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Characterization of P_{2x}-receptors in rabbit isolated ear artery

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1 The isolated central ear artery of the rabbit contracts in response to adenosine 5'-triphosphate (ATP) and analogues, effects proposed to be mediated by stimulation of P_{2x}-receptors. We have extended the characterization of the purinoceptor in this tissue by examining the effects of a series of receptor agonists. The study was designed in such a way as to avoid factors which normally limit attempts to classify receptors on the basis of agonist potency orders.

2 D-α,β-methylene ATP (D-α,β-meATP), D-β,γ-methylene ATP (D-β,γ-meATP), L-β,γ-methylene ATP (L-β,γ-meATP), 2-methylthio-D-ATP (2-MeSATP) and ATP produced concentration-related contractions of the ear artery with similar maximum responses, suggesting that they were full agonists. Selective desensitization of P_{2x}-receptors abolished or greatly reduced responses to D-α,β-meATP, L-β,γ-meATP, D-β,γ-meATP and 2-MeSATP. Responses to ATP were inhibited by desensitization but a significant resistant component was still apparent.

3 D-α,β-meATP was the most potent agonist tested (pA_{50} 6.47 ± 0.04) being 2138 times more potent than ATP and approximately 9 times more potent than L-β,γ-meATP. The agonist potency order was: D-α,β-meATP > L-β,γ-meATP > D-β,γ-meATP ≥ 2-MeSATP > ATP. This is generally consistent with the order proposed for P_{2x}-receptors. The relative potencies of P_{2x}-agonists in the rabbit ear artery show both similarities to and differences from data obtained in other smooth muscle preparations.

Introduction

It has been recognized for some time that adenosine 5'-triphosphate (ATP) has pharmacological properties in its own right and these are mediated at P_2-purinoceptors, as distinct from P_1-purinoceptors which are preferentially activated by adenosine (Burnstock, 1978). ATP can, depending upon the chosen experimental conditions, elicit either contractile or relaxant responses from smooth muscle preparations. In 1985, Burnstock & Kennedy proposed that there were sufficient data to support a provisional subclassification of P_2-purinoceptors, designating those receptors mediating contraction of guinea-pig vas deferens, urinary bladder and rabbit ear artery as the P_{2x}-subtype, and those mediating relaxation of guinea-pig taenia coli and various vascular tissues as P_{2y}-receptors; vasorelaxation usually resulted indirectly from the release of endothelial-derived relaxant factors from vascular endothelium (Gordon, 1986).

Given the absence of selective competitive receptor antagonists this sub-classification was based largely on the rank order of agonist potency for a series of structural analogues of ATP, with the order D-α,β-methylene ATP (D-α,β-meATP), D-β,γ-methylene ATP (D-β,γ-meATP) > ATP = 2-methylthio-D-ATP (2-MeSATP) being a general characteristic of the P_{2x}-subtype. The potential pitfalls of attempting to classify receptors using relative agonist potencies alone are well known (e.g. Collis, 1985). Concerns of specific relevance to the investigation of P_{2x}-receptors include the presence of other classes of purinoceptor e.g. P_1, P_{2y} in the same tissue, the use of unstable agonists and the production of poorly-defined agonist concentration-response curves.

Unlike certain visceral smooth muscle preparations, the rabbit isolated central ear artery responds to D-α,β-meATP with a 'classical' sigmoid log agonist concentration-effect curve (Kennedy & Burnstock, 1985), allowing correct definition of a quantitative measure of agonist potency at the 50% response level. This and other factors have led us to choose to characterize the P_2-receptor mediating smooth muscle contraction in this tissue. In doing so we have attempted, where possible, to avoid pitfalls like those listed above which would otherwise tend to reduce the validity of a classification based on relative agonist potencies.

