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Characterization of P_1-purinoceptors on rat duodenum and urinary bladder

J. Nicholls, [1]S.M.O. Hourani & I. Kitchen

Receptors & Cellular Regulation Research Group, School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH

1 The P_1-purinoceptors mediating relaxation of the rat duodenum and inhibition of contraction of the rat urinary bladder were characterized by use of adenosine and its analogues 5'-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA) and 2-*p*-((carboxyethyl)phenethylamino)-5'-carboxamidoadenosine (CGS 21680), as well as the A_1-selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). The stable analogue of adenosine 5'-triphosphate (ATP), adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP), was also used as previous work had indicated that it has a direct action on some P_1 receptors in addition to its P_2-purinoceptor activity.

2 In the rat duodenum, the order of potency of the adenosine agonists was NECA $\geqslant$ CPA > AMPPCP = adenosine > CGS 21680, and DPCPX antagonized CPA and AMPPCP at a concentration of 1 nM whereas equivalent antagonism of NECA and adenosine required a concentration of 1 μM. This suggests the presence of a mixture of A_1 and A_2 receptors in this tissue, with CPA and AMPPCP acting on the A_1 and NECA and adenosine acting on the A_2 receptors.

3 In the rat bladder, the order of potency of the adenosine agonists for inhibition of carbachol-induced contractions was NECA $\gg$ adenosine > CPA = CGS 21680, and a concentration of DPCPX of 1 μM was required to antagonize responses to NECA and adenosine. This suggests the presence of A_2 receptors in this tissue. ATP and AMPPCP each caused contractions which were not enhanced by DPCPX (1 μM) which suggests that in this tissue AMPPCP was acting only via P_2 receptors and had no P_1 agonist activity. That AMPPCP was active on the A_1 receptors in the duodenum but inactive on the A_2 receptors in the bladder implies that it has selectivity for the A_1 subtype.

4 That CGS 21680, which has been reported to bind selectively to the high affinity A_{2a} subclass of A_2 receptors, had a very low potency on the A_2 receptors in the duodenum and in the bladder suggests that these receptors are of the low affinity A_{2b} subclass.

Keywords: Adenosine; ATP; purinoceptors; duodenum; bladder

Introduction

Adenosine and adenine nucleotides have pharmacological actions on a variety of smooth muscle preparations, and these effects are mediated via receptors which have been classified as P_1- and P_2-purinoceptors, recognising adenosine and adenosine 5'-triphosphate (ATP) respectively (Burnstock, 1990; Kennedy, 1990). Studies with ATP are complicated by its rapid dephosphorylation by ectonucleotidases present on smooth muscle preparations, to give adenosine which has its own effects (Slakey *et al.*, 1990).

P_2-purinoceptors on smooth muscle have been subdivided into P_{2X}, usually mediating contraction, and P_{2Y}, usually mediating relaxation, and this classification is largely based on the different structure-activity relationships of ATP analogues in causing these two effects (Burnstock & Kennedy, 1985; Gordon, 1986; Kennedy, 1990; Cusack & Hourani, 1990). For P_{2X}-purinoceptors, methylene phosphonate analogues of ATP such as adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP) and adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP), which are resistant to dephosphorylation, are more potent than ATP which is equipotent with 2-substituted analogues such as 2-methylthioadenosine 5'-triphosphate (MeSATP), whereas on P_{2Y}-purinoceptors MeSATP is more potent than ATP which is more potent than AMPPCP or AMPCPP. However, these structure-activity relationships may be partly dependent on the different sensitivities to degradation of the analogues by ectonucleotidases, which appears to enhance their potency on some tissues but not on others (Welford *et al.*, 1986; 1987).

