British Journal of Pharmacology (1996) 117, 170–174

Characterization of P_1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae

J. Nicholls, V.R. Brownhill & [1]S.M.O. Hourani

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

1 P_1-purinoceptors mediating relaxation of the rat duodenum longitudinal muscle and contraction of the rat duodenum muscularis mucosae were characterized by the use of adenosine and its analogues, 5′-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyl-adenosine (CPA), N^6-(phenylisopropyl)adenosine (**R**-PIA), 2-chloroadenosine (2-CADO) and 2-*p*-((carboxyethyl)phenethylamino)-5′-carboxamidoadenosine (CGS21680), as well as the P_1-purinoceptor antagonist 8-phenyltheophylline (8-PT) and the A_1-selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX).

2 In the rat duodenum longitudinal muscle, the order of potency of the adenosine agonists was CPA > NECA > adenosine > CGS21680. DPCPX antagonized responses to CPA and NECA at a concentration of 1 nM suggesting that they are acting at A_1 receptors. A Schild plot versus CPA gave a slope near to unity (slope = 0.955) and a pA_2 of 9.8 confirming that CPA was acting via A_1 receptors. Schild analysis for DPCPX versus NECA, however, gave a slope of 0.674 suggesting that NECA was acting on both A_1 and A_2 receptors. CGS21680, a selective A_{2a} agonist, was much less potent than adenosine suggesting that the A_2 receptors are of the A_{2b} subtype.

3 In the rat duodenum muscularis mucosae, the order of potency of the adenosine agonists was NECA $\geq$ **R**-PIA = CPA > 2-CADO > adenosine, and DPCPX antagonized responses to CPA and NECA at a concentration of 1 μM. CGS21680, at a concentration of 10 μM, had no effect on this tissue. This suggests the presence of A_2 receptors in this tissue and that they are of the A_{2b} subtype.

4 These results are in agreement with previous studies in the whole duodenum showing the presence of A_1 and A_{2b} receptors causing relaxation, and this shows that the longitudinal muscle dominates the response of the whole tissue. In addition, a contractile A_{2b} receptor has been revealed on the muscularis mucosae, the first time this subtype has been reported to elicit an excitatory response in a smooth muscle preparation.

Keywords: Adenosine receptors; purinoceptors; rat duodenum; smooth muscle

Introduction

Adenosine has pharmacological actions on a variety of smooth muscle preparations and mediates its effects by acting at P_1-purinoceptors (Burnstock, 1978). The P_1-purinoceptor was originally subdivided into A_1 and A_2 (van Calker *et al.*, 1979), and in smooth muscle, adenosine usually causes a relaxation via A_2 receptors but can also contract some tissues via A_1 receptors (for review, see Collis & Hourani, 1993). Since this original P_1-purinoceptor classification, it has been shown that the A_2 receptor can be subdivided into A_{2a} (high affinity receptor) and A_{2b} (low affinity receptor) and that a third receptor type, the A_3 receptor, also exists (Fredholm *et al.*, 1994).

P_1-purinoceptors have been classified from the relative potency of agonists and antagonists. For the A_1-receptor, N^6-substituted analogues such as N^6-cyclopentyladenosine (CPA) and N^6-(phenylisopropyl)adenosine (**R**-PIA) are more potent than 5′-N-ethylcarboxamidoadenosine (NECA) and the C2 substituted analogues such as 2-chloroadenosine (2-CADO), and the A_1-selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) has a dissociation constant in the nanomolar range. On A_2 receptors NECA and 2-CADO are more potent than CPA and **R**-PIA, and DPCPX has a dissociation constant in the micromolar range (Bruns, 1990; Collis & Hourani, 1993). The A_{2a} and A_{2b} subtypes can be distinguished by the selective binding of some C2-substituted analogues such as 2-((carboxyethyl)phenethylamino)-5′-carboxamidoadenosine (CGS-21680) to the high affinity A_{2a} subclass of A_2 receptors (Jarvis *et al.*, 1989). For the rat, A_3 receptor radioligand binding experiments have shown that NECA is equipotent with **R**-PIA and that CGS21680 is a relatively weak agonist. The rat A_3 receptor is also highly resistant to certain xanthine antagonists such as DPCPX and 8-phenyltheophylline (8-PT) (van Galen *et al.*, 1994) but is sensitive to other xanthine antagonists such as BW-A522 (Fozard & Hannon, 1994).

