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ATP release and contraction mediated by different P2-receptor subtypes in guinea-pig ileal smooth muscle

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1 The present study was addressed to clarify the subtypes of P2-purinoceptor involved in ATP release and contraction evoked by α,β-methylene ATP (α,β-mATP) and other P2-agonists in guinea-pig ileum.

2 α,β-mATP 100 μM produced a transient and steep contraction followed by ATP release from tissue segments. These maximum responses appeared with different time-courses and their ED_{50} values were 5 and 25 μM, respectively. The maximum release of ATP by α,β-mATP was markedly reduced by 250 μM suramin, 30 μM pyridoxal-phosphate-6-azophenyl-2′,5′-disulphonic acid (PPADS) and 30 μM reactive blue 2 (RB-2), P2-receptor antagonists. However, the contractile response was inhibited by suramin, tetrodotoxin and atropine, but not by PPADS and RB-2.

3 Although the contraction caused by α,β-mATP was strongly diminished by Ca^{2+}-removal and nifedipine, and also by tetrodotoxin and atropine at 0.3 μM, the release of ATP was virtually unaffected by these procedures.

4 UTP, β,γ-methylene ATP (β,γ-mATP) and ADP at 100 μM elicited a moderate release of ATP. The release caused by UTP was virtually unaffected by RB-2. However, these P2-agonists failed to elicit a contraction of the segment.

5 The potency order of all the agonists tested for the release of ATP was α,β-mATP > UTP > β,γ-mATP > ADP.

6 In superfusion experiments with cultured smooth muscle cells from the ileum, α,β-mATP (100 μM) enhanced the release of ATP 5 fold above the basal value. This evoked release was inhibited by RB-2.

7 These findings suggest that ATP release and contraction induced by P2-agonists such as α,β-mATP in the guinea-pig ileum result mainly from stimulation of different P2-purinoceptors, P2Y-like purinoceptors on the smooth muscles and, probably, P2X-purinoceptors on cholinergic nerve terminals, respectively. However, the ATP release may also be mediated, in part, by P2U-receptors, because UTP caused RB-2-insensitive ATP release.

Keywords: Non-neuronal ATP release; contraction; different P2-purinoceptors; α,β-methylene ATP; guinea-pig ileum; cultured cells

Introduction

On the basis of functional characteristics and potency order of agonists, P2-purinoceptors have been divided into six subtypes, P2X, P2Y, P2U, P2T, P2Z and P2D (Fredholm *et al*., 1994). However, it is likely that neither agonists nor antagonists for the P2-receptor subtypes show specific effects.

Recent cloning studies presented evidence that P2X-receptors (ion channels) and P2Y-receptors (G-protein coupled) could be subclassified into $P2X_1$, $P2X_2 \cdots\cdots P2X_7$ and $P2Y_1$, $P2Y_2 \cdots\cdots P2Y_7$, respectively (Burnstock & King, 1996; Fredholm *et al*., 1997). Evidence showing functional roles of these new subtypes has not yet been gathered. α,β-Methylene adenosine 5′-triphosphate (α,β-mATP) has long been accepted as a typical P2X-receptor agonist because the ATP analogue contracts smooth muscles, e.g., vas deferens of guinea-pig, and the evoked contraction is antagonized by suramin (Fredholm *et al*., 1994). However, α,β-mATP has also been described as a weak P2Y-agonist (Satchell & Maguire, 1975; Fredholm *et al*., 1994). Therefore, the relationship between the functions of this agonist and its selectivity for P2X- and P2Y-receptors deserves careful attention. In previous studies, we found that administration of α,β-mATP caused ATP release and contraction simultaneously in ileal longitudinal muscle segments of guinea-pigs (Katsuragi *et al*., 1991; 1992; 1996). The evoked release of ATP was inhibited by phospholipase C inhibitors, e.g., spermine and neomycin, whereas, the contraction was virtually unaffected (Katsuragi *et al*., 1996). The findings suggest that the release of ATP and the contraction evoked by α,β-mATP are mediated via different subtypes of P2-receptor. Accordingly, the present study was addressed to elucidate the receptors mediating these responses to α,β-mATP, uridine 5′-triphosphate (UTP) and other P2-agonists in intact and, in part, cultured smooth muscles from guinea-pig ileum.

