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Pharmacological analysis of the interaction between purinoceptor agonists and antagonists in the guinea-pig taenia caecum

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1 In the absence of adenosine uptake inhibition, adenosine produced a concentration-dependent (threshold 30 μM) relaxation of the 5-methylfurmethide pre-contracted guinea-pig taenia caecum. The relaxation was not blocked by 8-phenyltheophylline (8-PT, 3 μM) or 1,3-dipropyl, 8-cyclopentylxanthine (DPCPX, 30 μM).

2 In the presence of the adenosine uptake inhibitor, dipyridamole (Dip, 3 μM), a biphasic adenosine concentration-effect curve was obtained (threshold 0.3 μM). The time course of the responses to adenosine in the absence of Dip was similar to that of the second phase responses in the presence of Dip and occurred over the same adenosine concentration-range. 5'-(N-ethyl) carboxamido-adenosine (NECA) concentration-effect curves (in the absence of Dip) were also biphasic. Only the first phases of the concentration-effect curves obtained with NECA and adenosine (plus Dip) were inhibited by 8-PT. The pA_2 values for 8-PT of 6.7 and 7.0 versus adenosine and NECA, respectively, were consistent with actions at P_1-purinoceptors. There was a trend towards an increase in the upper asymptote of the first phase of the NECA curve in the presence of increasing concentrations of 8-PT. The A_1-purinoceptor selective antagonist, DPCPX, also blocked only the first phase of the NECA concentration-effect curve and produced a significant increase in the upper asymptote. The pA_2 value (6.8) obtained was consistent with activation of A_2-subtype P_1-purinoceptors by the low concentrations of NECA.

3 There was no correlation between A_1-purinoceptor affinity and the propensity to cause the increase in the upper asymptote of the first phase of the NECA concentration-effect curves amongst a series of 9-methyl adenine analogues, suggesting that the amplification was not due to inhibition of an underlying A_1-purinoceptor-mediated contractile response.

4 DPCPX (10 μM) produced a significant increase in the upper asymptote of the NECA concentration-effect curve, but had no effect on isoprenaline curves whereas the phosphodiesterase inhibitor Ro 20-1724 (30 μM) produced a significant increase in the upper asymptote of both NECA and isoprenaline concentration-effect curves. Therefore the amplification of the first phase responses by DPCPX did not appear to be due to phosphodiesterase inhibition.

5 It was not possible to conclude whether second phase responses to adenosine and NECA were mediated by intracellular or extracellular sites of action. However, if intracellular sites of action were involved then adenosine did not apparently gain access by the Dip-sensitive uptake system.

Keywords: Adenosine; taenia caecum; receptor antagonism; purinoceptors

Introduction

Cell surface purinoceptors were first classified by Burnstock (1978) who proposed that purine receptors could be subdivided into two subtypes, one at which adenosine is most potent (P_1) and the other at which ATP is most potent (P_2). Responses mediated by the P_1-purinoceptor were found to be blocked by theophylline and those mediated by the P_2-purinoceptor were blocked, although not selectively, by high concentrations of quinidine 2-substituted imidazolines, 2'2-pyridyloisatogen or apamin. Subsequently, Van Calker *et al.* (1979) established the existence of two distinct P_1-purinoceptors. Those receptors mediating a decrease in adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels were termed A_1 and those mediating an increase, A_2. In addition to the contrasting effects on cyclic AMP levels, the subtypes could be distinguished by the agonist potency order of adenosine and two analogues, N-ethylcarboxamido-adenosine (NECA) and N^6-phenylisopropyladenosine (PIA). The potency order at the A_1-purinoceptor, PIA > adenosine > NECA, was reversed at the A_2-purinoceptor. The relative selectivity of the R-stereoisomer of PIA over the S-isomer was also noted to be greater at the A_1- than at the A_2-purinoceptor subtype.

It is well established that there are separate receptors for adenosine (P_1) and ATP (P_2) in the guinea-pig taenia caecum, both of which mediate relaxation (Spedding & Weetman, 1976; Brown & Burnstock, 1981; Satchell & Maguire, 1982). Satchell & Maguire (1982) showed that similar structural modifications of ATP and adenosine resulted in divergent effects and Brown & Burnstock (1981) also noted that theophylline, while inhibiting adenosine responses, failed to block the response to ATP and found the reverse to be the case for apamin, which has been classified as a non-specific blocker of ATP responses.