Previous functional studies on the rat duodenum showed that this tissue contained a mixture of A_1 and A_{2b} receptors both mediating relaxation (Nicholls *et al.*, 1992). The presence of A_1 receptors has also recently been confirmed by radioligand binding experiments with [^{3}H]-DPCPX (Peachey *et al.*, 1994). The significance of two P_1-purinoceptor subclasses both mediating an inhibitory response was unclear, and relaxations mediated by A_1 receptors have not been reported elsewhere. Several tissues, such as the rat colon and the guinea-pig trachea and aorta, have been shown to possess both A_1 and A_2 receptors; however, in each case the A_1 receptor mediated contraction while only the A_2 receptor mediated relaxation (Farmer *et al.*, 1988; Stoggall & Shaw, 1990; Bailey & Hourani, 1990; Bailey *et al.*, 1992). In the case of the rat colon, A_1 and A_2 receptors were localized at different sites, contractile A_1 receptors being present on the muscularis mucosae and relaxant A_{2b} receptors on the longitudinal muscle.

The aim of this work was to study P_1-purinoceptors on the two layers of the rat duodenum which contract in the longitudinal plane, the longitudinal muscle and the muscularis mucosae. We investigated whether the A_1 and A_2 receptors were localized at two distinct sites.

[1] Author for correspondence.

Methods

Male Wistar rats, University of Surrey strain, (200–250 g) were killed by cervical dislocation. The duodenum was dissected out by cutting at the base of the pyloris and 1.5 cm from this point, cleared of any connective tissue and placed over a fine bore (diameter: 5 mm) pipette and a longitudinal cut made. The two preparations were separated in the same way as previously described for the rat colon (Bailey & Hourani, 1990; 1992; Bailey *et al.*, 1992). The longitudinal muscle was separated by gently rubbing the layer with moist cotton wool, leaving a thick walled tube of mucosal tissue which contains the muscularis mucosae. The tissues were mounted in 4 ml organ baths by threads tied around each end, so that in the case of the muscularis mucosae the lumen was sealed, and maintained in Krebs solution 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. The Krebs solution was aerated with 95% O_2: 5% CO_2 and maintained at 35–36°C, and the tissues were equilibrated for at least 45 min before addition of drugs. A resting tension of 1 g was applied to the tissues and force was recorded isometrically with Grass FT03 transducers and displayed on Grass 79D polygraphs.

Concentration-response curves were obtained non-cumulatively, and for the longitudinal muscle the inhibitory responses were quantified by precontracting the tissues with carbachol (0.3 μM) before challenge with the purines. Contractions to carbachol were measured from the peak of spontaneous activity to the highest point of carbachol contraction. Relaxations were measured as the reduction in this peak height and expressed as % inhibition of carbachol contraction. For the muscularis mucosae preparation, contractions were observed by adding purine directly to the bath without precontracting the tissue and contractions were expressed as % of contraction induced by KCl (35 mM). The dose-cycle for both tissue types was 10–15 min, and no tachyphylaxis was observed to either carbachol or any of the purines used. After concentration-response curves to purines had been obtained, tissues were incubated for 30 min with DPCPX or 8-PT and the concentration-response curves were then repeated in the presence of the antagonist. In some muscularis mucosae preparations after concentration-response curves to CPA had been obtained, tissues were incubated with 25 μM indomethacin for 45 min and the concentration-response curves were repeated in the presence of indomethacin.