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Methods

Contraction and ATP release from tissue segments

Male guinea-pigs weighing 200 to 350 g were stunned and bled. The distal part (about 5 cm long) of the ileum was dissected 3 cm from the ileocaecum. The mucosal membranes including submucosal layers and circular muscles were mechanically removed from the longitudinal muscle layer as described previously (Katsuragi *et al*., 1990; 1991). The longitudinal strip (3 by 30 mm) was suspended in a bath (1 ml) filled with Krebs solution (32°C) of the following composition (mM): NaCl 122, KCl 5.2, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.6, D-glucose 11, Na_2EDTA 0.03 and ascorbic acid 0.1, bubbled with 95% O_2/5% CO_2. After 0.5 g initial tension had been applied to the preparation, the isometric tension was monitored simultaneously on a polygraph with a force transducer (Toyo-Baldwin, T-7-8-240). The longitudinal muscle segment was allowed to equilibrate for 20 min, washed three times quickly with fresh solution and then was kept for a further 30 min in this solution. A control sample (50 μl) was taken 5 min before the end of the 50 min equilibration period.

Thereafter, aliquots (50 μl) as test samples were collected 3, 15 and 25 min after the addition of agonists. After every sampling, an equivalent volume of bubbled Krebs solution (32°C) was added to the bath. At the end of the experiment, the segment was blotted with filter paper and weighed.

ATP release from cultured cells

The ileum was dissected from guinea-pigs of either sex within 2 days after birth. The longitudinal muscle segment was minced into small pieces (about 0.5 mm^3) in Dulbecco's phosphate buffered saline supplemented with 0.25 mg ml^{-1} collagenase and kept at 4°C overnight. After being kept in a 37°C incubator for 40 min, the pellet was collected by centrifugation (180 *g*) for 3 min and transferred to a dish containing the phosphate buffered saline with 0.062% trypsin. Following trituration, the content was transferred to a tube with 10% foetal calf serum and centrifuged for 5 min. The pellet was dispersed in a dish containing M-199 medium (Bodin *et al*., 1991) with foetal calf serum and the culture was started in the incubator (37°C) supplied with CO_2. The culture medium was not replaced with any other media during the culturing. After 5 to 7 days, the cell growth became confluent. Before use, the medium culturing the cells was replaced by 0.25% trypsin in phosphate buffered saline and kept for 3 min at 37°C. Fibroblast and other cells contaminating the smooth muscle cells were floated by a slight vibration and eliminated by enough rinsing with a fresh phosphate buffered saline. The morphological purity of the smooth muscle cells and the lack of neuronal cells in a dish was microscopically determined. The cultured cells were trapped in a Millipore filter holder (Swinnex-25) and were perfused at 0.5 ml min^{-1} with the oxygenated Krebs solution (37°C) by use of a peristaltic pump. After a 5 min equilibration period, the perfusate was collected every 90 s for 15 min. Then, an aliquot, 100 μl, was taken from a fraction medium for measurement of ATP.

The protein content of the smooth muscle cells was determined with the Bio-Rad protein assay kit II after an overnight incubation (4°C) of the filter in deionized water containing 0.1% Triton X-100.

ATP measurement

In order to determine endogenous ATP, every sample of medium (50 or 100 μl) was treated with 100 μl of ATP assay solution (Lucifel-LU; Kikkoman, Noda, Japan). The intensity of light produced by the reaction was measured with a luminometer as described previously (Katsuragi *et al*., 1991; 1996).

Treatments

Although we do not know whether uridine 5′-triphosphate (UTP) was contaminated with a trace of ATP or is a very weak substrate for the firefly enzyme, UTP *per se*, unlike other P2-agonists tested, reacted slightly with luciferin-luciferase. Therefore, the values of ATP released by UTP were finally estimated by subtracting the small value found when the corresponding concentration of UTP was tested in tissue-free medium. When ATP was determined from a sample medium containing suramin, 250 μl of the ATP assay solution, i.e., a larger volume than that normally used, was applied because of interference by suramin with luciferase activities. Ca^{2+}-free Krebs solution was prepared by removal of Ca^{2+} from the solution and, by addition of 1 mM EGTA.