P_1-purinoceptors have been subdivided into A_1 and A_2 according to the potency of adenosine analogues and their sensitivity to A_1-selective antagonists such as 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). On A_1 receptors, N^6-substituted analogues such as N^6-cyclopentyladenosine (CPA) are more potent than 5'-substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA), and DPCPX has a dissociation constant in the nanomolar range, whereas on A_2 receptors NECA is more potent than CPA and DPCPX has a dissociation constant in the micromolar range (Bruns, 1990; Kennedy, 1990). Because the affinities of adenosine agonists are generally less for A_2 than for A_1 receptors, NECA is not selective for A_2 receptors but has roughly equal potency on each subtype, but a new analogue, 2-*p*-((carboxyethyl)phenethylamino)-5'-carboxamidoadenosine (CGS 21680) has recently been developed and reported to be highly A_2-selective (Hutchison *et al.*, 1989; Jacobson, 1990). Although some problems have been encountered in the classification of P_1-purinoceptors in smooth muscle, as compounds which have been reported to be selective in the central nervous system do not always display selectivity in the periphery, DPCPX has been shown to discriminate clearly between the A_1 and A_2 subtypes in pharmacological studies (Collis *et al.*, 1989; Collis, 1990).

Little is known about the ontogeny of purinergic systems in smooth muscle, but we have studied the development of responses to adenosine and ATP in two tissues, the rat duodenum and urinary bladder. In each tissue responses to purines are present from birth, and in some cases the potency of the purines is considerably higher in the neonate than in the adult, indicating that purines may play an important role in neonatal tissues. The rat duodenum relaxes to ATP and the rat bladder contracts, and these responses, as expected, are mediated by P_{2Y}- and P_{2X}-purinoceptors respectively. In the neonatal rat duodenum however, ATP causes contraction rather than relaxation, and this contraction is also mediated by P_{2Y}-purinoceptors and is not due to secondary release of prostaglandins (Nicholls *et al.*, 1990). Adenosine is inhibitory

[1] Author for correspondence.

in both these tissues, but the subtype of P_1-purinoceptor which mediates its responses has not been defined so far. In the duodenum we have shown that AMPPCP acts unexpectedly via P_1 and not P_2-purinoceptors to cause relaxation, as its effects are inhibited by the P_1 antagonist 8-*p*-sulphophenyltheophylline (8-SPT), whereas the effects of ATP, MeSATP and AMPCPP are not inhibited (Hourani *et al.*, 1991). The ontogeny of responses of the duodenum to AMPPCP also mirrors that of adenosine rather than of ATP, and indeed AMPPCP is never observed to induce contraction (Nicholls *et al.*, 1990). We have also shown an anomolous P_1 effect of AMPPCP in another smooth muscle preparation, the rat colon muscularis mucosae (Bailey & Hourani, 1990), and to a lesser extent in the guinea-pig taenia caeci (Hourani *et al.*, 1991). The P_1 effect of AMPPCP in these tissues is direct and not a consequence of its breakdown to adenosine, as it is much more resistant to degradation than is ATP or MeSATP, which are not inhibited by 8-SPT (Bailey & Hourani, 1990; Hourani *et al.*, 1991). In the rat duodenum, a ten fold lower concentration of 8-SPT was required to inhibit the effects of AMPPCP than to inhibit the effects of adenosine, which could indicate that their effects were mediated by different P_1-purinoceptors as 8-SPT has been reported to be selective for A_1 receptors in the central nervous system (Bruns *et al.*, 1986), although it may not discriminate between the subtypes in smooth muscle (Collis *et al.*, 1987). We therefore decided to use the more selective antagonist DPCPX, together with the agonists NECA, CPA and CGS 21680, to characterize the P_1-purinoceptor subtypes in the rat duodenum and urinary bladder.

