Cross Desensitizations on Contractions by P2-Agonists of Guinea Pig Ileum

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ABSTRACT—The present study was designed to clarify the characteristics of contractions of guinea pig ileal longitudinal muscles evoked by α,β -methylene ATP as compared with those by other P2-agonists. α,β -Methylene ATP, ADP- β -S and 2-methylthio ATP as P2-agonists produced remarkable phasic contractions of the segment in a suramin-sensitive- and reactive blue-2-insensitive manner. However, ADP- β -S and 2-methylthio ATP, unlike α,β -methylene ATP, showed a biphasic contraction accompanied by a second sustained phase. Their second sustained contractions were notably suppressed by 30 μ M reactive blue-2, probably being a component mediated by P2Y-purinoceptor. The phasic contractile response to α,β -methylene ATP, but not ADP- β -S and 2-methylthio ATP, was largely reduced by tetrodotoxin and atropine, indicating that the contraction is due to acetylcholine released from the cholinergic nerves. At 100 μ M, α,β -methylene ATP inhibited the phasic contractions caused by a low concentration of itself, but not those induced by ADP- β -S and 2-methylthio ATP, presumably serving as a desensitizer of the P2-receptor. Although β,γ -methylene ATP per se showed little contraction, it prevented the contraction evoked by α,β -methylene ATP, but not those by ADP- β -S and 2-methylthio ATP. The contraction evoked by 100 μ M 2-methylthio ATP was attenuated in the presence of ADP- β -S at 10 and 30 μ M. From separate cross-interactions between two groups of P2-agonists, there seems to be different subtypes of P2X-purinoceptors in the pre- and postsynapse in producing phasic contractions, but not sustained contractions that are mediated by, presumably, the P2Y-purinoceptor of the ileum.

Keywords: Contraction, α,β -Methylene ATP, β,γ -Methylene ATP, ADP- β -S, Guinea pig ileum

Recent cloning studies have shown that P2-purinoceptors can be divided into two major subtypes, P2X receptors of ion channel types and P2Y receptors of G-protein coupled types, each having seven further subtypes (1, 2). When a c-DNA of some P2X-receptor subtype was transfected to *Xenopus* oocytes, α,β -methylene ATP, compared to 2-methylthio ATP, has been documented to be much less potent as an agonist in activating the ligand-gated ionic channels coupled with P2X-receptor subtypes from P2X1 to P2X6 (3–5). In the previous category based on functional activities, e.g., contraction of smooth muscles, however, α,β -methylene ATP and 2-methylthio ATP or ADP- β -S had been considered to be typical agonists for P2X- and P2Y-purinoceptors, respectively (6–8). At present, therefore, the concept of agonists for P2X and P2Y receptors seems not to be

clearly established and further studies are required to understand the properties of P2-purinoceptors.

There is some evidence that acetylcholine release from the ileal cholinergic nerves is suppressed by α,β -methylene ATP, but not by 2-methylthio ATP, via mediation of presynaptic P2Y-like receptors (8). We also found that α,β -methylene ATP caused not only an increase of postsynaptic ATP release, but also a decrease of acetylcholine release via electrostimulation from guinea pig ileum. In the study, it was concluded that α,β -methylene ATP caused a P2-receptor-mediated release of ATP from the smooth muscles, then endogenous ATP released postjunctionally was metabolized to adenosine, and finally, the adenosine triggered presynaptically A1-purinoceptor-sensitive neuromodulation of cholinergic transmission, thus being conducted by so-called transsynaptic neuromodulation (9).

Apart from such roles of α,β -methylene ATP in

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neuromodulation, the P2-agonist per se appears to elicit acetylcholine release from the cholinergic nerve terminals. Moody and Burnstock (10) first reported that α,β -methylene ATP contracted the ileal smooth muscles of the guinea pig in a tetrodotoxin and atropine-sensitive manner (11). The neuronal event with the agonist was further supported by the findings that the α,β -methylene ATP-evoked contraction was mediated by stimulation of the presynaptic P2X-purinoceptors (12).