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Spasmogenic effects of series of P_2-purinoceptor agonists, putatively P_{2x}-receptor-mediated, have been described for a number of different tissues e.g. guinea-pig bladder (Cusack & Hourani, 1984), rat portal vein (Reilly & Burnstock, 1987), rabbit mesenteric artery (Burnstock & Warland, 1987) and human pulmonary artery (Liu *et al.*, 1989a). The other purpose of our study was to contribute to the accumulating body of data on these spasmogenic effects, which may allow tentative between-tissue comparisons to be made with respect to the relative potencies of key agonist probes. With this in mind we have included L-β,γ-methylene ATP (L-β,γ-meATP), demonstrated to be a stable, selective P_{2x}-receptor agonist and the most potent agent of this type tested in the guinea-pig bladder (Hourani *et al.*, 1986). A preliminary account of some of this work has been presented to the British Pharmacological Society (O'Connor *et al.*, 1990).

Methods

Tissue preparation

Male New-Zealand White rabbits (2.5–3 kg) were killed by an overdose of pentobarbitone (300 mg i.v.). The ears were removed and the central ear artery dissected out after insertion of a scored polythene cannula (0.75 mm e.d.). The cannula serves as an aid to dissection and as a means of removing the vascular endothelium. The artery was cut into 5–10 mm rings and each ring mounted horizontally on fine tungsten wire hooks in a 20 ml organ bath under isometric conditions. The baths contained Krebs solution of the following composition (mM): NaCl 117.56, NaH_2PO_4 0.89, $NaHCO_3$ 25.0, $MgSO_4$ 1.18, glucose 11.1, KCl 5.36 and $CaCl_2$ 2.55. The tissues were maintained at 37°C and gassed continually with 95% O_2/5% CO_2. Indomethacin (2.8×10^{-6} M) was included in the Krebs to eliminate the influence of products of cyclo-oxygenase. Tissues were set up under an initial tension of 0.5–1 g and allowed to equilibrate for 1 h.

Experimental protocols

Preliminary studies Various preliminary experiments were undertaken for the purpose of establishing the protocol to be used in the study proper.

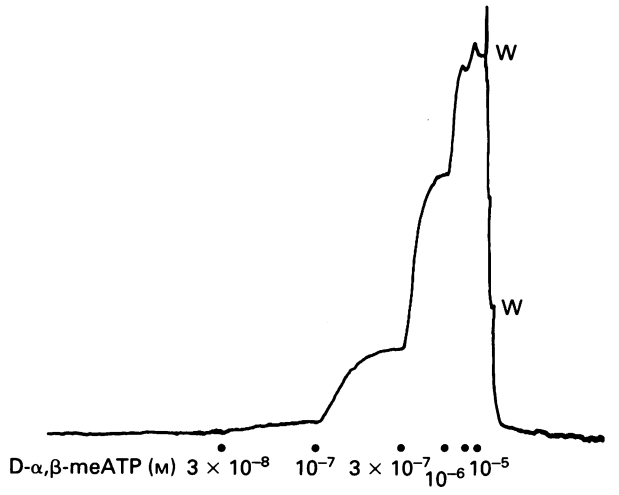

Figure 1 Reproduction of a trace showing a typical cumulative concentration-effect curve to D-α,β-methylene ATP in an isolated, endothelial-denuded, rabbit ear artery. W denotes washing of the tissue.

(a) To determine the validity of using cumulative agonist concentration-effect (E/[A]) curves, D-α,β-meATP and D-β,γ-meATP were examined with a view to comparing E/[A] curves derived from single and cumulative administration of agonist. Single exposure curves were generated using a 40 min dose interval, which had previously been shown to allow stable responses to a submaximal concentration of D-α,β-meATP. Single exposure and cumulative curves were constructed in separate tissue from the same animal.

(b) To establish that agonist sensitivity did not vary significantly throughout the course of the intended protocol, three consecutive cumulative E/[A] curves to D-α,β-meATP were generated at 70 min intervals.

(c) The selective P_1-receptor antagonist 8-sulphophenyltheophylline (8-SPT, Gustafsson, 1984) was included in all experiments in order to eliminate agonist-induced relaxations mediated directly or indirectly at adenosine receptors. A preliminary study was undertaken to confirm that the chosen concentration of 8-SPT (3×10^{-4} M) did not influence responses to a stable selective P_{2x}-receptor agonist (D-α,β-meATP).