Methods

Adult male Wistar rats (200–250 g) were killed by cervical dislocation, and the duodenum and urinary bladder dissected out and responses quantified as described in Nicholls *et al.* (1990). Briefly, the tissues were mounted in 10 ml organ baths containing Krebs buffer of the following composition (mM): NaCl 118, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11, aerated with 95% O_2/5% CO_2 and maintained at 35°C. A resting tension of 1 g was applied to the tissues, and isometric responses were recorded with a Grass FT03 transducer and displayed on a Grass 79D polygraph. Tissues were equilibrated for 45 min before addition of drugs, concentration-response curves were obtained non-cumulatively and potency estimates (EC_{50} values) were obtained from linear regression analysis of the ascending, linear portion of the individual concentration-response curves. For the duodenum, inhibitory responses were quantified by pre-contracting the tissue with 0.1 μM carbachol before challenging with agonist, and the relaxation expressed as % inhibition of this carbachol-induced contraction. For the bladder, excitatory responses to ATP and to AMPPCP were expressed as % of the maximal contraction induced by KCl (120–180 mM). The inhibitory effects of adenosine and its analogues were expressed as % inhibition of carbachol contraction, where the purines were added 30 s before challenge with carbachol (3 μM). In each tissue, after control concentration-response curves had been obtained, the tissues were incubated for 30 min with DPCPX, and the concentration-response curves repeated in the presence of the antagonist. Dose-ratios were calculated from the ratio of the EC_{50} values in the absence and presence of antagonist, and dissociation constants (K_D values) were calculated as the molar concentration of the antagonist divided by the dose-ratio − 1. In some experiments with the duodenum, hexamethonium (10 μM) was used as a nicotinic antagonist with the same protocol as DPCPX, and dimethylphenylpiperazinium (DMPP) was used as a nicotinic agonist.

Materials

Adenosine, ATP, AMPPCP, NECA, DMPP, carbachol and hexamethonium were obtained from Sigma UK, Ltd, and DPCPX, CPA and CGS 21680 from Research Biochemicals Inc. CPA (1 mM) was dissolved in 2% ethanol and DPCPX (1 mM) in 2% aqueous dimethylsulphoxide (DMSO) containing 6 mM NaOH. After dilution corresponding to the final bath concentration of the substances used, these solvents had no effect on the responses of the tissues.

Results

Rat duodenum

Adenosine, AMPPCP, CPA, NECA and CGS 21680 all relaxed the carbachol-contracted rat duodenum, the order of potency being NECA ≥ CPA > AMPPCP = adenosine > CGS 21680, and the EC_{50} values of these compounds were 0.4 μM, 0.7 μM, 18 μM, 28.5 μM and 98 μM respectively (Figure 1). DPCPX (1 nM) caused a parallel shift to the right of the concentration-response curves to AMPPCP and CPA, giving dose-ratios of 6.7 and 5.7, corresponding to K_D values of 0.18 nM and 0.21 nM, respectively (Figures 2a,b), but had no effect on the concentration-response curves to adenosine or NECA (Figures 2c,d). At 1 μM, DPCPX did cause shifts in the concentration-response curves to adenosine and NECA, giving dose-ratios of 6.3 and 6.7, corresponding to K_D values of 0.19 μM and 0.18 μM, respectively (Figures 2e,f). DMPP (0.1–100 μM) caused relaxation of the rat duodenum, followed at high concentrations by contraction, and these effects were inhibited by hexamethonium (10 μM), which had no effect on the contractions induced by carbachol, or on the concentration-response curves to AMPPCP or to CPA (results not shown).

Rat urinary bladder

Adenosine and NECA each inhibited contractions of the rat bladder induced by carbachol (3 μM), and the order of potency was NECA ≫ adenosine, the EC_{50} values being 1.6 μM and 92 μM respectively (Figure 3a). CPA and CGS 21680 (10 μM) also weakly inhibited carbachol-induced contractions, but EC_{50} values could not be obtained due to their low potency (results not shown). DPCPX (1 μM) caused parallel shifts to the right of the concentration-response curves for NECA and adenosine, giving dose-ratios of 5.7 and 6.3, corresponding to K_D values of 0.21 μM and 0.19 μM, respectively (Figure 3a). ATP and AMPPCP each contracted the rat urinary bladder, and these contractions were not enhanced by DPCPX (1 μM) (Figure 3b).