The present study was designed to clarify the characteristics of the contraction of guinea pig ileal segments elicited by α,β -methylene ATP as compared with those by other P2-agonists, 2-methylthio ATP and ADP- β -S.

MATERIALS AND METHODS

Measurement of tension development

Male guinea pigs weighing 200 to 350 g were stunned and bled. The distal part (about 5-cm-long) of the ileum was dissected at 3 cm from the ileocecum. The mucosal membranes or submucosal layers, including circular muscles, were carefully removed from the longitudinal muscle layer with the aid of fine tweezers and scissors under the microscope. The longitudinal strip (3 by 30 mm) was suspended in a bath (1 ml) filled with Krebs' solution (32°C) of the following composition: 122 mM NaCl, 5.2 mM KCl, 2.4 mM CaCl_2 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25.6 mM NaHCO_3 , 11 mM D-glucose, 0.03 mM Na_2EDTA and 0.1 mM ascorbic acid, bubbled with 95% O_2 / 5% CO_2 . After loading of 0.5 g initial tension, the isometric tension of

Fig. 1. Concentrations-contraction curves for ADP- β -S, 2-methylthio ATP (2-m-sATP) and α,β -methylene ATP (α,β -mATP) in ileal segments of the guinea pig. $\circ$, α,β -mATP; $\bullet$, ADP- β -S; $\blacktriangle$, 2-m-sATP. Values are expressed as the mean $\pm$ S.E.M. Administrations of an agonist were carried out in a 20 min-interval, and thereafter, the segment was rinsed after arrival at the maximum response.

the preparation was monitored simultaneously on a polygraph with a force transducer (T-7-8-240; Toyo-Baldwin, Tokyo). After a 40 min-equilibration, an agonist was added to the bath and then, washed-out sufficiently. Fifty minutes later, the second application of the agonist was

Fig. 2. Effects of suramin and reactive blue-2 (RB-2) on contractions evoked by P2-receptor agonists. For details, see the legend of Fig. 1. Antagonists were administered 30 min before the agonist. * $P < 0.01$ vs control (Dunnett's test).

Fig. 3. Effects of reactive blue-2 (RB-2) on contraction evoked by ADP- β -S. A typical recording from four experiments is shown.

carried out. The responses to the repeated applications of agonist were confirmed to be reproducible as shown in Fig. 4. The first phasic contraction peaked immediately following applications of agonists was evaluated as an evoked response. The values were expressed as percent of the control or mN contraction.

Drugs

The drugs used were as follows: α,β -methylene ATP Li^+ salt, β,γ -methylene ATP Na^+ salt, ADP- β -S trilithium salt, reactive blue-2, physostigmine hemisulfate salt (Sigma, St. Louis, MO, USA); 2-methylthio ATP tetrasodium salt, (Research Biochemical International, Natick, MA, USA); crystalline tetrodotoxin (Sankyo, Tokyo); atropine sulfate (E. Merck, Darmstadt, Germany); and

suramin (gift from Bayer, Wuppertal, Germany).

Statistics

The significance difference between two means and the significance difference between multiple means were analyzed by Student's *t*-test and by one way ANOVA followed by Dunnett's test, respectively. A value of $P < 0.05$ was considered to be significant.

RESULTS

Contractile activities of P2-receptor agonists

P2-receptor agonists such as ADP- β -S and α,β -methylene ATP contracted the ileal segment of the guinea pig. The rank order of potency in the phasic contractile am-

Fig. 4. Effects of tetrodotoxin (TTX) and atropine on contractions evoked by P2-agonists. For details, see the legend of Fig. 1. Antagonists were administered 30 min before the agonist. * $P < 0.01$ vs control (Dunnett's test).

plitude evaluated with the agonists, at 100 μM , was $\text{ADP-}\beta\text{-S} > 2\text{-methylthio ATP} > \alpha,\beta\text{-methylene ATP} \gg \text{ATP} > \text{UTP} > \text{ADP} > \beta,\gamma\text{-methylene ATP}$ (data not shown). $\alpha,\beta\text{-Methylene ATP}$ exhibited a steep and transient contraction, whereas $\text{ADP-}\beta\text{-S}$ and 2-methylthio ATP showed the transient phasic contraction and the subsequent sustained contraction. Concentration-contraction curves for $\text{ADP-}\beta\text{-S}$, 2-methylthio ATP and $\alpha,\beta\text{-methylene ATP}$ are illustrated in Fig. 1.