Dose-ratios were calculated from the ratio of the individual EC_{40} values (concentration of agonist producing 40% of the response) in the absence and presence of antagonist. For NECA on rat duodenum longitudinal muscle, EC_{40} values were estimated from extrapolation of the concentration-response curves. Apparent pA_2 values were calculated as the negative logarithm of the molar concentration of the antagonist divided by the dose-ratio − 1.

Materials

Adenosine, NECA, carbachol, 2-CADO, R-PIA, indomethacin and 8-PT were obtained from Sigma Chemicals., U.K. CPA, CGS21680 and DPCPX were obtained from Research Biochemicals Inc., and the buffer salts were of analytical grade and were obtained from BDH.

CPA (1 mM) was dissolved in 2% ethanol, DPCPX (1 mM) in 20% ethanol or in 6% aqueous dimethylsulphoxide (DMSO) containing 6 mM NaOH, and indomethacin (2.5 mM) was initially dissolved in ethanol and made up to 1/19 v/v ethanol/ 0.1M phosphate buffer. The pH of the indomethacin was 7.4 and the drug should therefore be stable for 24 h (Curry & Brown, 1982).

Results

Longitudinal muscle

On the longitudinal muscle preparation adenosine, NECA and CPA all relaxed the carbachol-contracted tissue, the order of potency being CPA > NECA > adenosine > CGS21680 with mean EC_{40} values of 0.1 μM, 0.3 μM, 8.8 μM and 41.8 μM respectively ($n = 4–6$) (Figure 1). DPCPX (1 nM) inhibited responses to CPA and NECA, giving dose-ratios of 7.5 and 3.9, corresponding to apparent pA_2 values of 9.8 and 9.5 respec-

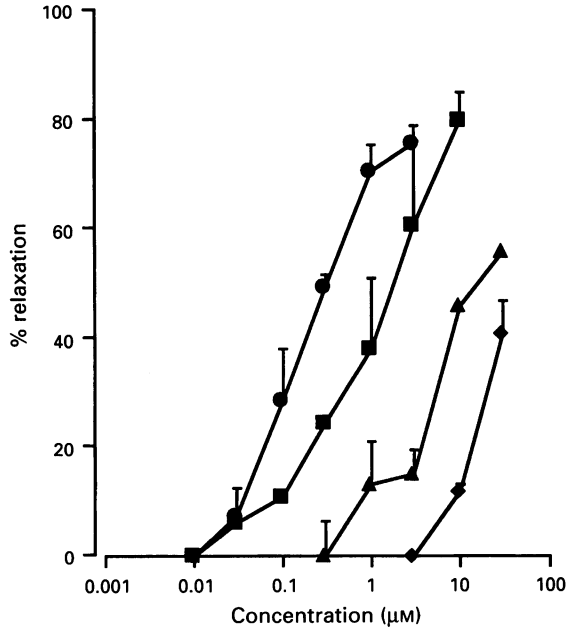

Figure 1 Relaxations of rat duodenum longitudinal muscle induced by CPA (●), NECA (■), adenosine (▲) and CGS21680 (◆). Each point is the mean with s.e.mean of at least four determinations. For abbreviations see text.