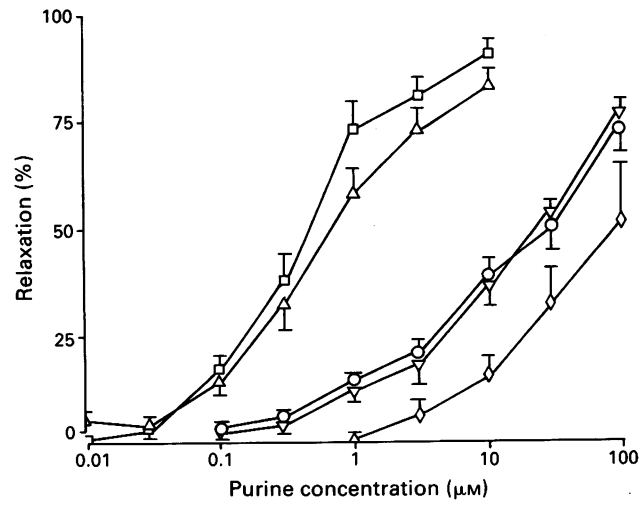

Figure 1 Relaxation of the rat duodenum induced by NECA (□), CPA (△), AMPPCP (▽), adenosine (○) and CGS 21680 (◇). Each point is the mean of at least nine determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

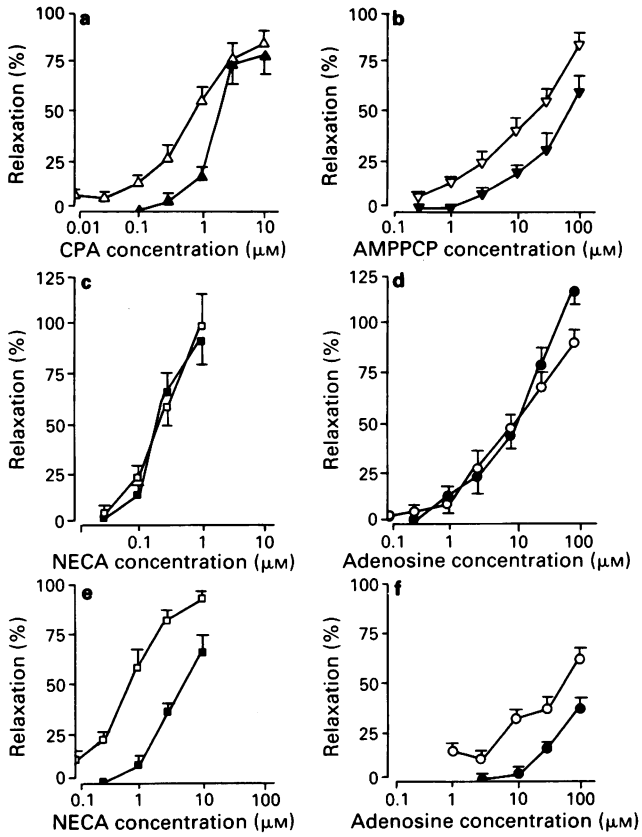

Figure 2 Relaxation of the rat duodenum induced by purines in the absence (open symbols) or presence (closed symbols) of DPCPX. (a) CPA ± DPCPX (1 nM), (b) AMPPCP ± DPCPX (1 nM), (c) NECA ± DPCPX (1 nM), (d) adenosine ± DPCPX (1 nM), (e) NECA ± DPCPX (1 μM), (f) adenosine ± DPCPX (1 μM). Each point is the mean of at least seven determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