(d) An earlier study (Kennedy & Burnstock, 1985) suggested that the smooth muscle of the central ear artery of the rabbit lacks relaxant P_{2y}-receptors. To confirm this we have looked for relaxant properties of the selective P_{2y}-agonist 2-MeSATP in endothelial-denuded preparations contracted with histamine (10^{-6} M).

(e) Exposure to a supramaximal concentration of D-α,β-meATP (3×10^{-5} M, 15 min) was used to desensitize/occupy P_{2x}-receptors and thereby investigate the mechanism by which ATP analogues contract this tissue. The selectivity of this intervention was examined by testing it against cumulative E/[A] curves produced by other spasmogens (potassium chloride, histamine and phenylephrine) in the ear artery.

Comparison of P_{2x}-receptor agonists The relative potencies of a series of selected P_{2x}-agonists and the mechanism by which each contracted the ear artery were investigated using the following protocol.

Tissues were contracted with 80 mM K^+ and 10^{-6} M acetylcholine was added once the contraction had stabilized to confirm functional denudation of endothelium. After washing, the tissues were incubated with 3×10^{-4} M 8-SPT for 45 min and a E/[A] curve was constructed to the agonist internal standard, D-α,β-meATP, by cumulative additions at 0.5 $\log_{10}$ unit increments. After washing, 8-SPT was re-administered and, 70 min after the first curve, a cumulative E/[A] curve was generated for the P_{2x}-agonist under test. The tissues were washed again, 8-SPT re-administered and D-α,β-meATP added at a supramaximal concentration (3×10^{-5} M) to produce desensitization/occupancy of P_{2x}-receptors. Once the contraction had faded (15 min) and in the continued presence of D-α,β-meATP the E/[A] curve to the agonist under test was repeated to confirm mechanism of action.

Data analysis

Contractions were expressed as a percentage of the maximum response (α) produced by the internal standard, D-α,β-meATP, in the first E/[A] curve in each tissue. Negative log molar agonist concentrations producing 50% of the maximum response (pA_{50} values) were used throughout as the index of agonist potency. These were calculated by fitting each E/[A] curve data set to a logistic function of the form;

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m}$$

in which α and m are asymptote and slope parameters, respectively, E is effect and [A] is agonist concentration.

Mean ± s.e. pA_{50} values were derived by meaning the values obtained in single tissues each taken from a different animal, with n = number of rabbits. Where appropriate, the statistical significance of differences between group pA_{50} values was determined by one-way analysis of variance with $P < 0.05$ considered to be significant.

Drugs

Drugs were obtained from the following sources; acetylcholine, adenosine 5'-triphosphate, D-α,β-methylene ATP, D-β,γ-methylene ATP, histamine, indomethacin and phenylephrine (Sigma, Poole, U.K.); 2-methylthio-D-ATP and 8-sulphophenyltheophylline (Research Biochemicals Inc., St. Albans, U.K.; L-β,γ-methylene ATP was synthesized by P.A. Cage and S.F. Hunt in the Department of Medicinal Chemistry, Fisons, Loughborough. Indomethacin was dissolved initially in 10% Na_2CO_3, all other drugs were dissolved in distilled water.

Results

Preliminary studies

(a) Agonist potencies derived from cumulative E/[A] curves did not differ significantly from those values obtained from single exposure curves for both of the examples chosen. pA_{50} values were: D-α,β-meATP 6.49 ± 0.07 (cumulative) and 6.57 ± 0.04 (single exposure), $n = 5$; D-β,γ-meATP 4.36 ± 0.12 (cumulative) and 4.26 ± 0.11 (single exposure), $n = 3$. Cumulative E/[A] curves were therefore used throughout the rest of the study.

(b) Cumulative E/[A] curves to D-α,β-meATP repeated at 70 min intervals showed no alteration in responsiveness to the agonist. pA_{50} values for first, second and third curves were; 6.36 ± 0.05, 6.36 ± 0.06 and 6.35 ± 0.07, respectively, $n = 3$.

(c) Inclusion of 8-SPT did not affect the potency of D-α,β-meATP. pA_{50} values were; 6.47 ± 0.04 (vehicle) and 6.44 ± 0.06 (in the presence of 3×10^{-4} M 8-SPT), $n = 4$.