Drugs

The drugs used were: α,β-methylene ATP Li^+ salt, β,γ-methylene ATP Na^+ salt, UTP Na^+ salt, ADP Na^+ salt, nifedipine, reactive blue-2 (Sigma, St. Louis, MO, U.S.A.), pyridoxal-phosphate-6-azophenyl-2′,5′-disulphonic acid tetrasodium salt (PPADS) (Tocris Cookson, Bristol, U.K.) and suramin (gift from Bayer, Wuppertal, Germany).

Statistics

Difference between multiple means was tested for statistical significance by one way analysis of variance (ANOVA) followed by Dunnett's test. A value of $P<0.05$ was considered to be significant.

Results

In the ileal longitudinal muscles of guinea-pigs, α,β-methylene ATP (α,β-mATP, 100 μM), a stable ATP analogue, produced ATP release accompanying a transient and steep contraction as described previously (Katsuragi *et al*., 1991). A concentration-response curve for ATP release by α,β-mATP was compared with its concentration-contractible response curve. The tissue was contracted by α,β-mATP in concentrations ranging from 0.1 to 100 μM, whereas ATP was released by the agonist in a narrow concentration range between 10 and 100 μM (Figure 1). The ED_{50} values of the contractile and release responses amounted to approximately 5 and 25 μM, respectively. Furthermore, the time-course of the contraction and the release of ATP evoked by 100 μM α,β-mATP was evaluated. The contraction culminated immediately after the administration of α,β-mATP. On the other hand, the release gradually reached its maximum level 2–3 min after the administration of the drug, as described previously (Katsuragi *et al*., 1991; 1996). The values for the basal and the maximum release of ATP were 18.32 ± 1.44 pmol ml^{-1} g^{-1} wet weight ($n=6$) and 57.52 ± 5.99 pmol ml^{-1} g^{-1} wet weight ($n=26$), respectively. The maximum release of ATP was markedly diminished by 250 μM suramin, a non-selective antagonist for P2X and P2Y receptors and 30 μM PPADS, a P2-antagonist. In addition, the release was also inhibited by 30 μM reactive blue 2 (RB-2, a P2Y-antagonist) (Figure 2a). The contractile response to α,β-mATP was usually monophasic, but occasionally a biphasic contraction was seen, as shown previously (Kennedy & Humphrey, 1994). In the case of the biphasic contraction, an initial transient and large contraction followed by a slow

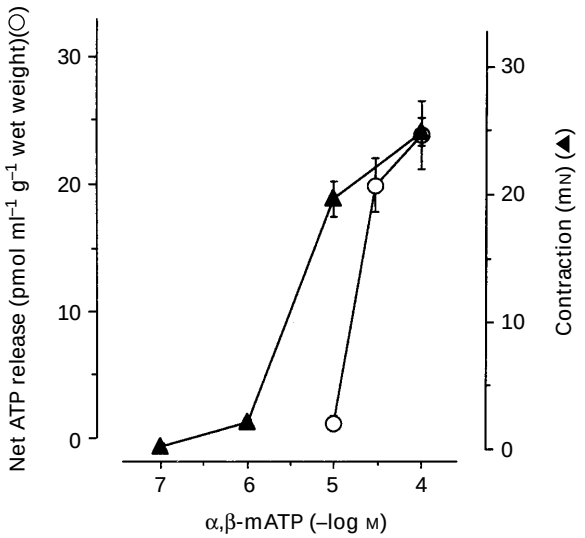

Figure 1 Concentration-response relationship for ATP release and contraction evoked by α,β-methylene ATP (α,β-mATP) in guinea-pig ileal longitudinal muscle segments. Net release of ATP (evoked release of ATP − basal release of ATP) was obtained 3 min after exposure to α,β-methylene ATP and is expressed as mean in pmol ml^{-1} g^{-1} wet weight ($n=5-12$). Contraction was measured from the maximum amplitude obtained immediately after exposure to α,β-methylene ATP and expressed as mean in mN ($n=7-8$). Vertical lines show s.e.mean.

contraction were observed. Thus, the amplitude of the response to the P2-agonist was determined from the transient large contraction in all cases. The evoked contraction was antagonized by 250 μM suramin, 0.3 μM tetrodotoxin and 0.3 μM atropine, but not by PPADS and RB-2 at 30 μM (Figures 2b and 3b). Furthermore, the contraction was completely abolished by Ca^{2+}-removal from the medium and 0.1 μM nifedipine, a voltage gated Ca^{2+}-channel blocker (Figure 3b). However, interestingly, the release of ATP was almost unaffected by these procedures (Figure 3a).