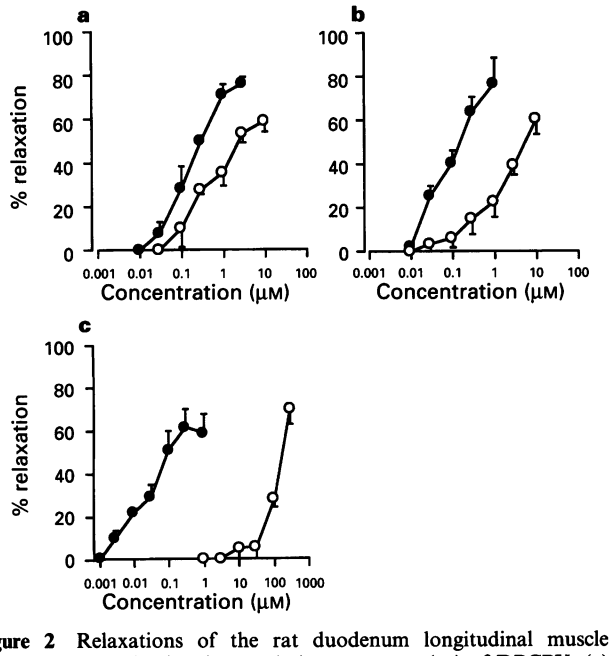

Figure 2 Relaxations of the rat duodenum longitudinal muscle induced by CPA in the absence (●) or presence (○) of DPCPX, (a) 1 nM, (b) 10 nM, (c) 100 nM. Each point is the mean with s.e.mean of at least four determinations. For abbreviations see text.

tively (Figures 2a and 3a). DPCPX (10 nM) caused a parallel shift to the right of the concentration-response curves to CPA and NECA, giving dose-ratios of 34.9 and 8.9, corresponding to apparent pA_2 values of 9.5 and 8.9 respectively (Figures 2b and 3b). DPCPX (100 nM) gave an even greater shift to the right of the concentration-response curves to CPA and NECA with dose-ratios of 527.9 and 67.3, corresponding to apparent pA_2 values of 9.7 and 8.8 (Figures 2c and 3c). DPCPX (1 μM) shifted the concentration-response curve to NECA further to the right, giving an estimated dose-ratio of 254.8, corresponding to an apparent pA_2 value of 8.4 (Figure 3d). Vehicle alone had no effect on the concentration-response curves to CPA or NECA (results not shown). Schild analysis of the results with DPCPX versus CPA gave a linear plot ($r=0.990$) with a slope of 0.955, corresponding to a pA_2 of 9.8. For DPCPX versus NECA, Schild analysis gave a linear plot ($r=0.991$) with a slope of 0.674 (Figure 4). Since the slope was much lower than unity, no pA_2 could be calculated.

Muscularis mucosae

Adenosine, NECA, CPA, R-PIA and 2-CADO all contracted the rat duodenum muscularis mucosae and the order of potency was NECA $\geq$ R-PIA = CPA > 2-CADO > adenosine with mean EC_{40} values of 0.2 μM, 0.5 μM, 0.5 μM, 2.1 μM and 33.5 μM respectively ($n=4-6$) (Figure 5). CGS21680 (10 μM) had no effect on this tissue (results not shown). DPCPX (10 nM) had no effect on the concentration-response curve to CPA (results not shown) but DPCPX (1 μM) caused a small parallel shift to the right of the concentration-response curves to CPA and NECA, giving dose-ratios of 3.8 and 4.9, corresponding to apparent pA_2 values of 6.6 and 6.4 respectively (Figure 6a,b). 8-PT (10 μM) caused a parallel shift to the right of the concentration-response curves to CPA and NECA giving dose-ratios of 36.6 and 38.5, corresponding to apparent pA_2 values of 6.6 (Figure 6c,d). Indomethacin (25 μM) had no effect on CPA-induced contractions, with concentration-response curves to CPA in the absence and presence of indomethacin (25 μM) giving mean EC_{40} values of 0.05 μM and 0.04 μM respectively (results not shown).

