP was completely abolished and that of 2-ClATP strongly inhibited.

3 The non-selective P2-receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 100 μM) did not affect the relaxation induced by ATP, 2-MeSATP, and 2-ClATP.

4 The A_{2A}-adenosine receptor antagonist ZM 241385 inhibited the ATP-induced relaxation in a concentration-dependent manner (1–100 nM). In the presence of 100 nM ZM 241385, the relaxing effects of 2-MeSATP and 2-ClATP were also inhibited.

5 ADP, AMP and adenosine also produced concentration-dependent inhibition of spontaneous contractions. The relaxing effects of AMP and adenosine were insensitive to $\alpha\beta$-MeATP (200 μM) but were inhibited by ZM 241385 (100 nM).

6 Simultaneous measurements of contraction and ecto-ATPase activity estimated by the degradation of [γ-^{32}P]-ATP showed that muscular strips rapidly (10–60 s) hydrolyzed ATP. This ecto-ATPase activity was abolished in the presence of EDTA and was inhibited by $57 \pm 11\%$ ($n=3$) by 200 μM $\alpha\beta$-MeATP.

7 These results suggest that ATP and other P2-receptor agonists are relaxant in rat portal vein smooth muscle, because ectonucleotidase activity leads to the formation of adenosine which activates A_{2A}-receptors.

Keywords: Smooth muscle; P2-purinoceptors; A_{2A}-receptors; ecto-ATPases

Introduction

ATP is a well recognized extracellular messenger that exerts a variety of effects on numerous cell types by activating P2-nucleotide receptors (North & Barnard, 1997). In blood vessels, ATP has multiple effects in both arteries and veins (Dubyak & El-Moatassim, 1993). ATP-induced vasoconstriction has been ascribed to the activation of P2X-nucleotide receptor subtypes located on the surface of vascular smooth muscle cells (Reilly & Burnstock, 1987; Pacaud *et al*., 1994). ATP also mediates vasodilatation involving both endothelium-dependent and endothelium-independent mechanisms (Ravelic & Burnstock, 1991; Dubyak & El-Moatassim, 1993). The endothelium-dependent relaxation is clearly related to the presence of $P2Y_1$- and $P2Y_2$-purinoceptor subtypes in endothelial cells (Ravelic & Burnstock, 1996; Pirotton *et al*., 1996). In contrast, the P2Y-purinoceptor subtype(s) of vascular smooth muscle cells involved in the endothelium-independent relaxation has not been firmly identified. In addition to these unidentified relaxing P2Y-nucleotide receptor subtypes, vascular smooth muscle also expresses relaxing P1-purinoceptors, probably the A_2 subtype (Tucker & Linden, 1993; Corr & Burnstock, 1994).

Extracellular membrane-bound nucleotidases (ectonucleotidases) which are present in most tissues (Ziganshin *et al*., 1994; Plesner, 1995) sequentially dephosphorylate ATP to ADP, AMP and adenosine and it is generally assumed that these enzymes terminate the neurotransmitter action of ATP (Westfall *et al*., 1996). Several ectonucleotidases, including ecto-ATPase from chick smooth muscle have been cloned (Kirley, 1997). Recently, in addition to these membrane-bound enzymes, soluble nucleotidases, which break down ATP to adenosine have been shown to be released with ATP by sympathetic nerves innervating the guinea pig vas deferens (Todorov *et al*., 1997; Kennedy *et al*., 1997). AMP and adenosine formed from ATP breakdown by nucleotidases might subsequently produce their own effects via P1-purinoceptors.

The relationship between ectonucleotidase activity and the vasoactive effects of ATP is unclear. However, two recent observations suggest that the influence of ectonucleotidase activity on the response to P2-purinoceptor agonists has to be evaluated. (1) The development of inhibitors of ecto-ATPase activity have led to the observation that the potency of P2-nucleotide receptor agonists was greatly influenced by breakdown by ectonucleotidases (Kennedy & Leff, 1995). (2) Several P2-nucleotide receptor agonists and antagonists (Chen *et al*., 1996; Ziganshin *et al*., 1996; Chen & Lin, 1997) have been shown to have inhibitory action on ectonucleotidase activity

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which could partly account for their action in pharmacological experiments.

The aim of the present study was, therefore, to examine the endothelium-independent relaxation induced by ATP and other P2Y-nucleotide receptor agonists and to define the influence of ectonucleotidase activity on this relaxing effect in rat portal vein smooth muscle.

Methods

Wistar rats (150 g) were stunned and then killed by cervical dislocation. Portal veins were removed and placed into normal physiological solution (PSS). The veins were cleaned of adherent connective tissue and opened longitudinally. The endothelium was carefully removed by gently rubbing the intimal surface with the tip of small forceps. The cleaned veins were then cut into longitudinal strips (5–7 mm long, 1 mm wide).

Contraction measurement

The small strips of portal veins were tied at each end with a single silk thread to the tips of two hooks, one of which was connected to a force transducer (AE 801, SensoNor, Norway). Strips were placed in a well on a bubble plate (Horiuti, 1988) filled with PSS (230 μl) and stretched to about 1.3 resting length. The preparations were left to equilibrate for 1 h and washed at 20 min intervals. The solution was rapidly changed by sliding the plate to an adjacent well. All the experiments were performed at room temperature which prevented rapid evaporation and maintained constant conditions in the course of the experiments. The absence of endothelium was confirmed in each strips by the inability of carbachol (10 μM) to relax phenylephrine (1 μM)-induced contraction. Successive concentration-response curves to purinoceptor agonists were separated by a minimal time interval of 20 min.

To quantify the effects of purinoceptor agonists, the mean amplitude of spontaneous contractions was determined over a 3 min period before and during agonist application. The relaxing effect of purinoceptor agonists was then expressed as the percentage of the mean amplitude under control conditions.

Ecto-ATPase assay

Ecto-ATPase activity of muscular strips was determined by measuring the amount of [^{32}P]-P_i released from [γ-^{32}P]-ATP. After the 1 h equilibration period in 230 μl PSS (see previous section), the strips were successively transferred in fresh PSS containing 100 μM ATP supplemented with 3.5 μCi ml^{-1} [γ-^{32}P]-ATP for 10, 20, 30, 45 and 60 s. Medium (200 μl) taken from each well was transferred to an Eppendorf containing activated charcoal (100 mg ml^{-1} in 0.5 N HCl), then centrifuged at 15,000 r.p.m. for 10 min. Radioactivity in the supernatant (200 μl) was then measured by using a β-counter. The strips were washed three times, then equilibrated in PSS in the presence or absence of 200 μM $\alpha\beta$-Me ATP. After 45 min, the degradation of [γ-^{32}P]-ATP was measured again. For each strip, ecto-ATPase activity was estimated by plotting the amount of released [^{32}P]-P_i in control and in the presence of $\alpha\beta$-Me ATP against time. The amount of released [^{32}P]-P_i was expressed as a percentage of the [^{32}P]-P_i release under control conditions over a period of 60 s. No change was observed when two measurements were successively performed under control conditions. Low Ca^{2+}-Mg^{2+} solution was prepared by adding 4 mM EDTA to PSS.