In a further study, in addition to α,β-mATP, the effects of other P2-agonists on both responses were examined in the tissue segments. The release of ATP was considerably enhanced by 100 μM UTP. However, the evoked release was virtually unaffected by RB-2 at 30 μM. β,γ-Methylene ATP (β,γ-mATP) and ADP at 100 μM caused a moderate release of ATP (Figure 4a). From these findings, the potency order of the P2-agonists tested at releasing ATP was α,β-mATP > UTP > β,γ-mATP > ADP. On the other hand, unlike α,β-mATP, neither UTP, β,γ-mATP nor ADP elicited a measurable contraction of the ileal segments (Figure 4b).

In perfused cultured smooth muscle cells from the ileum, α,β-mATP (100 μM) enhanced ATP release by approximately 5 fold from the basal release (Figure 5a). The evoked release of ATP was notably reduced in the presence of 30 μM RB-2 (Figure 5b).

Discussion

For a long time α,β-mATP has been considered to be a typical P2X-purinoceptor agonist as it causes a contractile response in the mesenteric artery (Burnstock & Warland, 1987) and ear artery (Leff *et al*., 1990) of rabbit and in the urinary bladder (Hoyle *et al*., 1990) of guinea-pig. However, several studies have suggested that α,β-mATP may also act as a weak agonist for P2Y-purinoceptors (Satchell & Maguire, 1975; Fredholm *et al*., 1994). In the present study, the ileal longitudinal muscle segments of guinea-pig responded to 100 μM α,β-mATP with a contraction and ATP release. The release of ATP evoked by the P2-agonist seems to be dissimilar in characteristics from the evoked contraction on the basis of the concentration-response curve data and the time-course until a maximum response. Furthermore, the dependence on extracellular Ca^{2+} between the contraction and ATP release was quite opposite, as seen from the different effects of Ca^{2+}-removal and nifedipine on both responses. In addition, the contraction by the P2-agonist was sensitive to tetrodotoxin and atropine, whereas the release of ATP was insensitive to either blocker.

The contractile response to α,β-mATP was strongly antagonized by suramin, tetrodotoxin and atropine, but not by PPADS and RB-2, whereas the evoked release of ATP was suppressed by all of the P2-antagonists tested. So far, no specific antagonists for P2X, P2Y and P2U-purinoceptors have been found. It is well-documented that suramin shows antagonistic effects for P2Y as well as for P2X-receptors (Hoyle *et al*., 1990; Fredholm *et al*., 1994). Another P2-receptor antagonist, RB-2 has been shown to exhibit relatively specific blockade of P2Y-purinoceptors in a narrow concentration range, e.g., 1 to 30 μM, at most 50 μM (Hoyle, 1992). For example, recent studies provide evidence that RB-2 (0.3 – 50 μM) inhibits agonist-induced K^+-currents in microglial cultured