Effects of purinoceptor antagonists, tetrodotoxin and atropine on the evoked contractions

The contraction evoked by 100 μM $\alpha,\beta\text{-methylene ATP}$ was almost completely antagonized by 250 μM suramin,

a nonspecific P2-receptor antagonist, but not by 30 μM reactive blue-2, a P2Y-receptor antagonist (13) indicating that the contraction may be mediated by P2X-like purinoceptors. Similarly, the biphasic contractile responses to $\text{ADP-}\beta\text{-S}$ and 2-methylthio ATP at the concentration of 100 μM were moderately inhibited by suramin as shown in Fig. 2 and their responses at 3 μM were completely abolished (data not shown), thus indicating a competitive antagonism. However, although the first phasic contractions evoked by $\text{ADP-}\beta\text{-S}$ and 2-methylthio ATP were virtually unaffected by 30 μM reactive blue-2, the second sustained contractions were markedly suppressed by the same procedure. A typical illustration of the effect of reactive blue-2 on the contraction evoked by

Fig. 5. Effects of $\beta,\gamma\text{-methylene ATP}$ ($\beta,\gamma\text{-mATP}$) on contractions evoked by $\alpha,\beta\text{-methylene ATP}$, $\text{ADP-}\beta\text{-S}$, 2-methylthio ATP and ATP. For details, see the legend of Fig. 1. $\beta,\gamma\text{-mATP}$ was administered 30 min before the agonist. * $P < 0.001$ vs control (Student's t -test).

Fig. 6. Typical illustrations of contractions evoked by α,β -methylene ATP (A) and ADP- β -S (B) in the absence (a) and presence (b) of β,γ -methylene ATP. β,γ -Methylene ATP was administered in the same way as described in Fig. 3.

30 μ M ADP- β -S is shown in Fig. 3. In some cases, reactive blue-2 alone, but not suramin, exhibited a small sustained contraction. The response to ATP tended to be attenuated not only by suramin, but also by reactive blue-2. The transient contractile response to 100 μ M α,β -methylene ATP was intensely prevented by both 0.3 μ M tetrodotoxin and 0.3 μ M atropine, thus, resulting from acetylcholine released via stimulation of presynaptic P2-purinoceptors. However, the contractions caused by other agonists, ADP- β -S, 2-methylthio ATP and ATP, were unaffected or tended to be slightly attenuated in the presence of tetrodotoxin and atropine (Fig. 4).

Mutual interference among P2-agonists for their contractile activities

β,γ -Methylene ATP alone has only a very low contractile intrinsic activity. However, in the presence of 100 μ M of the ATP analog, the large phasic, not the small tonic, contractions caused by α,β -methylene ATP at 10, 30 and 100 μ M were significantly inhibited, suggesting that the tonic component is of different nature from the phasic one. On the other hand, the contractions by ADP- β -S (10 and 100 μ M), 2-methylthio ATP (100 μ M) and ATP (100 μ M) could not be inhibited by β,γ -methylene ATP even at 100 μ M (Fig. 5). Typical illustrations of such effects of β,γ -methylene ATP on the contractions evoked by α,β -methylene ATP and ADP- β -S are shown in Fig. 6. α,β -Methylene ATP has been documented to be a desensitizer for P2X-purinoceptors (14, 15). Preaddition of the agonist at 100 μ M showed a notable inhibition of the contractile response to 10 μ M α,β -methylene ATP per se, whereas it failed to attenuate the response to ADP- β -S and 2-methylthio ATP at 10 μ M (Fig. 7). In the concentrations of 10 μ M and 30 μ M, ADP- β -S clearly prevented

Fig. 7. Effects of α,β -methylene ATP on contractions evoked by α,β -methylene ATP, ADP- β -S and 2-methylthio ATP. Values are expressed as the mean $\pm$ S.E.M. of percent contraction (control: 100%) from experimental numbers in columns. α,β -Methylene ATP was added to the bath 30 min before the agonist. * $P < 0.001$ vs control (Student's t -test).