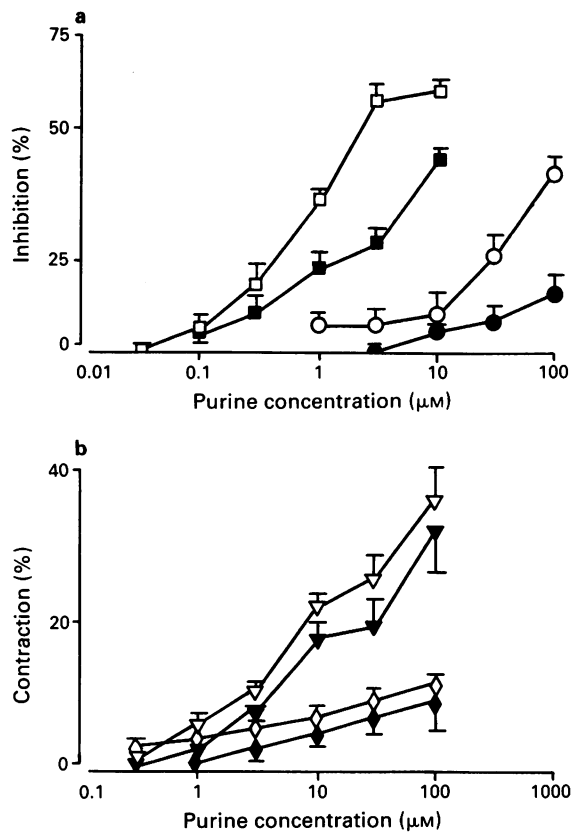

Figure 3 Effects of purines on rat urinary bladder in the absence (open symbols) or presence (closed symbols) of DPCPX (1 μM). (a) Inhibitory actions of NECA (□, ■) and adenosine (○, ●) on carbachol-induced contractions. (b) Contractile effects of AMPPCP (▽, ▼) and ATP (◇, ◆). Each point is the mean of at least five determinations, and the vertical bars show s.e.mean. For abbreviations, see text.

Discussion

These results show that in the duodenum all the adenosine receptor agonists used caused relaxation, but that the order of potency did not fall clearly into one of the two expected patterns for A_1 or A_2 receptors, as NECA and CPA were almost equipotent. Similar results have been interpreted to imply the existence of a third class of adenosine receptor, which has been called A_3 (Ribeiro & Sebastiao, 1986), but this suggestion has not gained wide acceptance. Our results with DPCPX show that whereas CPA and AMPPCP were inhibited by an A_1-selective concentration of DPCPX (1 nM), much higher concentrations (1 μM) were required to inhibit adenosine or NECA. This suggests that the duodenum contains a mixture of A_1 and A_2 receptors, with NECA and adenosine acting on the A_2 population and being inhibited by DPCPX with a dissociation constant in the micromolar range, whereas CPA and AMPPCP act on the A_1 receptors and are inhibited by DPCPX with a dissociation constant in the nanomolar range. The log concentration-response curve to CPA was somewhat steeper in the presence of 1 nM DPCPX, suggesting that at high concentrations CPA may be acting on the A_2 receptors as well. It is possible that in other tissues where NECA and CPA are equipotent, the same explanation may apply, and that a mixture of A_1 and A_2 receptors may account for the proposed A_3 subclass.

The significance of the presence of two P_1-purinoceptor subclasses both mediating an inhibitory response is unclear, as in general in smooth muscle A_2 receptors mediate relaxation whereas A_1 receptors mediate presynaptic inhibition of transmitter release or postsynaptic contractile responses (White, 1988; Kennedy, 1990). Although in several tissues, such as the guinea-pig trachea and aorta, the presence of both subclasses of P_1-purinoceptor has been reported, in each case the A_1 receptor mediates contraction while only the A_2 mediates relaxation (Farmer *et al.*, 1988; Stoggall & Shaw, 1990). For two receptor subtypes to mediate the same response in one tissue preparation is unusual, and it is not at all clear why such an apparently redundant system should have evolved. One possible explanation for the relaxant A_1 effect we have observed is that the carbachol used to precontract the tissues could have been stimulating ganglionic nicotinic receptors to cause release of acetylcholine or some other excitatory transmitter, with the A_1 receptors being situated presynaptically to oppose this release. However, the initial response to the nicotinic agonist DMPP was relaxation rather than contraction, as found by Manzini *et al.* (1985) who suggested that this is due to ganglionic stimulation of purinergic nerves. Hexamethonium at a concentration sufficient to block nicotinic receptors (10 μM) inhibited these responses to DMPP but did not inhibit the carbachol-induced contractions, suggesting that carbachol was acting directly on the muscle. In addition, hexamethonium did not affect the responses to the A_1 agonists, which again suggests that they were not acting on presynaptic receptors.