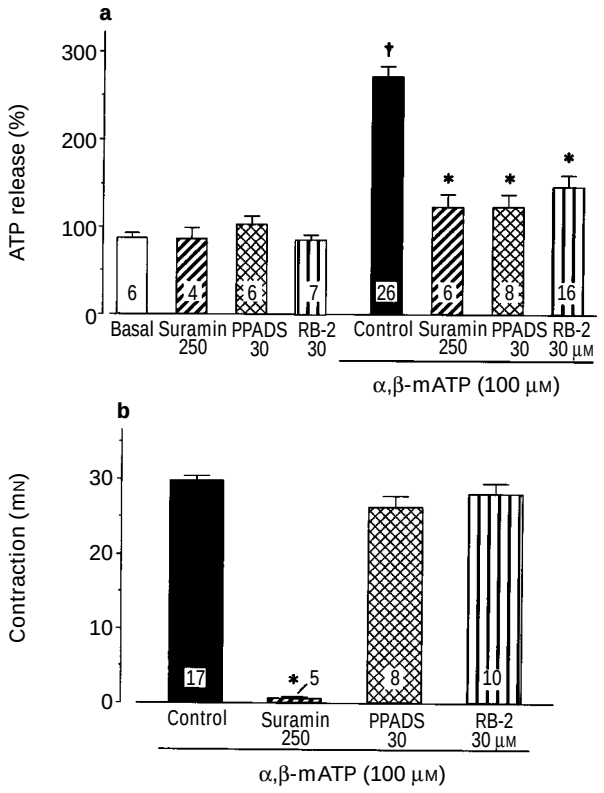

Figure 2 Effects of P2- antagonists on maximum ATP release (a) and contraction (b) evoked by α,β-methylene ATP (α,β-mATP). Values are expressed as mean ± s.e.mean of % release of ATP or of mN contraction. In (a), the 100% value was obtained by measuring ATP 5 min before administration of α,β-methylene ATP. Values of basal and evoked release of ATP were measured 3 min after addition of the agonist. Antagonists were added to the bath 15 min before the agonist. Numbers in columns are the numbers of experiments. $^{+}P<0.01$ vs basal. $^{*}P<0.01$ vs control.

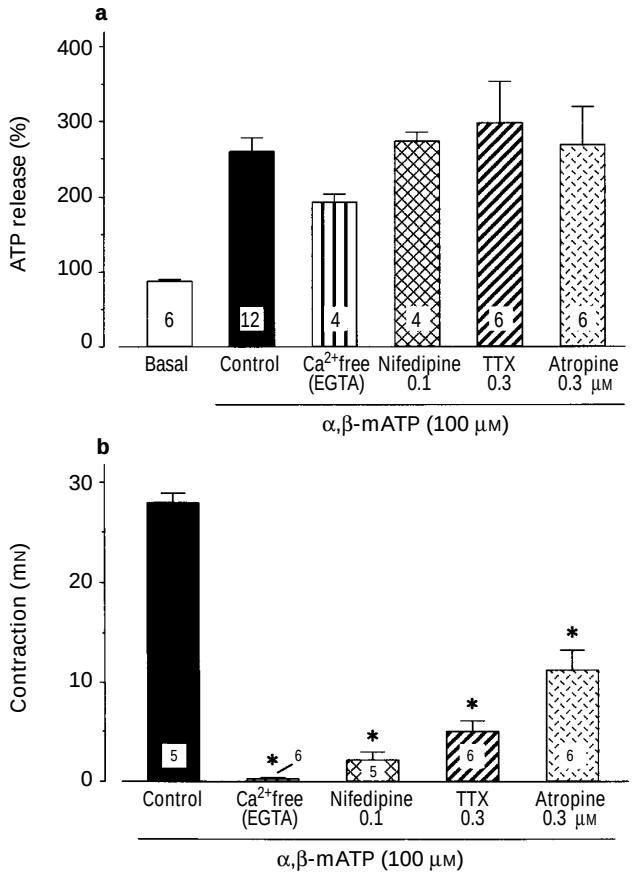

Figure 3 Effects of Ca^{2+}-removal, nifedipine, tetrodotoxin (TTX) and atropine on maximum responses of ATP release (a) and contraction (b) evoked by α,β-methylene ATP (α,β-mATP). For details see legend of Figure 2. Ca^{2+}-free solution was prepared by removal of Ca^{2+} from the solution and addition of 1 mM EGTA. Nifedipine and atropine were added 15 min and tetrodotoxin 30 min before the agonist. $^{*}P<0.01$ vs control.