Fig. 8. Effects of ADP- β -S on 2-methylthio ATP-evoked contractions. For details, see the legend of Fig. 1. ADP- β -S was administered 30 min before 2-methylthio ATP. * $P < 0.01$ vs control (Dunnett's test).

the contractions evoked by 100 μ M 2-methylthio ATP (Fig. 8).

DISCUSSION

In the present study, α,β -methylene ATP, ADP- β -S and 2-methylthio ATP caused remarkable contractions of ileal longitudinal muscles from the guinea pig. Among them, ADP- β -S and 2-methylthio ATP exhibited a biphasic contraction consisting of a first phasic component and second sustained component. The transient phasic contractions evoked by these agonists were suppressed by 250 μ M suramin, although the sensitivity for suramin of the response to α,β -methylene ATP was considerably higher than those to the other two agonists. Reactive blue-2 up to 50 μ M seems to be a specific antagonist for P2Y-purinoceptors (13). The antagonist failed to prevent the phasic contraction evoked by these agonists. It is noteworthy that, however, the second sustained response to ADP- β -S and 2-methylthio ATP was strongly suppressed in the presence of reactive blue-2. Therefore, the phasic and tonic contractions of the ileal smooth muscles evoked by these agonists may be mediated by stimulation of P2X and P2Y purinoceptors, respectively.

β,γ -Methylene ATP hindered the contractile response to α,β -methylene ATP, but not to ADP- β -S and 2-methylthio ATP. Thus, it is highly possible that β,γ -methylene ATP with a low intrinsic activity in the contraction plays a role as a partial agonist rather than a desensitizer for the P2X-like purinoceptor stimulated by α,β -methylene ATP (11). Interestingly, the contraction evoked by α,β -methylene ATP, not by other two agonists, was prevented by treatments with tetrodotoxin

and atropine of the segment, suggesting that the evoked contraction is due to acetylcholine released from the cholinergic nerves (10–12). Exactly, α,β -methylene ATP per se, like DMPP known as an acetylcholine releaser (16), tended to enhance the release of acetylcholine from the ileal cholinergic nerves (data not shown). Furthermore, 100 μ M α,β -methylene ATP reduced the contraction evoked by the agonist itself, at 10 and 30 μ M, in desensitizing P2X-purinoceptors, as is well known (14, 15). However, α,β -methylene ATP failed to attenuate the contractions caused by ADP- β -S and 2-methylthio ATP. In contrast, the contraction evoked by 100 μ M 2-methylthio ATP was prevented in the presence of 10 μ M and 30 μ M ADP- β -S. On the basis of the findings showing mutual interactions between two P2-agonists, α,β -methylene ATP and β,γ -methylene ATP seem to bind to a common P2X-receptor on ileal cholinergic neurons, which is tentatively named the "P2Xn-receptors", whereas ADP- β -S and 2-methylthio ATP may stimulate a P2X-like receptor on ileal smooth muscle, which is similarly named the "P2Xm-receptors".

Exposure to ATP, a representative P2-agonist, at 100 μ M, responded to a small contraction in a atropine and tetrodotoxin-insensitive manner as reported by Moody and Burnstock (10). However, in the present study, it still remains uncertain why ATP, dissimilar to α,β -methylene ATP, had no influence on the cholinergic nerve activity.

Hitherto, seven c-DNAs encoding P2X-receptor subtypes have been cloned as reviewed by Burnstock and King (1). Cloning of the purinoceptors from P2X2 to P2X6 among them was carried out by using the cDNA library from mammalian neuronal tissues, e.g., celiac, cervical and dorsal root ganglia and the hippocampus of the rat (5, 17–19). In the patch-clamp studies, *Xenopus* oocytes and HEK 293 cells transfected with c-DNAs of P2X2 and P2X3 subtypes of P2X-purinoceptors exhibited measurable inward currents across the ligand-gated cation channels via administration of the corresponding P2X-agonists. Accordingly, the findings suggest that, from the data obtained with β,γ -methylene ATP, the α,β -methylene ATP sensitive-P2Xn-receptor on ileal cholinergic nerves might be a subtype differentiated from the P2X-purinoceptor responding to ADP- β -S and 2-methylthio ATP. The hypothesis should be elucidated in further cloning studies.