(d) In tissues contracted with histamine, 2-MeSATP (3×10^{-8} M–3×10^{-5} M) did not produce relaxations ($n = 3$). Concentrations above 3×10^{-6} M caused further contraction of the tissues.

(e) Continuous exposure to a desensitizing concentration of D-α,β-meATP did not change the sensitivity of the ear artery to a series of other spasmogens. pA_{50} values obtained for each agent under control and desensitized conditions were as follows: potassium chloride 1.51 ± 0.04, 1.54 ± 0.03, $n = 4$; histamine 6.28 ± 0.09, 6.19 ± 0.04, $n = 4$; phenylephrine 6.99 ± 0.11, 7.02 ± 0.11, $n = 4$.

Comparison of P_{2x}-receptor agonists

A reproduction of an experimental trace showing a typical cumulative response curve to D-α,β-meATP is illustrated in Figure 1. Of the other agonists tested L-β,γ-meATP and 2-MeSATP showed qualitatively similar responses, while those to ATP and D-β,γ-meATP were somewhat less tonic in nature.

Figure 2 shows E/[A] curves for each of the agonists tested in the study (second curves of the protocol). It is apparent that all the compounds tested produced curves which had similar slopes and were sigmoid in nature. In addition, all appeared to be full agonists, as judged by the similarity of their maximum responses (α). Mean α, pA_{50} values and relative potencies are shown in Table 1. D-α,β-meATP was, by a clear margin, the most potent agent tested, showing a potency 2138 times greater than ATP and 9 times greater than the next most potent analogue, L-β,γ-meATP. The relative order of agonist potencies was: D-α,β-meATP > L-β,γ-meATP > D-β,γ-meATP ≥ 2-MeSATP > ATP.

The effect of desensitization/occupancy of P_{2x}-receptors, following exposure to a supramaximal concentration of D-α,β-meATP, on the contractile responses to each agonist is shown in Figure 3. Responses to D-α,β-meATP and L-β,γ-meATP were abolished by this intervention over a wide concentration range. The effects of D-β,γ-meATP and 2-MeSATP were also effectively eliminated, since residual responses amounted to approximately 10% and 19% respectively at the highest concentrations tested. Only ATP showed a significant resistant spasmogenic response, although the E/[A] curve was clearly right-shifted and depressed following desensitization.

Discussion

In this study of P_{2x}-receptors in the rabbit ear artery, we have tried to avoid some of the problems commonly encountered when attempting to classify receptor types by use of agonist potency orders. ATP and analogues may activate P_1-receptors

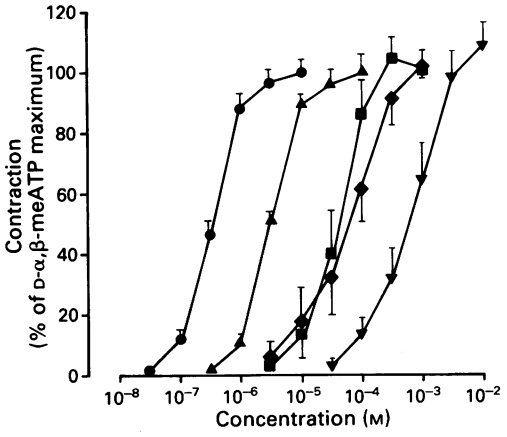

Figure 2 Cumulative log agonist concentration-effect curves for contractions of the rabbit ear artery to D-α,β-methylene ATP (D-α,β-meATP, ●), L-β,γ-meATP (▲), D-β,γ-meATP (■), 2-methylthio-D-ATP (◆) and ATP (▼). Points are means and vertical lines show s.e.mean, $n = 5$, except 2-MeSATP ($n = 3$).

Table 1 Potencies of P_{2x}-receptor agonists to contract the rabbit isolated ear artery

Compound	n	α	pA_{50}	*Relative potency*
D-α,β-meATP	5	100 ± 4	6.47 ± 0.04	2138
L-β,γ-meATP	5	100 ± 4	5.52 ± 0.04	240
D-β,γ-meATP	5	106 ± 6	4.37 ± 0.12	17
2-MeSATP	3	110 ± 7	4.15 ± 0.16	10
ATP	5	116 ± 7	3.14 ± 0.14	1

All data taken from the second E/[A] curve in each tissue.