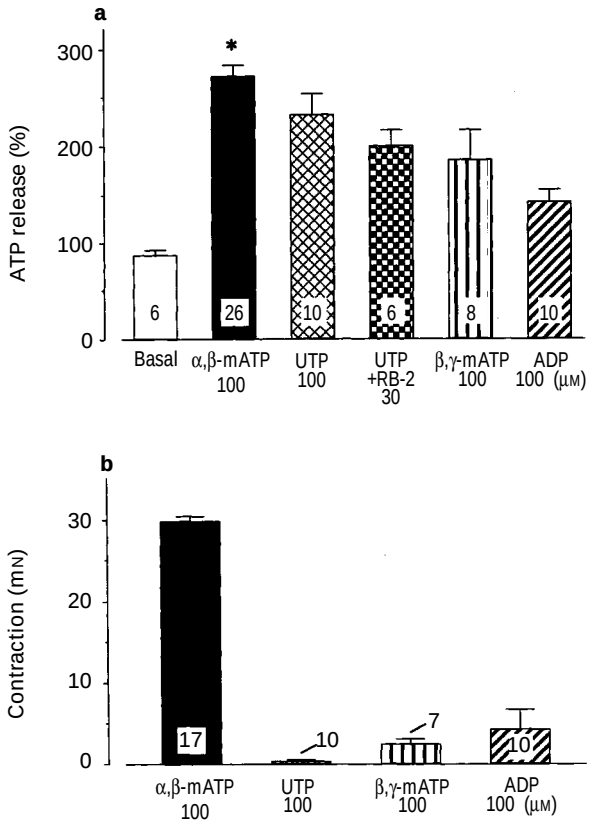

Figure 4 ATP release (a) and contraction (b) evoked by P2-purinoceptor agonists. Reactive blue 2 (RB-2) was added 15 min before UTP. β,γ-mATP: β,γ-methylene ATP. For details see legend of Figure 2. The same data with α,β-methylene ATP (α,β-mATP) shown in Figure 2 are again illustrated for reference. $^*P<0.01$ vs basal.

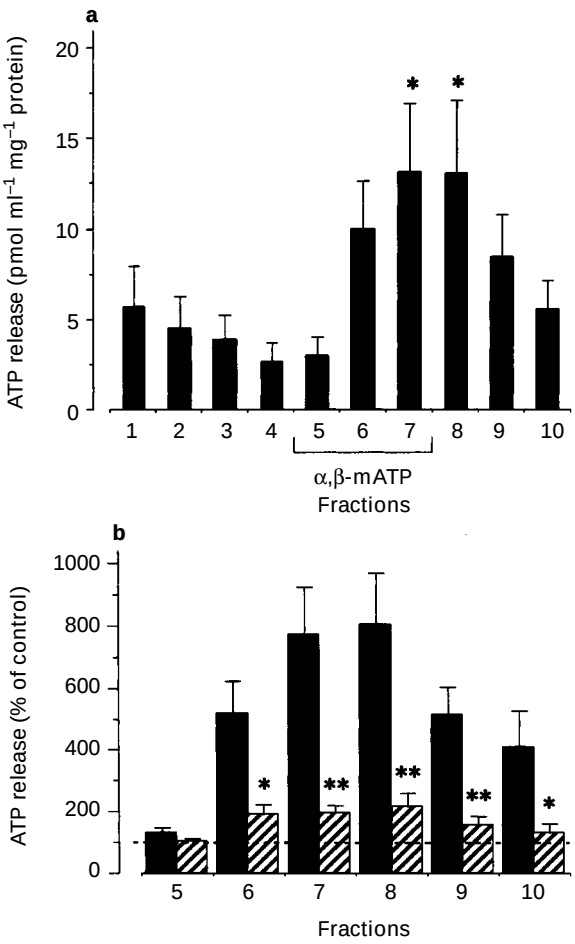

Figure 5 ATP release from perfused cultured ileal smooth muscle cells evoked by α,β-methylene ATP (α,β-mATP) in the absence (a) and presence (b) of reactive blue 2 (RB-2). (a) α,β-methylene ATP 100 μM was present from 5th to 7th fractions. Values are expressed as mean$\pm$s.e.mean ($n=13$). (b) Reactive blue 2 30 μM (hatched columns, $n=9$) was introduced into the perfusate 10 min before 100 μM α,β-methylene ATP (solid columns, $n=13$). Values are expressed as mean$\pm$s.e.mean of % release from 4th fraction (100%). (a) $^*P<0.05$ vs control (4th fraction). (b) $^*P<0.05$, $^{**}P<0.01$ vs corresponding control.