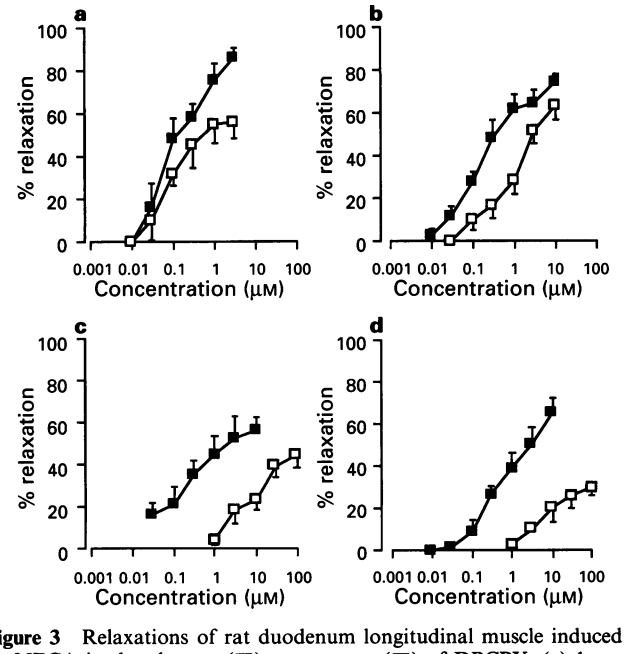

Figure 3 Relaxations of rat duodenum longitudinal muscle induced by NECA in the absence (■) or presence (□) of DPCPX, (a) 1 nM, (b) 10 nM, (c) 100 nM, (d) 1 μM. Each point is the mean with s.e.mean of at least four determinations. For abbreviations see text.

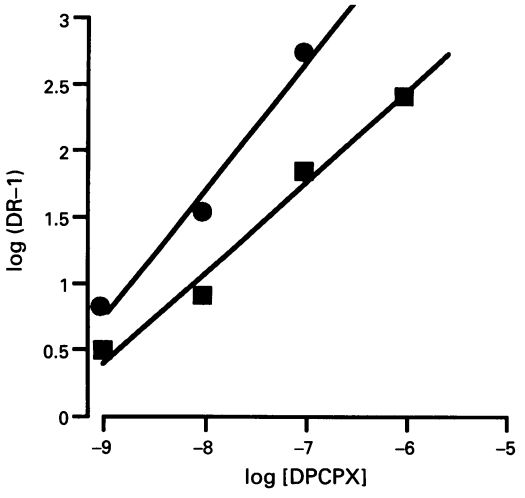

Figure 4 Schild plots of the effect of DPCPX against CPA (●) and NECA (■) in rat duodenum longitudinal muscle, from results shown in Figures 2 and 3. Each point is the mean of at least four determinations. For abbreviations see text.

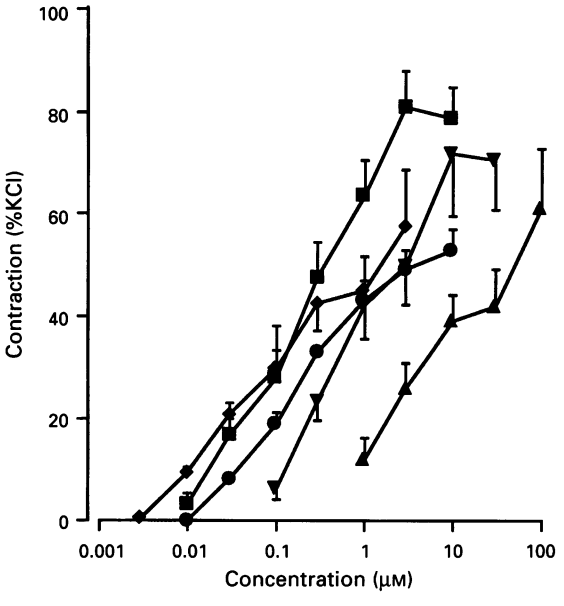

Figure 5 Contraction of rat duodenum muscularis mucosae induced by NECA (■), R-PIA (◆), CPA (●), 2-CADO (▼) and adenosine (▲). Each point is the mean with s.e.mean of at least four determinations. For abbreviations see text.