Solutions

The normal physiological saline solution (PSS) contained (in mM): NaCl 130, KCl 5.6, $MgCl_2$ 1, $CaCl_2$ 2, glucose 11 and Tris 10; pH 7.4 with HCl. All experiments were performed at room temperature (25°C).

Statistics

All results are expressed as the mean ± s.e. of mean with n being the number of independent experiments. Significance was tested by means of Student's t test. $P<0.05$ was considered significant.

Chemicals and drugs

Adenosine 5′-triphosphate (ATP; disodium salt), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), adenosine and $\alpha\beta$-methyleneATP ($\alpha\beta$-MeATP) were purchased from Sigma (Saint Quentin Fallavier, France). 2-methylthioATP (2-MeSATP), 2-chloroATP (2-ClATP) and pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) were obtained from Research Biochemicals International (Natick, MA). ZM 241385 was purchased from Tocris Cookson (Bristol, U.K.). [γ-^{32}P]-ATP (4500 Ci mmol^{-1}) was purchased from ICN Pharmaceutical (Irvine, CA).

Results

Relaxing effect of ATP and analogues (2-ClATP and 2-MeSATP)

Smooth muscle strips from rat portal vein displayed spontaneous rhythmic mechanical activity. Recordings

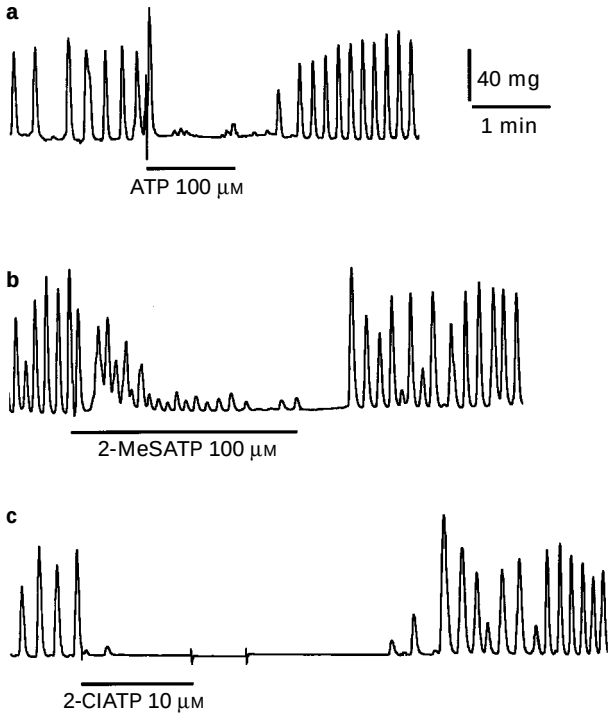

Figure 1 Effect of ATP and analogues on the spontaneous contractile activity of endothelium-denuded rat portal vein strips. ATP (100 μM, a), 2-MeSATP (100 μM, b) and 2-ClATP (10 μM, c) applied for the indicated period abolished the spontaneous contractions.

obtained with an expanded time scale show that after a transient contracting effect, ATP (100 μM) and 2-MeSATP (100 μM) suppressed the spontaneous contractions (Figure 1a,b). The rhythmic contractile activity recovered in less than 1 min after washout. 2-ClATP (10 μM) had no contractile effect and was more potent at abolishing the spontaneous contractions (Figure 1c). The inhibitory action of 2-ClATP was maintained and the spontaneous contractile activity was restored only several minutes after the removal of 2-ClATP. The contracting effect of ATP desensitized by repetitive stimulations whereas similar relaxing responses could be obtained by repetitive applications of ATP, even at high concentration (100 μM; Figure 2a). The relaxing effect of ATP, 2-ClATP and 2-MeSATP was concentration-dependent (Figure 2b–d). The rank order of agonist potency defined from the half-inhibitory concentrations was 2-ClATP (2.7 ± 0.5 μM, $n=7$) > ATP (12.9 ± 1.1 μM, $n=9$) $\geqslant$ 2-MeSATP (21.9 ± 4.8 μM, $n=4$).

$\alpha\beta$-MeATP inhibits the relaxing effect of ATP, 2-MeSATP and 2-ClATP

$\alpha\beta$-MeATP (200 μM) transiently induced an increase in the basal tone, the amplitude and the frequency of spontaneous contractions. Prolonged application of $\alpha\beta$-MeATP led to the desensitization of the response and after 15–30 min, both basal tone and spontaneous contractile activity had returned to initial level. These effects of $\alpha\beta$-MeATP are similar to those previously obtained and ascribed to P2X-purinoceptor activation (Reilly & Burnstock, 1987). After treatment of strips with $\alpha\beta$-MeATP for 45 min, the effects of ATP (100 μM) were totally abolished (Figure 2a,b). $\alpha\beta$-MeATP (200 μM,