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REFERENCES

- 1 Burnstock G and King BF: Numbering of cloned P2 purinoceptors. *Drug Dev Res* **38**, 67–71 (1996)
- 2 Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson KA, Schwabe U and Williams M: Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol Sci* **18**, 79–82 (1997)
- 3 Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A and Buell G: A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature* **371**, 516–519 (1994)
- 4 Brake AJ, Wagenbach MJ and Julius D: New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371**, 519–523 (1994)
- 5 Bo X, Zhang Y, Nassar M, Burnstock G and Schoepfer R: A P2X purinoceptor cDNA conferring a novel pharmacological profile. *FEBS Lett* **375**, 129–133 (1995)
- 6 Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P and Williams M: Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46**, 143–156 (1994)
- 7 Abbracchio MP and Burnstock G: Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* **64**, 445–475 (1994)
- 8 Von Kügelgen I, Schöffel E and Starke K: Inhibition by nucleotides acting at presynaptic P2-receptors of sympathetic neuro-effector transmission in the mouse isolated vas deferens. *Naunyn Schmiedeberg's Arch Pharmacol* **340**, 522–532 (1989)
- 9 Katsuragi T, Shirakabe K, Soejima O, Tokunaga T, Matsuo K, Sato C and Furukawa T: Possible transsynaptic cholinergic neuromodulation by ATP released from ileal longitudinal muscles of guinea pigs. *Life Sci* **53**, 911–918 (1993)
- 10 Moody CJ and Burnstock G: Evidence for the presence of P1-purinoceptors on cholinergic nerve terminals in the guinea-pig ileum. *Eur J Pharmacol* **77**, 1–9 (1982)
- 11 Kennedy I and Humphrey PPA: Evidence for the presence of two types of P2 purinoceptor in the guinea-pig ileal longitudinal smooth muscle preparation. *Eur J Pharmacol* **261**, 273–280 (1994)
- 12 Matsuo K, Katsuragi T, Fujiki S, Sato C and Furukawa T: ATP release and contraction mediated by different P2-receptor subtypes in guinea-pig ileal smooth muscle. *Br J Pharmacol* **121**, 1744–1748 (1997)
- 13 Reilly WM, Saville VL and Burnstock G: An assessment of the antagonistic activity of reactive blue 2 at P1- and P2-purinoceptors: supporting evidence for purinergic innervation of the rabbit portal vein. *Eur J Pharmacol* **140**, 47–53 (1987)
- 14 Sneddon P and Burnstock G: Inhibition of excitatory junction potentials in guinea-pig vas deferens by α,β -methylene-ATP: further evidence for ATP and noradrenaline as cotransmitters. *Eur J Pharmacol* **100**, 85–90 (1984)
- 15 Katsuragi T, Kuratomi L and Furukawa T: Clonidine-evoked selective P1-purinoceptor antagonism of contraction of guinea-pig urinary bladder. *Eur J Pharmacol* **112**, 119–122 (1986)
- 16 Soejima O, Katsuragi T and Furukawa T: Opposite modulation by muscarinic M1 and M3 receptors of acetylcholine release from guinea pig ileum as measured directly. *Eur J Pharmacol* **249**, 1–6 (1993)
- 17 Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G and Wood JN: A P2X purinoceptor expressed by a subset of sensory neurons. *Nature* **377**, 428–431 (1995)
- 18 Buell G, Lewis C, Collo G, North RA and Surprenant A: An antagonist-insensitive P2X receptor expressed in epithelia an brain. *EMBO J* **15**, 55–62 (1996)
- 19 Collo G, North RA, Kawashima E, Merlo-Pich E, Neidhart S, Surprenant A and Buell G: Cloning of P2X5, P2X6 receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J Neurosci* **16**, 2495–2507 (1996)