Discussion

The results show that on the rat duodenum longitudinal muscle all the adenosine receptor agonists used caused relaxation. The order of potency of the P_1-purinoceptor agonists was CPA > NECA > adenosine > CGS21680 and DPCPX had nanomolar affinity for this receptor suggesting that the agonists are acting on A_1 receptors (Collis & Hourani, 1993). The presence of a relaxant A_1 receptor is unusual as adenosine normally relaxes smooth muscle via A_2 receptors (White, 1988; Olsson & Pearson, 1990; Collis & Hourani, 1993) and in fact the rat duodenum is the only smooth muscle preparation to have been shown to relax directly via A_1 receptors (Nicholls *et al.*, 1992).

Previous studies on intact rat duodenum showed that a mixture of A_1 and A_2 receptors were present both causing relaxation of the tissue and that NECA and adenosine acted via

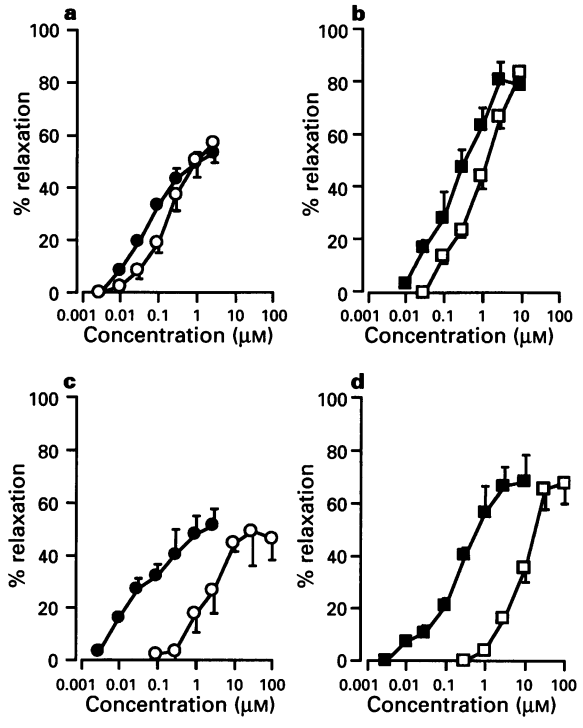

Figure 6 Contraction of rat duodenum muscularis mucosae induced by P_1-agonists in the absence (solid symbols) or presence (open symbols) of DPCPX or 8-PT. (a) CPA±DPCPX (1 μM); (b) NECA±DPCPX (1 μM); CPA±8-PT (10 μM); (d) NECA±8-PT (10 μM). Each point is the mean with s.e.mean of at least four determinations. For abbreviations see text.

the A_2 receptors whilst CPA acted on A_1 receptors (Nicholls *et al.*, 1992). In the current studies of the longitudinal muscle, NECA initially appeared to be acting via A_1 receptors as DPCPX at 1 nM and 10 nM shifted the concentration-response curve to the right with an apparent pA_2 of around 9. However, qualitatively the relaxations of the intact rat duodenum were similar to those observed in the rat duodenum longitudinal muscle, suggesting that when the whole tissue was examined responses of the longitudinal muscle dominate. It therefore seemed likely that NECA would also act via A_2 receptors on this tissue. Indeed, Schild analysis showed that DPCPX versus CPA gave a slope that did not differ significantly from unity suggesting that CPA was acting on one receptor type, and since the pA_2 for DPCPX versus CPA was 9.8 this confirms that CPA was acting via A_1 receptors. Schild analysis for DPCPX versus NECA, however, gave a slope that was much lower than unity suggesting that NECA was acting on a mixed population of receptors, presumably A_1 and A_2, to mediate relaxation of the rat duodenum longitudinal muscle. Since CGS21680 was much less potent than adenosine on this tissue it would suggest that the A_2 receptors are of the A_{2b}-subtype.