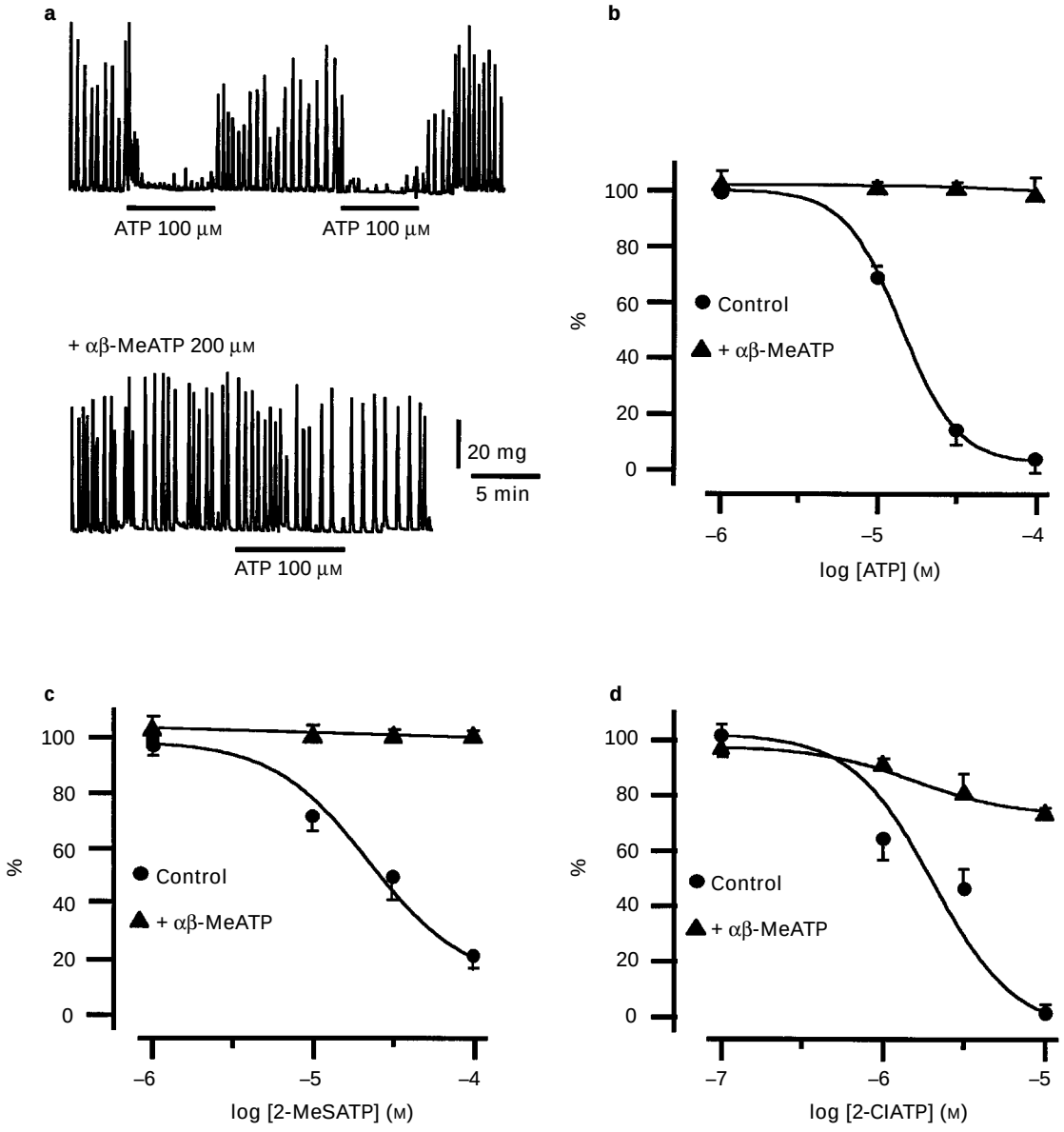

Figure 2 Concentration-dependence and inhibition by $\alpha\beta$-MeATP of ATP-, 2-MeSATP- and 2-ClATP-induced relaxation. (a) Typical traces showing that ATP-induced relaxation could be repetitively evoked by consecutive applications of ATP (top) and was inhibited by $\alpha\beta$-MeATP (200 μM) added 45 min before ATP application (bottom). (b–d) Concentration-dependence of the inhibition of spontaneous contractions induced by ATP (b), 2-MeSATP (c) and 2-ClATP (d) obtained in the absence or in the presence of 200 μM $\alpha\beta$-MeATP. The results are expressed as the percentage of the mean amplitude of spontaneous contractions measured under control conditions. Each point represents mean and vertical lines show s.e.mean of 3–9 experiments.

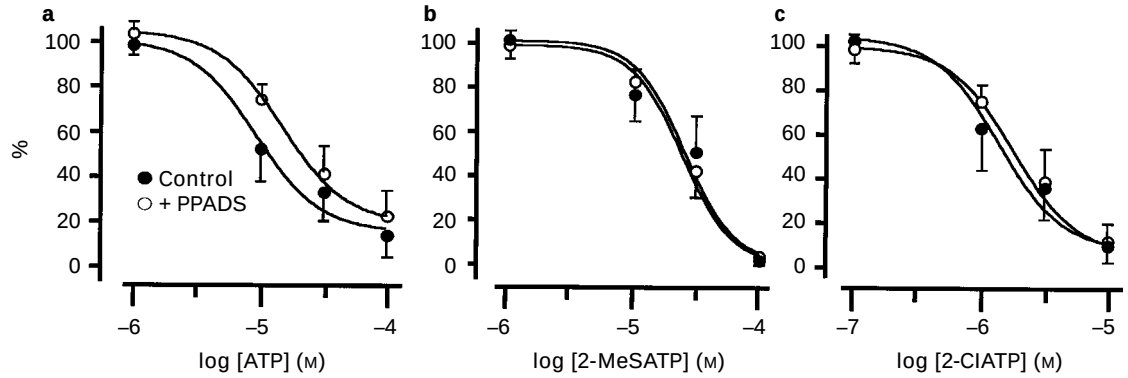

Figure 3 Effect of PPADS on the relaxing effect of ATP, 2-MeSATP and 2-ClATP. Concentration-dependence of the inhibition of spontaneous contractions induced by ATP (a), 2-MeSATP (b) and 2-ClATP (c) obtained in the absence or in the presence of 100 μM PPADS. The results are expressed as the percentage of the mean amplitude of spontaneous contractions measured under control conditions, in the absence of agonist. Each point represents mean and vertical lines show s.e.mean of 4 experiments.