The P_1-purinoceptor present in the taenia caecum was first classified as A_2 on the basis of the potency order of adenosine analogues and the virtual absence of stereoselectivity of PIA (Burnstock *et al.*, 1984). The data presented in that study included the effects of a single concentration of the purinoceptor antagonist, 8-phenyltheophylline (8-PT), on the agonist concentration-effect curves obtained using NECA, 2-chloro-adenosine, L-PIA, D-PIA, cyclohexyladenosine (CHA) and adenosine. The results show that the degree of rightward shift obtained with 8-PT (10 μM) was agonist-dependent. The concentration-ratios ranged from a highly

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significant value of 45 for NECA to non-significant displacements for L-PIA, CHA and adenosine. These results are not consistent with expectations for the competitive antagonism of a homogeneous population of purinoceptors. In an attempt to expose any receptor heterogeneity, we began by performing experiments on the taenia caecum preparation using adenosine and NECA as agonists and a wide range of concentrations of the antagonists, 8-PT and 1,3-dipropyl, 8-cyclopentyl xanthine (DPCPX). The agonist response time-courses and the agonist concentration-effect curves obtained revealed complexity in the actions of both the agonists and antagonists.

Methods

Guinea-pig taenia caecum assay

Lengths of taenia caecum (1.5–2 cm), dissected from male guinea pigs (Dunkin Hartley, 250–350 g), were suspended in 20 ml organ baths containing de Jalons solutions maintained at 31 ± 0.5°C (mM composition: Na^+ 160, K^+ 5.63, Cl^- 160.71, Ca^{2+} 0.54, HCO_3^- 5.95, glucose 2.78). Responses were measured isotonically following addition of a 1.5 g load, a single wash by bath fluid replacement and a 60 min stabilisation period. Test compounds were applied for a 60 min incubation period after the response to a concentration (0.1 μM) of an ACh M-receptor agonist, 5-methylfurmethide (5-mef), reached a plateau. This response was approximately 80% of the maximum response which could be obtained with 5-mef. In preliminary experiments (data not shown) the response to 5-mef was shown to be maintained for at least 180 min which is sufficient time to obtain a fully-defined relaxatory agonist concentration-effect curve. Relaxatory responses were expressed as mm of experimental trace where the recording system amplification was set so that 10 mm was equivalent to 1.3 mm change in tissue length.

Guinea-pig left atrium assay

Purinoceptor-mediated negative inotropic responses were studied under isometric conditions in isolated left atria from male guinea pigs (Dunkin Hartley, 250–350 g). Left atria were rapidly excised and suspended under 1 g tension in 20 ml organ baths containing Krebs-Henseleit solution (mM, composition: Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, $H_2PO_4^-$ 1.2, HCO_3^- 24.9, SO_4^{2-} 1.2, glucose 11) maintained at 37°C and gassed with 95%O_2:5%CO_2. The preparations were electrically stimulated with square wave pulses (1 Hz frequency, 1 ms pulse duration, 120% threshold V) via both a punctate and field platinum electrode. Preparations were washed four times during a 60 min stabilisation period prior to 60 min incubation with test compounds or vehicle. Responses were expressed as % change in basal force (g) of contraction.

Protocols

Single agonist concentration-effect curves were obtained by cumulative dosing in each preparation. Six preparations were used simultaneously and treatments were allocated on a randomised block design so that, as far as possible, each organ bath received each treatment.

Data analysis

Where possible, individual agonist curve data were fitted to the Hill equation,

$$E = \frac{\alpha \cdot [A]^{n_h}}{[A]_{50}{}^{n_h} + [A]^{n_h}}$$

to provide estimates of midpoint slope parameter (n_H), midpoint location ($\log[A]_{50}$) and upper asymptote (α), as described previously (Black & Shankley, 1985). The effect of drug treatment on these parameters was assessed by one-way analysis of variance, paired t test or Bonferroni modified t test for multiple comparisons (Wallenstein *et al.*, 1980), as appropriate. P values of less than 0.05 were considered to be significant. When it was not possible to fit the data to the Hill equation, individual $\log[A]_{50}$ values were estimated by linear interpolation between the data points either side of the half maximal effect level.

When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel rightward shift of the agonist concentration-effect curves with no change in upper asymptote, pK_B values were obtained by fitting the individual $\log[A]_{50}$ values obtained in the absence and presence of antagonist to a derivation of the Schild equation as described previously (Black *et al.*, 1985). When the criteria were not satisfied or not fully testable, a pA_2 value was estimated from the dose-ratio obtained with the lowest antagonist concentration which produced a significant rightward shift in all the replicate experiments.