The results with the rat bladder show that the P_1-purinoceptor mediating inhibition of contraction in this tissue is of the A_2 class, as NECA was much more potent than CPA and a high concentration (1 μM) of DPCPX was required to antagonize adenosine or NECA, an A_1-selective concentration (1 nM) having no effect. The log concentration-response curve to NECA was somewhat flattened by DPCPX (1 μM), suggesting that it may not be having a solely competitive effect here. ATP and AMPPCP, which act at

P_{2X}-purinoceptors in this tissue to cause contraction, do not appear to have any action on the A_2 receptors in this tissue, as their contractions were not enhanced by blockade of P_1-purinoceptors by DPCPX. The relaxation induced by AMPPCP in the rat duodenum was however antagonized by an A_1-selective concentration of DPCPX, which confirms our previous conclusions (Hourani *et al.*, 1991) that this agonist, although it is an ATP analogue which is resistant to degradation, acts entirely and directly via P_1-purinoceptors in this tissue, and has no P_{2Y} effect. That AMPPCP has apparently no effect on the A_2 receptors in the bladder whereas it is clearly an A_1 agonist in the duodenum, suggests that it is selective for A_1 receptors and has little A_2 agonist activity. This suggestion would be compatible with its rather weak P_1 effect in the guinea-pig taenia caeci (Hourani *et al.*, 1991), which also contains A_2 receptors (Burnstock & Kennedy, 1985).

One surprising finding was the lack of potency of the A_2-selective agonist CGS 21680 in these tissues. In both the duodenum and the bladder it was much less potent than NECA, whereas it binds to rat brain A_2 receptors with an affinity roughly equal to NECA (Jarvis *et al.*, 1989), and is roughly equipotent with NECA in relaxing a number of blood vessels (Balwierczak *et al.*, 1991). A_2 receptors have been subdivided into A_{2a}, which have a high affinity and are found in the striatum, and A_{2b} which have low affinity and are found elsewhere in the brain and in fibroblasts (Bruns *et al.*, 1986; Lupica *et al.*, 1990). CGS 21680 appears to bind only to the A_{2a} sites in rat brain (Jarvis *et al.*, 1989), and one possible explanation for the lack of potency of CGS 21680 in the rat duodenum and bladder may therefore be that the A_2 receptors in these tissues are of the A_{2b} subtype. CGS 21680 has also been found to have very weak relaxant effects compared to NECA in the dog saphenous vein and guinea-pig aorta, and this has been interpreted as indicating the presence of A_{2b} receptors in these tissues (Hargreaves *et al.*, 1991), although in the absence of any selective antagonists this cannot be confirmed.

In conclusion, we have shown that the P_1-purinoceptors on the rat duodenum are a mixture of A_1 and A_2, each mediating relaxation, and that the inhibitory P_1-purinoceptors on the rat bladder are of the A_2 subtype. The stable ATP analogue, AMPPCP, appears to act directly on the A_1 receptors but not on the A_2, in addition to its potent action on the contractile P_{2X} receptors in the bladder. In each tissue the A_{2a}-selective agonist CGS 21680 has a very low potency, suggesting that the A_2 receptors may be of the low affinity A_{2b} subtype.

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