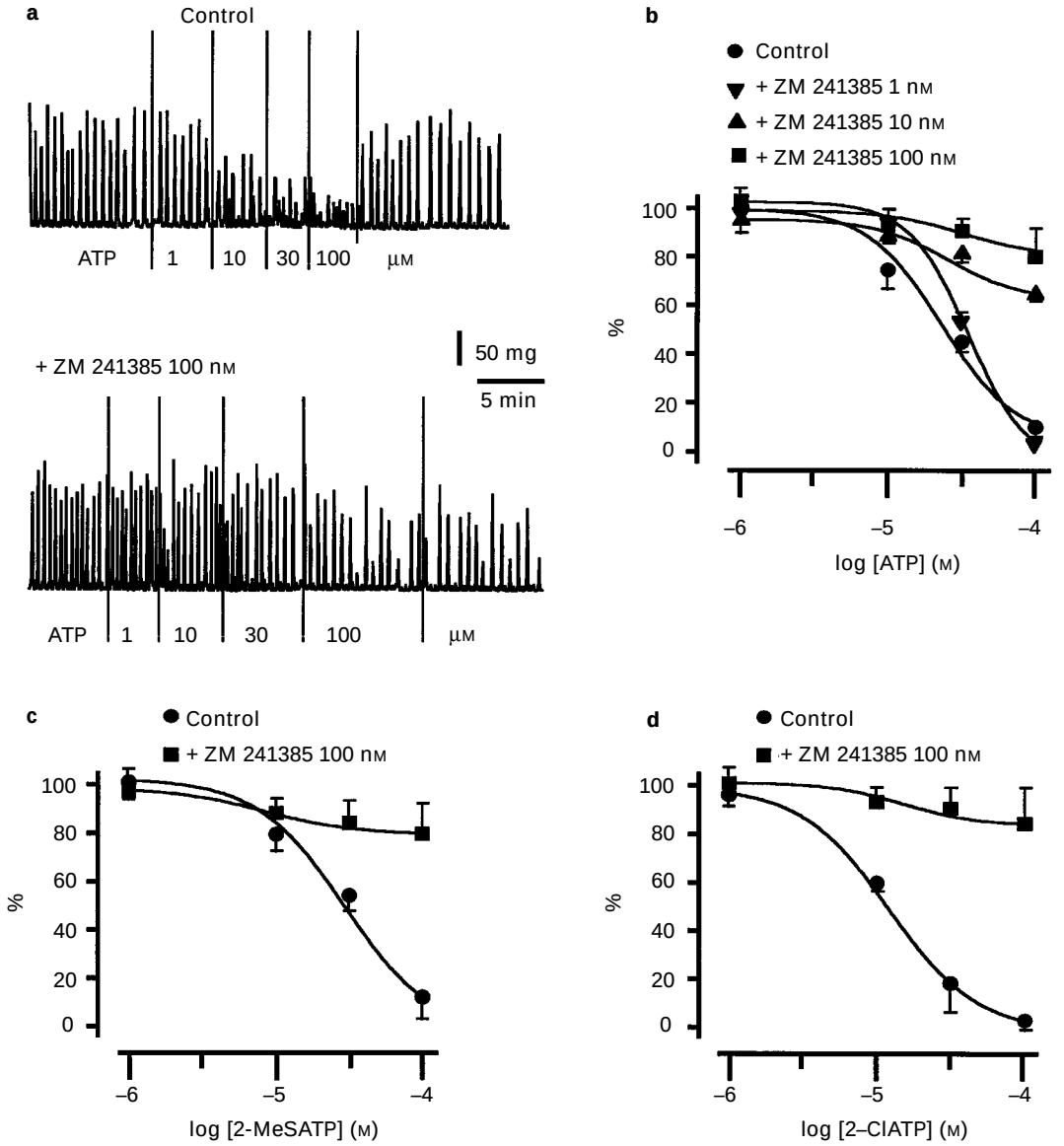

Figure 4 Effect of ZM 241385 on the relaxing effect of ATP, 2-MeSATP and 2-ClATP. (a) Typical traces showing that the gradual inhibition of spontaneous contractions by increasing ATP concentrations (top) was abolished in the presence of ZM 241385 (100 nM) added 30 min before (bottom). (b–d) Concentration-dependence of the inhibition of spontaneous contractions induced by ATP (b), 2-MeSATP (c) and 2-ClATP (d) obtained in the absence or in the presence of 100 nM ZM 241385. (b) Also shows concentration-response curve to ATP obtained in the presence of 1 nM and 10 nM ZM 241385. The results are expressed as the percentage of the mean amplitude of spontaneous contractions measured under control conditions, in the absence of agonist. Each point represents mean and vertical lines show s.e.mean of 3–5 experiments.

45 min) also inhibited the relaxing effect of 2-MeSATP and 2-ClATP (Figure 2c,d).

Lack of effect of PPADS on the ATP and ATP analogue-induced relaxation

PPADS has been shown to be an antagonist of P2-receptors (Lambrecht, 1996; Charlton *et al*., 1996). Concentration-response curves for the relaxing effect of ATP, 2-MeSATP and 2-ClATP were not significantly modified in the presence of PPADS (100 μM, 30 min; Figure 3). The half-inhibitory concentrations were 14.4 ± 2.9 μM in control and 19.6 ± 4.2 μM in the presence of PPADS for ATP ($n=4$, $P>0.3$); 28.5 ± 7.9 μM in control and 31.8 ± 13.7 μM ($n=4$, $P>0.8$) in the presence of PPADS for 2-MeSATP and 2.6 ± 0.3 μM in control and 2.1 ± 0.6 μM ($n=4$, $P>0.06$) in the presence of PPADS for 2-ClATP.