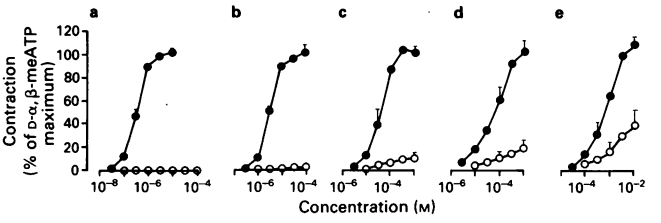

Figure 3 The effect of desensitization with D-α,β-methylene ATP (D-α,β-meATP) on contractile responses of the rabbit ear artery to (a) D-α,β-meATP, (b) L-β,γ-meATP, (c) D-β,γ-meATP, (d) 2-methylthio-D-ATP and (e) ATP. (●) Control responses and (○) responses after desensitization. Points are means and vertical lines show s.e.mean, $n = 5$, except 2-MeSATP ($n = 3$).

resulting in smooth muscle relaxation, as has been shown previously in the ear artery (Kennedy & Burnstock, 1985). This effect, which could interfere with accurate assessment of spasmogenic potency, may occur either directly, or indirectly following degradation to an ADP analogue or adenosine. We have recently illustrated the potential importance of this property by demonstrating that the relaxant effects of the P_{2y}-agonist, ADP-β-F, in the rabbit jugular vein are primarily mediated at P_1-receptors (Wood *et al.*, 1989). In the present study 8-SPT was included throughout to abolish effects of P_1-receptor activation at a concentration which did not affect responsiveness to the stable P_{2x}-analogue D-α,β-meATP. 8-SPT is a potent antagonist at P_1-receptors (pA_2 against N-ethylcarboxamidoadenosine in rabbit jugular vein 6.42, unpublished observation) and is reputed to lack the phosphodiesterase inhibitory properties associated with other compounds of this type, because of poor cell penetration (Gustafsson, 1984). Potential interference from P_{2y}-receptor stimulation was also addressed. Although present in visceral smooth muscle, in the majority of vascular tissues these are exclusively of endothelial location, so denudation is an effective means of eliminating their influence. The rabbit central ear artery appears unusual, in that P_{2y}-receptors have been shown to be absent from both vascular endothelium and smooth muscle (Kennedy & Burnstock, 1985), and we have confirmed that denuded toned preparations do not relax to 2-MeSATP. Of the agonists tested in this study, D-α,β-meATP, L-β,γ-meATP and D-β,γ-meATP have been found to be relatively resistant to degradation by ectonucleotidases (Welford *et al.*, 1987) and therefore are acceptable for receptor classification purposes. Clearly the same does not apply to ATP and 2-MeSATP which are readily dephosphorylated (Welford *et al.*, 1987), although by blocking P_1-receptors in this study we have minimized the consequences of any degradation. Attractive features of the E/[A] curves produced by P_{2x}-agonists in the rabbit ear artery are their sigmoid form, similar slopes and clearly defined maxima. Such characteristics allow agonist potency data to be interpreted with confidence. This contrasts with the biphasic curves observed for contraction of guinea-pig vas deferens (Fedan *et al.*, 1982) and guinea-pig bladder (Cusack & Hourani, 1984) and the poorly defined curves obtained in certain vascular tissues, for example, rat aorta (White *et al.*, 1985), rat pulmonary artery (Liu *et al.*, 1989b) and human small pulmonary vessels (Liu *et al.*, 1989a). Finally, although we have taken measures to increase the validity of the classification of P_{2x}-receptors in this tissue, it should be remembered that the observed potency of an agonist reflects both its affinity for the receptor and its efficacy. Tissue-related changes in receptor density or coupling efficiency can result in significant variation in responsiveness to compounds of different efficacies. Calculation of agonist affinity, as possible amongst a series of partial agonists, would provide the definitive agonist-based classification of P_{2x}-receptors.