The rat duodenum muscularis mucosae preparation contracted to the P_1-purinoceptor agonists with a potency order for the analogues of NECA ≥ **R**-PIA = CPA > 2-CADO > adenosine, although it should be noted that concentration-response curves to CPA and **R**-PIA did not achieve the same maximal response as NECA and 2-CADO within the concentration-range tested. This agonist potency order did not fall clearly into either the A_1 or A_2 classification as NECA and **R**-PIA were almost equipotent, and in fact the potency order was more like that reported for an A_3 receptor (Zhou *et al.*, 1992). However it is known that on the rat A_3 receptor, CGS21680 has a K_i of approximately 0.5 μM and that the receptor is highly resistant to DPCPX and 8-PT even at concentrations of 10 μM and 100 μM respectively (van Galen *et al.*, 1994). The results in this study indicate that the contractile P_1-purinoceptor on the rat duodenum muscularis mucosae is not an A_3 receptor as CGS21680 at 10 μM had no contractile effect and both DPCPX (1 μM) and 8-PT (10 μM) inhibited responses to NECA and CPA, the pA_2 derived for 8-PT being consistent with A_1 or A_2 receptors. Since DPCPX (1 μM) gave about a 4 fold shift in the concentration-response curves to NECA and CPA it would suggest that the agonists are acting via A_2 receptors, in spite of the high potency of the N^6-substituted analogues, and the observation that CGS21680 (10 μM) had no effect on this tissue would suggest that the A_2 receptors are of the A_{2b} subtype (Lupica *et al.*, 1990).

The findings of a relaxant P_1-purinoceptor on the rat duodenum longitudinal muscle and a contractile P_1-purinoceptor on the muscularis mucosae are consistent with our previous studies in the rat colon where adenosine analogues cause relaxation of the longitudinal muscle and contraction of the muscularis mucosae (Bailey & Hourani, 1990; 1992; Bailey *et al.*, 1992). However, whereas in the rat colon contraction and relaxation are mediated by A_1 and A_{2b} receptors respectively, unusually in the rat duodenum muscularis mucosae, contractions were mediated via A_2 receptors and not A_1 receptors as would normally be expected, and in the rat duodenum longitudinal muscle, relaxations were predominantly mediated by A_1 not A_2 receptors. The receptor subtypes mediating the responses in the two layers of the duodenum are therefore the opposite of those mediating the same responses in the colon. In some tissues such as the guinea-pig taenia caeci, purines (namely ATP) have been shown to cause prostaglandin-mediated rebound contractions following relaxations (Burnstock *et al.*, 1975). However, the contractions in the rat duodenum muscularis mucosae were never preceded by relaxations and were not mediated by prostaglandins as they were not blocked by indomethacin.

In conclusion, we have shown that a mixture of A_1 and A_{2b} receptors mediate relaxation of the rat duodenum longitudinal muscle, with CPA acting on the A_1 subtype and NECA acting on both A_1 and A_{2b} receptors. The relaxant A_2 receptors however are only clearly observed when the A_1 receptors have been blocked by DPCPX. On the rat duodenum muscularis mucosae, adenosine analogues cause contraction by acting at P_1-purinoceptors of the A_{2b} subtype. In the rat whole duodenum it appears that the responses of the longitudinal muscle dominate, as in our previous work no contractile A_{2b} receptors were detected (Nicholls *et al.*, 1992).

V.R.B. is a University of Surrey Scholar. We thank the Wellcome Trust for a research grant for J.N.

References

BAILEY, S.J., HICKMAN, D. & HOURANI, S.M.O. (1992). Characterization of the P_1-purinoceptors mediating contraction of the rat colon muscularis mucosae. *Br. J. Pharmacol.*, **105**, 400–404.

BAILEY, S.J. & HOURANI, S.M.O. (1990). A study of the purinoceptors mediating contraction in the rat colon. *Br. J. Pharmacol.*, **100**, 753–756.

BAILEY, S.J. & HOURANI, S.M.O. (1992). Effects of purines on the longitudinal muscle of the rat colon. *Br. J. Pharmacol.*, **105**, 885–892.