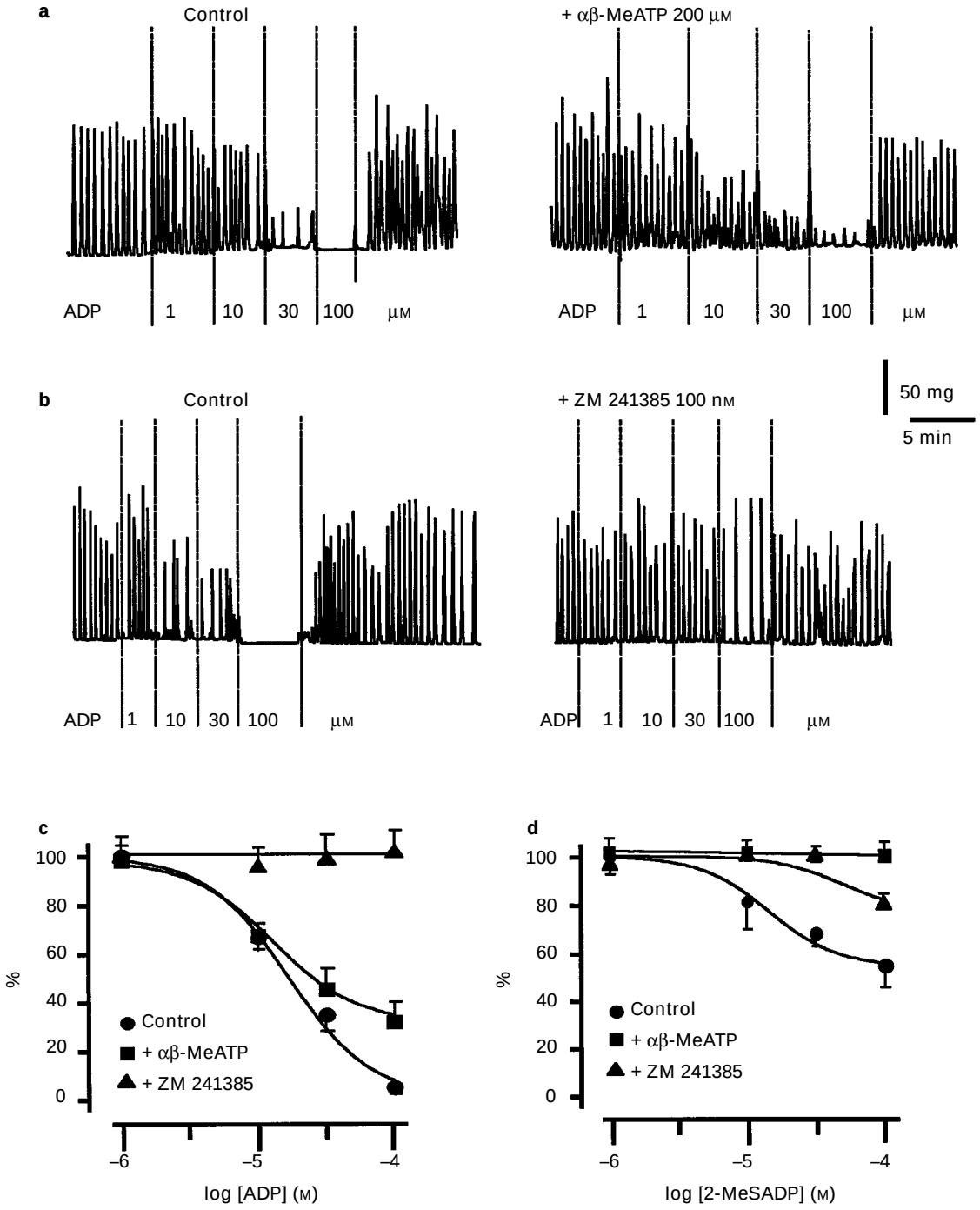

Figure 5 Relaxing effect of ADP and 2-MeSADP. (a,b) Typical traces showing that the gradual inhibition of spontaneous contractions by increasing ADP concentrations (left) was partially inhibited in the presence of 200 μM $\alpha\beta$-MeATP (a, right) and was abolished in the presence of 100 nM ZM 241385 (b, right) added 30 min before. (c,d) Concentration-dependence of the inhibition of spontaneous contractions induced by ADP (c) and 2-MeSADP (d) obtained under control conditions, in the presence of 200 μM $\alpha\beta$-MeATP or in the presence of 100 nM ZM 241385. The results are expressed as the percentage of the mean amplitude of spontaneous contractions measured under control conditions, in the absence of agonist. Each point represents mean and vertical lines show s.e.mean of 4–7 experiments.

Inhibition of the P2-purinoceptor agonist-induced relaxation by ZM 241385

ZM 241385 is a highly selective A_{2A}-adenosine receptor antagonist (Poucher *et al*., 1995). ZM 241385 inhibited the ATP-induced relaxation in a concentration-dependent manner (1–100 nM, Figure 4a,b). In the presence of 100 nM ZM 241385, the relaxing effect of 2-MeSATP (1–100 μM) and 2-ClATP (0.1–10 μM) was also nearly abolished (Figure 4c, d). In contrast, neither the P2X-induced contracting effect of $\alpha\beta$-MeATP in portal vein, nor the P2Y-mediated rise in intracellular Ca^{2+} concentration induced by UTP in rat aortic