Receptor desensitization/occupancy produced by sustained exposure to a supramaximal concentration of D-α,β-meATP was used to establish the extent to which agonist-induced contractions could be attributed to activation of P_{2x}-receptors. In

the absence of a selective receptor antagonist, D-α,β-meATP desensitization was first introduced to investigate the non-adrenergic, non-cholinergic (NANC) component of guinea-pig bladder nerve stimulation (Kasakov & Burnstock, 1983), and has since been used extensively for the purpose of characterizing effects mediated at P_{2x}-receptors (e.g. Liu *et al.*, 1989b). The selectivity of this intervention in our hands was established by the demonstration that it eliminated subsequent contractile responses to D-α,β-meATP over a 1000 fold concentration range, without influencing responses to histamine, phenylephrine and potassium. This desensitization protocol effectively abolished responses to D-α,β-meATP, L-β,γ-meATP, D-β,γ-meATP and 2-MeSATP confirming their mechanism of action. Responses to ATP, although inhibited, did show a component which was resistant to desensitization. This may have introduced a slight over-estimate of its potency at P_{2x}-receptors as based on the calculated pA_{50} value, but this does not significantly alter relative agonist potencies. Other investigators have shown small desensitization-resistant contractions to ATP in the rabbit ear artery (Kennedy *et al.*, 1986; von Kugelgen *et al.*, 1987). The resistant component appears more prominent in our study because we have gone to higher concentrations of ATP in order to define fully its E/[A] curve. The mechanism responsible is not clear, although an effect at pyrimidine-recognising receptors, as shown in the rabbit ear artery for uridine 5'-triphosphate (von Kugelgen *et al.*, 1987), is a possibility.

The absolute potencies of D-α,β-meATP and ATP found in the present study show excellent agreement with those obtained earlier for the rabbit ear artery (Kennedy & Burnstock, 1985). The relative order of agonist potency found in this study; D-α,β-meATP > L-β,γ-meATP > D-β,γ-meATP ≥ 2-MeSATP > ATP, is broadly consistent with the order designated as characteristic of P_{2x}-receptors (Burnstock & Kennedy, 1985, see Introduction). Another criterion of Burnstock's classification, selective desensitization by D-α,β-meATP, has also been fulfilled. A number of points arising from the present data are worthy of note. For example, the Burnstock classification does not distinguish between D-α,β-meATP and D-β,γ-meATP, yet in this tissue they differed in potency by more than 100 fold, a result comparable with that found for rabbit mesenteric artery (Burnstock & Warland 1987), but apparently dissimilar to the guinea-pig bladder where these analogues have similar potency (Cusack *et al.*, 1987; Welford *et al.*, 1987). The activity of L-β,γ-meATP is interesting in two respects. Firstly, because it showed 14 fold greater potency than its D-isomer. This is at odds with the general stereochemical preference for D-ribose forms attributed to P_2-purinoceptors (Burnstock & Kennedy, 1985; Gordon, 1986). Of possibly greater importance is the observation that L-β,γ-meATP was 9 fold less potent than D-α,β-meATP in the ear artery, in contrast to the guinea-pig bladder where it has been described as the most potent P_{2x}-receptor agonist tested (Hourani *et al.*, 1986). Overall, the order of agonist potency observed in the rabbit ear artery is similar to that described for rat portal vein longitudinal muscle (Reilly & Burnstock, 1987), but quite different from that demonstrated in guinea-pig bladder where L-β,γ-meATP is the most potent agonist and D-α,β-meATP and D-β,γ-meATP have similar potency (Cusack & Hourani, 1984; Welford *et al.*, 1987).

In view of the foregoing discussion on the limitations of attempting to classify receptors by use of relative agonist potencies alone, the significance of the apparent differences highlighted between this and other studies investigating P_{2x}-receptor characteristics in various tissues in not easy to assess. It is certainly conceivable that they merely reflect tissue-related factors and variations in experimental protocols, and in particular the extent to which individual studies have managed to eliminate possible sources of error. Alternatively, they may be the first indicators of a genuine difference between the receptors involved. Further quantitative studies of the P_{2x}-receptors mediating spasmogenic effects would appear warranted, particularly those comparing vascular and visceral smooth muscle preparations.

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