BRUNS, R.F. (1990). Adenosine receptors: roles and pharmacology. *Ann. N.Y. Acad. Sci.*, **603**, 211–226.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: a Multidisciplinary Approach*. ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G., COCKS, T., PADDLE, B. & STASZEWSKA-BARCZAK, J. (1975). Evidence that prostaglandin is responsible for the 'rebound contraction' following stimulation of non-adrenergic, non-cholinergic ('Purinergic') inhibitory nerves. *Eur. J. Pharmacol.*, **31,** 360–362.

COLLIS, M.G. & HOURANI, S.M.O. (1993). Adenosine receptor subtypes. *Trends. Pharmacol. Sci.*, **14,** 360–366.

CURRY, S.H. & BROWN, E.A. (1982). Preparation and stability of indomethacin solutions. *Can. J. Physiol. Pharmacol.*, **60,** 988–992.

FARMER, S.G., CANNING, B.J. & WILKINS, D.E. (1988). Adenosine receptor-mediated contraction and relaxation of guinea pig isolated tracheal smooth muscle: Effects of adenosine antagonists. *Br. J. Pharmacol.*, **95,** 371–378.

FOZARD, J.R. & HANNON, J.P. (1994). BW-A522 blocks adenosine A_3 receptor-mediated hypotensive responses in the rat. *Eur. J. Pharmacol.*, **252,** R5–6.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DALY, J.W., HARDEN, K., JACOBSON, K.A., LEFF, P. & WILLIAMS, M. (1994). VI. Nomenclature and Classification of Purinoceptors. *Pharmacol. Rev.*, **46,** 143–156.

JARVIS, M.F., SCHULZ, R., HUTCHINSON, A.J., DO, U.H., SILLS, M.A. & WILLIAMS, M. (1989). [^{3}H]CGS21680, a selective A_2 adenosine receptor agonist, directly labels A_2 receptors in rat brain. *J. Pharmacol. Exp. Ther.*, **251,** 888–893.

LUPICA, C.R., CASS, W.A., ZAHNISER, N.R. & DUNWIDDIE, T.V. (1990). Effects of the selective adenosine A_2 receptor agonist CGS21680 on *in vitro* electrophysiology, cAMP formation and dopamine release in rat hippocampus and striatum. *J. Pharmacol. Exp. Ther.*, **252,** 1134–1141.

NICHOLLS, J., HOURANI, S.M.O. & KITCHEN, I. (1992). Characterization of P_1-purinoceptors on rat duodenum and urinary bladder. *Br. J. Pharmacol.*, **105,** 639–642.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoceptors. *Pharmacol. Rev.*, **70,** 761–845.

PEACHEY, J.A., HOURANI, S.M.O. & KITCHEN, I. (1994). The binding of 1,3-[^{3}H]-dipropyl-8-cyclopentylxanthine to adenosine A_1 receptors in rat smooth muscle preparations. *Br. J. Pharmacol.*, **113,** 1249–1256.

STOGGALL, S.M. & SHAW, J.S. (1990). The coexistence of adenosine A_1 and A_2 receptors in guinea-pig aorta. *Eur. J. Pharmacol.*, **190,** 329–335.

VAN CALKER, D., MÜLLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33,** 999–1005.

VAN GALEN, P.J.M., VAN BERGEN, A.H., GALLO-RODRIGUEZ, C., MELMAN, N., OLAH, M.E., IJZERMAN, O.P., STILES, G.L. & JACOBSON, K.A. (1994). A binding site model and structure-activity relationships for the rat A_3 adenosine receptor. *Mol. Pharmacol.*, **45,** 1101–1111.

WHITE, T.D. (1988). Role of adenine compounds in autonomic neurotransmission. *Pharmacol. Ther.*, **38,** 129–168.

ZHOU, Q-Y., LI, C., OLAH, M.E., JOHNSON, R.A., STILES, G.L. & CIVELLI, O. (1992). Molecular cloning and characterization of an adenosine receptor: The A_3 adenosine receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 7432–7436.

(Received May 11, 1995
Revised August 20, 1995
Accepted September 18, 1995)