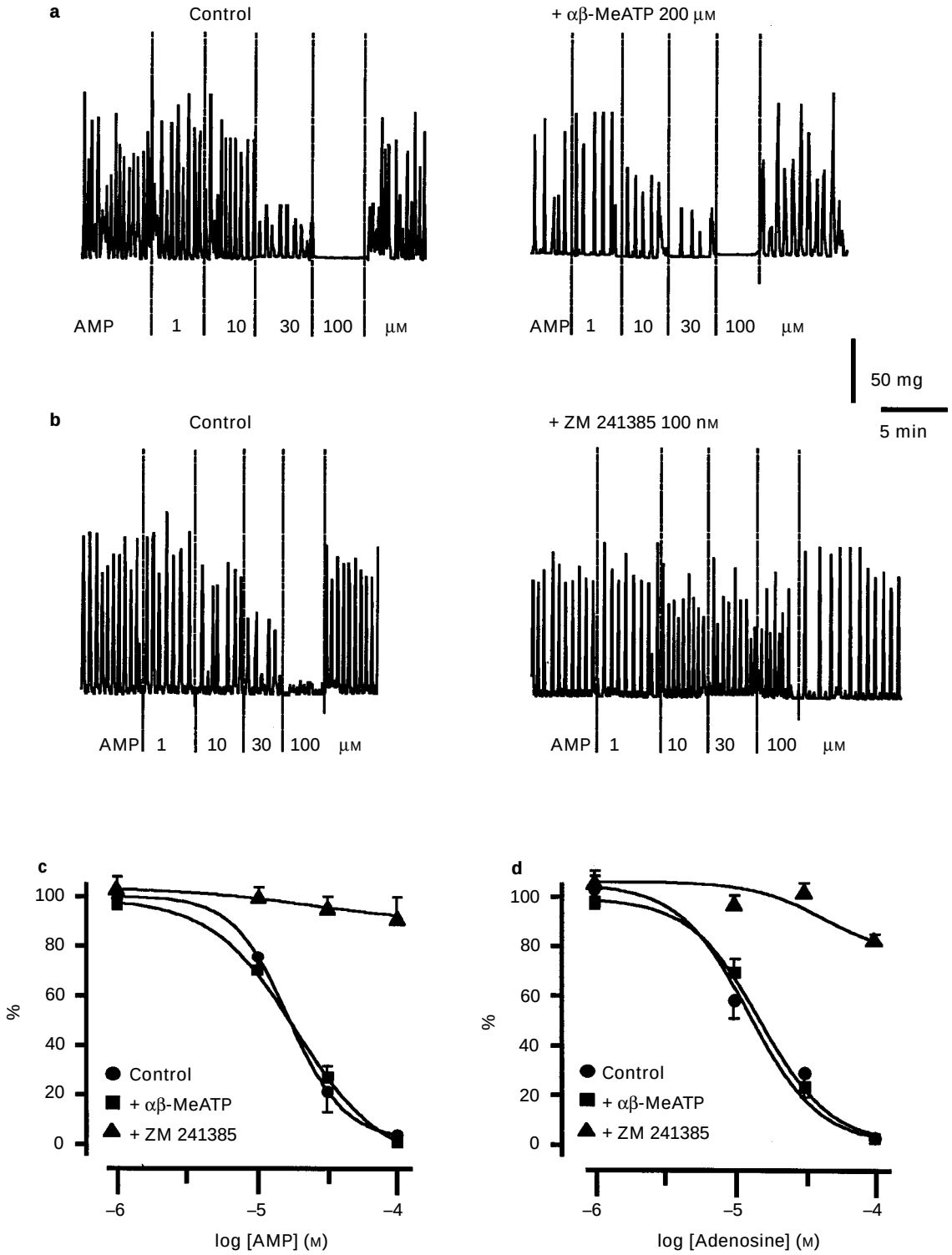

Figure 6 Relaxing effect of AMP and adenosine. (a,b) Typical traces showing that the gradual inhibition of spontaneous contractions by increasing AMP concentrations (left) was not modified in the presence of 200 μM $\alpha\beta$-MeATP (a, right) but was abolished in the presence of 100 nM ZM 241385 (b, right) added 30 min before. (c, d) Concentration-dependence of the inhibition of spontaneous contractions induced by AMP (c) and adenosine (d) obtained under control conditions, in the presence of 200 μM $\alpha\beta$-MeATP or in the presence of 100 nM ZM 241385. The results are expressed as the percentage of the mean amplitude of spontaneous contractions measured under control conditions, in the absence of agonist. Each point represents mean and vertical lines show s.e.mean of 4–7 experiments.

myocytes (Pacaud *et al*., 1995), nor the P2Y-mediated relaxation induced by ATP in longitudinal intestinal smooth muscle of rat ileum (Pacaud *et al*., 1996), were affected by 1 μM ZM 241385, suggesting that P2-receptor-mediated responses were not affected by the A_{2A}-receptor antagonist (not shown).

Effects of ADP, AMP and adenosine

ADP, 2-MeSADP, AMP and adenosine also produced concentration-dependent inhibition of spontaneous contractions (Figures 5 and 6). ADP (100 μM) completely abolished the automatic contractions and the concentration of ADP producing half-inhibition was estimated to 16.2 ± 1.9 μM ($n=6$; Figure 5a,c). 2-MeSADP produced only a partial inhibition of spontaneous contractions ($43 \pm 13\%$ ($n=4$) at 100 μM 2-MeSADP) and the half-maximal inhibition was obtained at a concentration of 13.8 ± 3.4 μM ($n=4$). AMP (100 μM) and adenosine (100 μM) also produced complete inhibition of the spontaneous contractile activity (Figure 6). Concentrations producing half-maximal inhibition were 14.3 ± 3.2 μM ($n=7$) and 14.9 ± 4.0 μM ($n=7$) for AMP and adenosine, respectively (Figure 6c,d).

$\alpha\beta$-MeATP (200 μM) partially and completely inhibited the relaxing effect of ADP and 2-MeSADP, respectively (Figure 5a,c,d). In contrast, $\alpha\beta$-MeATP (200 μM) did not affect the relaxing effect of AMP ($n=4$, $P>0.9$) and adenosine ($n=4$, $P>0.4$) (Figure 6a,c,d).

The relaxing effects induced by ADP, 2-MeSADP, AMP and adenosine were all inhibited by 100 nM ZM 241385 (Figure 5b,c,d and Figure 6b,c,d; $n=4$ for each agonist), suggesting that they were mediated through the activation of A_{2A}-receptor subtypes.

Inhibition of ecto-ATPase activity of portal vein strips by $\alpha\beta$-MeATP

The inhibition of ATP-induced relaxing effect by ZM 241385 together with the presence of adenosine-induced ZM 241385-sensitive relaxation suggest that A_{2A}-receptors were present in smooth muscle strips of portal vein and that they were involved in the ATP-induced relaxation. It could thus be hypothesized that ATP was broken down by ectonucleotidases of smooth muscle cells into products responsible for the observed relaxing effect of ATP. We therefore examined whether smooth muscle strips of portal vein displayed an ATPase activity. Incubation of muscular strips with [γ-^{32}P]-ATP demonstrated that they rapidly released [^{32}P]-P_i (Figure 7a). This phenomenon was almost linear with time (0–60 s) and was dependent on divalent cations as it was completely inhibited in the presence of EDTA (4 mM, Figure 7a). Figure 7b clearly shows that during the measurement of ATPase activity, performed in the presence of 100 μM ATP, the rhythmic contractile activity was completely inhibited. The ATPase activity estimated from the slope of the curve was inhibited by $57 \pm 11\%$ ($n=3$, $P<0.05$) after a 45 min treatment with $\alpha\beta$-MeATP (200 μM, Figure 7a). This inhibition was accompanied by a suppression of the relaxing effect of ATP (Figure 7b). These results indicate that portal vein smooth muscle possesses ATPase activity that can be inhibited by $\alpha\beta$-MeATP.

Discussion

The present study showed that in portal vein smooth muscle, P2-receptor agonists (ATP, 2-MeSATP and 2-ClATP) produces an endothelium-independent relaxing action via their breakdown by ecto-ATPase and the consequent activation of A_{2A}-receptors.

This relaxing action of P2-receptor agonists was inhibited by $\alpha\beta$-MeATP. We found that portal vein smooth muscle rapidly hydrolyzed ATP and this ecto-ATPase activity was inhibited by $\alpha\beta$-MeATP. Consistent with these results,