cells (Nörenberg *et al*., 1994) and megakaryocytes (Uneyama *et al*., 1994) from rat. Similarly, ATP-induced increase of dopamine in rat striatum was blocked by 20 μM RB-2 as well as by 1 μM suramin (Zhang *et al*., 1995). From results with the vas deferens and urinary bladder, it has been suggested that PPADS is a selective P2X-purinoceptor antagonist (Lambrecht *et al*., 1992; Ziganshin *et al*., 1993; Usune *et al*., 1996). However, there are marked tissue differences in the blocking effects of PPADS for P2-receptor mediated-responses. In rat duodenum, relaxations evoked by 2-methylthio ATP, a P2Y-receptor agonist, and α,β-mATP were antagonized by PPADS (3–30 μM) (Windscheif *et al*., 1995). In bovine aortic endothelial cells, accumulation of [^{3}H]-inositol polyphosphate mediated by 2-methylthio ATP, but not by UTP, was markedly attenuated by 10–30 μM PPADS (Brown *et al*., 1995). In addition, 30 μM PPADS has been found to antagonize competitively the activation of phospholipase C by 2-methylthio ATP in turkey erythrocytes (Boyer *et al*., 1994). Similarly, an increase of cytosolic Ca^{2+} concentration by 2-methylthio ATP in myocytes isolated from rat ileal smooth muscles was notably inhibited by 5 to 10 μM PPADS (Pacaud *et al*., 1996). These findings provide evidence that PPADS acts as a P2Y-receptor antagonist as well as a P2X-receptor antagonist. Therefore the present findings also suggest that PPADS, like RB-2, functions as, presumably, a P2Y-purinoceptor antagonist in this tissue. Unfortunately, we could not evaluate the effect of 2-methylthio ATP on ATP release because the agonist *per se* was highly reactive to luciferin-luciferase. However, the possibility that the release of ATP evoked by α,β-mATP may be mediated by P2Y-like purinoceptors is supported by a previous study showing that α,β-mATP causes an accumulation of inositol-1,4,5-trisphosphate together with ATP release in guinea-pig ileum (Katsuragi *et al*., 1996).

Taken together, these results on the contractile response and the release of ATP evoked by α,β-mATP in the ileal longitudinal muscle segments are interpreted as follows: the contraction and the release of ATP may result from stimulation of P2X-like and P2Y-like purinoceptors, respectively. Since the evoked contraction was inhibited by tetrodotoxin and atropine, α,β-mATP seems to act, probably, on P2X-purinoceptors situated on cholinergic nerve endings and, subsequently, to release acetylcholine causing an atropine-sensitive contraction of the smooth muscles as shown previously (Moody & Burnstock, 1982; Sperlagh & Vizi, 1991; Kennedy & Humphrey, 1994). The steepness of the concentration-contractile response curve for α,β-mATP indicates the characteristic depolarization by the P2-agonist of the cell membrane of cholinergic nerves. On the other hand, the evoked release of ATP was virtually unaffected by tetrodotoxin and atropine. We have shown that 1 μM acetylcholine and 10 μM bethanechol are capable of causing ATP release from guinea-pig ileal segments (Katsuragi *et al*., 1990; 1992a). The reason for the lack of effect of tetrodotoxin and atropine on the release of ATP may be that the endogeneous acetylcholine released by α,β-mATP is at an extremely low concentration compared to the 1 μM acetylcholine added exogenously in the studies mentioned above. The contractions elicited by P2-purinoceptor agonists are unlikely, at least, to be mediated by P2U-receptors because of the lack of a

contractile response to 100 μM UTP. However, since the release of ATP induced by UTP was not affected by the presence of RB-2, the involvement of P2U-purinoceptors in causing ATP release from the ileum cannot be excluded. In general, either muscular or nervous tissue in the ileal segments seem likely as the origin of the evoked release of ATP. However, the finding that the release of ATP by α,β-mATP was virtually unaffected by Ca^{2+}-removal and nifedipine argues against the possibility of a neuronal origin. Also, in the perfusion study with the cultured ileal smooth muscle cells, the α,β-mATP-elicited release of ATP was sensitive to RB-2. Accordingly, the purinoceptor-mediated release of ATP seems to come mainly from smooth muscle cells, at least, in the cultured cells. The physiological role of ATP release remains to be clarified in future investigations.

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