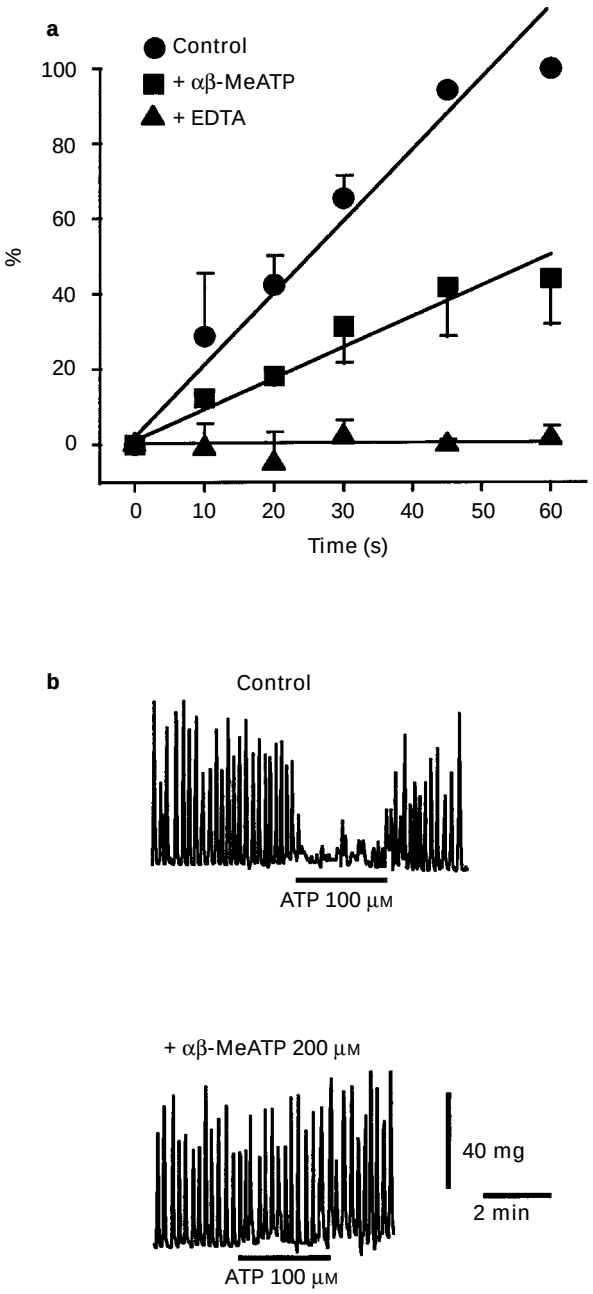

Figure 7 Inhibition of ecto-ATPase activity of smooth muscle strips from portal vein by $\alpha\beta$-MeATP. (a) Time-dependent [α-^{32}P]-ATP dephosphorylation by ecto-ATPase activity of muscular strips measured under control conditions, in the presence of EDTA (4 mM) or after preincubation of the strips with 200 μM $\alpha\beta$-MeATP as described in Methods. The concentration of ATP used was 100 μM. The amount of [^{32}P]-P_i released under each condition is expressed as a percentage of the [^{32}P]-P_i release by the same strips under control conditions over a period of 60 s ($n=3$). (b) Typical traces illustrating the contractile activity during the measurement of ecto-ATPase activity under control conditions (top) and in the presence of 200 μM $\alpha\beta$-MeATP (bottom) in the same strip.

inhibitory effects of $\alpha\beta$-MeATP have also been observed for ecto-ATPase of bovine pulmonary endothelial cells (Chen & Lin, 1997). Recently, it has been shown that inhibition of ecto-ATPase activity greatly increased responses mediated through activation of P2-receptors by neuronally released or exogenous ATP (Westfall *et al*., 1996; 1997; Chen & Lin, 1997). Therefore, if P2-receptor activation is responsible for the P2-receptor agonist-induced endothelium-independent relaxation, the inhibition of ecto-ATPase activity by $\alpha\beta$-MeATP would be expected to potentiate the relaxing action of ATP and its analogues. In fact, the observed inhibition of ATP-, 2-MeSATP- and 2-ClATP-induced relaxation in the presence of $\alpha\beta$-MeATP argues against the involvement of P2-receptor subtypes. This was further supported by the lack of effect of the non-selective P2-receptor antagonist PPADS (Lambrecht, 1996) on the relaxing effect of ATP, 2-MeSATP and 2-ClATP. However, as PPADS has also been shown to be an ecto-ATPase inhibitor (Chen *et al*., 1996), at least a partial inhibitory effect might be expected. This absence of effect of PPADS could be due to its lower efficiency to inhibit ecto-ATPase activity than that of $\alpha\beta$-MeATP (Chen *et al*., 1996; Chen & Lin, 1997).

We thus suggest that ATP-induced relaxation was not mediated by ATP itself but resulted from ATP degradation by ecto-ATPase of smooth muscle and the consequent activation of the P1-receptor. The inhibitory action of ZM 241385 supports this hypothesis. ZM 241385 (100 nM) inhibited the relaxing effect of adenosine and AMP but also that of ATP, 2-ClATP, 2-MeSATP, ADP and 2-MeSADP suggesting that they all mediated their action through activation of the A_{2A}-adenosine receptor.

A direct interaction of $\alpha\beta$-MeATP with the receptor mediating the vasorelaxing effect of ATP and its analogues could be ruled out since the ZM 241385-sensitive relaxation induced by adenosine and AMP was not affected by $\alpha\beta$-MeATP. The inhibition of the relaxing effect of ATP and analogues caused by $\alpha\beta$-MeATP is thus likely to be related to its inhibitory effect on ecto-ATPase activity. By inhibiting dephosphorylation of ATP, $\alpha\beta$-MeATP could prevent accumulation of active breakdown products in the vicinity of the A_{2A}-adenosine receptor.

The relaxing effect of ADP was only slightly inhibited by $\alpha\beta$-MeATP. Two different explanations could account for this result: (i) ADP may directly interact with A_{2A}-receptors in addition to AMP and adenosine produced by ADP hydrolysis, (ii) ATP and ADP may be hydrolyzed by different enzymes so that hydrolysis of ADP by an $\alpha\beta$-MeATP-insensitive enzyme could produce AMP and adenosine even in the presence of $\alpha\beta$-MeATP. This second explanation is more probable as it has been shown that ADP does not directly activate the A_{2A}-receptor by itself (Pirotton & Boeynaems, 1993). The complete inhibition of the ATP-induced relaxation in the presence of $\alpha\beta$-MeATP indicated that there was no accumulation of ADP under these conditions, suggesting that the $\alpha\beta$-MeATP-sensitive step in the ATP degradation is the formation of ADP. Alternatively, AMP could be directly produced from ATP hydrolysis by an ATP diphosphohydrolase (Yagi *et al*., 1991) that was inhibited by $\alpha\beta$-MeATP.

In conclusion, this study shows for the first time that only P1-receptors are involved in the P2-receptor agonist-induced endothelium-independent relaxation in portal vein smooth muscle. We have no evidence for the presence of P2-receptors mediating endothelium-independent relaxation. In the light of these data, other P2Y-receptor effects on blood vessels should be re-evaluated as potential A_{2A}-receptor effects.

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References

CHARLTON, S.J., BROWN, C.A., WEISMAN, G.A., TURNER, J.T., ERB, L. & BOARDER, M.R. (1996). PPADS and suramin as antagonists at cloned P2Y- and P2U-purinoceptors. *Br. J. Pharmacol.,* **118,** 704–710.

CHEN, B.C., LEE, C.M. & LIN, W.W. (1996). Inhibition of ecto-ATPase by PPADS, suramin and reactive blue in endothelial cells, C_6 glioma cells and RAW 264.7 macrophages. *Br. J. Pharmacol.,* **119,** 1628–1634.

CHEN, B.C. & LIN, W.W. (1997). Inhibition of ecto-ATPase by the P_2 purinoceptor agonists, ATPγS, $\alpha\beta$-methylene-ATP, and AMP-PNP, in endothelial cells. *Biochem. Biophys. Res. Commun.,* **233,** 442–446.

CORR, L. & BURNSTOCK, G. (1994). Analysis of P2-purinoceptor subtypes on the smooth muscle and endothelium of rabbit coronary artery. *J. Cardiovasc. Pharmacol.,* **23,** 709–715.

DUBYAK, G.R. & EL-MOATASSIM, C. (1993). Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol. (Cell Physiol.),* **265,** C577–C606.

HORIUTI, K. (1988). Mechanism of contracture on cooling of caffeine-treated frog skeletal muscle fibres. *J. Physiol.,* **398,** 131–148.

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

KENNEDY, C., TODOROV, L.D., MIHAYLOVA-TODOROVA, S. & SNEDDON, P. (1997). Release of soluble nucleotidases: a novel mechanism for neurotransmitter inactivation? *Trends Pharmacol. Sci.,* **18,** 263–266.

KIRLEY, T.L. (1997). Complementary DNA cloning and sequencing of the chicken muscle ecto-ATPase. *J. Biol. Chem.,* **272,** 1076–1081.

LAMBRECHT, G. (1996). Design and pharmacology of selective P2-purinoceptor antagonists. *J. Auton. Pharmacol.,* **16,** 341–344.

NORTH, R.A. & BARNARD, E.A. (1997). Nucleotide receptors. *Curr. Opin. Neurobiol.,* **7,** 346–357.

PACAUD, P., FEOLDE, E., FRELIN, C. & LOIRAND, G. (1996). Characterization of the P_{2Y}-purinoceptor involved in the ATP-induced rise in cytosolic Ca^{2+} concentration in rat ileal myocytes. *Br. J. Pharmacol.,* **118,** 2213–2219.

PACAUD, P., GREGOIRE, G. & LOIRAND, G. (1994). Releases of Ca^{2+} from intracellular store in smooth muscle cells of rat portal vein by ATP-induced Ca^{2+} entry. *Br. J. Pharmacol.,* **113,** 457–462.

PACAUD, P., MALAM-SOULEY, R., LOIRAND, G. & DESGRANGES, C. (1995). ATP raises $[Ca^{2+}]_i$ via different P_2-receptor subtypes in freshly isolated and cultured aortic myocytes. *Am. J. Physiol.,* **269,** H30–H36.

PIROTTON, C., COMMUNI, D., MOTTE, S., JANSSENS, R. & BOEYNAEMS, J.M. (1996). Endothelial P2-purinoceptors: subtypes and signal transduction. *J. Auton. Pharmacol.,* **16,** 353–356.

PIROTTON, S. & BOEYNAEMS, J.M. (1993). Evidence that ATP, ADP and AMP are not ligands of the striatal adenosine A2A receptors. *Eur. J. Pharmacol.,* **241,** 55–61.

PLESNER, L. (1995). Ecto-ATPases: identities and functions. *Int. Rev. Cytol.,* **158,** 141–214.

POUCHER, S.M., KEDDIE, J.R., SINGH, P., STOGGALL, S.M., CAULKETT, P.W.R., JONES, G. & COLLIS, M.G. (1995). The *in vitro* pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist. *Br. J. Pharmacol.,* **115,** 1096–1102.

RAVELIC, V. & BURNSTOCK, G. (1991). Roles of P2-purinoceptors in the cardiovascular system. *Circulation,* **84,** 1–14.

RAVELIC, V. & BURNSTOCK, G. (1996). Discrimination by PPADS between endothelial P2Y- and P2U-purinoceptors in the rat isolated mesenteric arterial bed. *Br. J. Pharmacol.,* **118,** 428–434.

REILLY, W.M. & BURNSTOCK, G. (1987). The effect of ATP analogues on the spontaneous electrical and mechanical activity of portal vein longitudinal muscle. *Eur. J. Pharmacol.,* **138,** 319–325.

TODOROV, L.D., MIHAYLOVA-TODOROVA, S., WESTFALL, T.D., SNEDDON, P., KENNEDY, C., BJUR, R.A. & WESTFALL, D.P. (1997). Neuronal release of soluble nucleotidases and their role in neurotransmitter inactivation. *Nature,* **387,** 76–79.

TUCKER, A.L. & LINDEN, J. (1993). Cloned receptors and cardiovascular responses to adenosine. *Cardiovasc. Res.,* **27,** 62–67.

WESTFALL, T.D., KENNEDY, C. & SNEDDON, P. (1996). Enhancement of sympathetic purinergic neurotransmission in the guinea-pig isolated vas deferens by the novel ecto-ATPase inhibitor ARL 67156. *Br. J. Pharmacol.,* **117,** 867–872.

WESTFALL, T.D., KENNEDY, C. & SNEDDON, P. (1997). The ecto-ATPase inhibitor ARL 67156 enhances parasympathetic neurotransmission in the guinea-pig urinary bladder. *Eur. J. Pharmacol.,* **329,** 169–173.

YAGI, K., SHINBO, M., HASHIZUME, M., SHIMBA, L.S., KURIMURA, S. & MIURA, Y. (1991). ATP diphosphohydrolase is responsible for ecto-ATPase and ecto-ADPase activities in bovine aorta endothelial and smooth muscle cells. *Biochem. Biophys. Res. Commun.,* **180,** 1200–1206.

ZIGANSHIN, A.U., HOYLE, C.H.V. & BURNSTOCK, G. (1994). Ecto-enzymes and metabolism of extracellular ATP. *Drug Dev. Res.,* **32,** 134–146.

ZIGANSHIN, A.U., ZIGANSHINA, L.E., KING, B.F., PINTOR, J. & BURNSTOCK, G. (1996). Effects of P2-purinoceptor antagonists on degradation of adenine nucleotides by ecto-nucleotidases in folliculated oocytes of *Xenopus laevis*. *Biochem. Pharmacol.,* **51,** 897–901.

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