Compounds

The following compounds were used: Adenosine, 5'-(N-ethyl)carboxamido adenosine (NECA), 8-phenyltheophylline (8-PT), dipyridamole (Dip), isoprenaline (all from Sigma Chemicals Ltd.), 1,3-dipropyl, 8-cyclopentylxanthine (DPCPX, Cambridge Research Biochemicals Ltd), 5-methylfurmethide (5-Mef, a gift from Wellcome Research Laboratories, Beckenham, Kent) and Ro 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, a gift from Hoffman La Roche, A.G.). The following compounds were supplied by Discovery Therapeutics Inc., Richmond, VA, USA N-0838 (9-methyl adenine), N-0837 (N^6-(3-pentyl), 9-methyl adenine), N-0840 (N^6-cyclopentyl, 9-methyl adenine), N-0861 (N^6-(endo-2-norbornyl), 9-methyl adenine) and N-0946 (N^6-(endo-2-norbornyl)-8-cyclopentyl),9-methyl adenine). NECA, adenosine and 5-Mef were dissolved in water. 8-PT and DPCPX were prepared in 80% ethanol plus 0.2 N NaOH at 20 mM. Dip, Ro 20-1724, N-0838, N-0837, N-0840, N-0946 and N-0861 were dissolved in a minimum volume (< 10 μl)) of absolute ethanol and made up to a concentration of 20 mM with distilled water. Isoprenaline was prepared in stoichiometric ascorbic acid as an antioxidant. Thereafter all dilutions of compounds were made in distilled water. In all experiments a vehicle control was included which corresponded to the highest dose of test compound administered.

Results

Effects of adenosine

In preliminary experiments, it was found that adenosine reduced the basal tone of some, but not all, the taenia caecum preparations examined. Therefore, tissues were precontracted with 5-Mef (0.1 μM). Under these conditions, adenosine, in the absence of adenosine uptake blockade, consistently produced concentration-dependent (30 μM to 3 mM) relaxation. The maximum relaxation ranged between 80 and 180% of the 5-Mef contraction varying considerably between, but not within, experiments. It was not possible to define the upper asymptote of the relaxant concentration-effect curves because of the high concentrations of adenosine involved which were at the limit of its solubility. The responses to adenosine were not blocked by the P_1-receptor non-selective antagonist, 8-PT (3 μM, Figure 1a) or the P_1-purinoceptor, A_1-subtype selective antagonist, DPCPX (30 μM, Figure 1b). These concentrations of the antagonists correspond to approximately 10 and 300 fold their reported

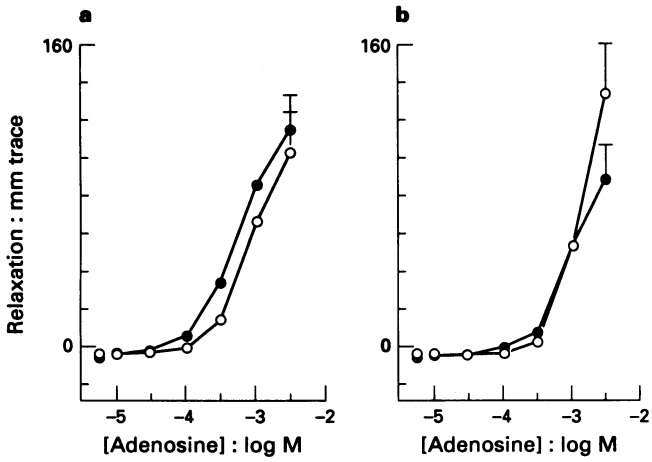

Figure 1 Adenosine concentration-effect curves obtained in the absence (●) and presence (○) of (a) 3 μM 8-PT and (b) 30 μM DPCPX on the guinea-pig taenia caecum assay ($n = 5/6 \pm$ s.e.mean). For abbreviations, see text.

K_B values at A_2-purinoceptors and 10 and 10,000 fold their reported K_B values at A_1-purinoceptors, respectively (Griffith *et al.*, 1981; Martinson *et al.*, 1987). Therefore, the action of adenosine in the absence of uptake blockade was apparently not mediated by A_1- or A_2-purinoceptors.

In the presence of increasing concentrations of the adenosine uptake blocker, dipyridamole (Dip, 0.3–3 μM), adenosine produced concentration-dependent relaxations at progressively lower concentrations (Figure 2a). In the presence of 3 μM Dip, the adenosine concentration-effect curve looked as though it was biphasic and spanned a concentration range of ~4.5 log cycles. Further evidence for the biphasic nature of the curve was provided by the observation that the responses to a concentration (3 μM) of adenosine in the presence of 3 μM Dip, which produced approximately half the first phase maximum, took about 5 min to reach a plateau whereas the second phase response to 1 mM adenosine took about 15 min to reach a plateau. The slow time courses of the individual responses in the second phase of the adenosine curve obtained in the presence of 3 μM Dip appeared to be similar to those obtained throughout the curve obtained in the absence of Dip (Figure 2b). It was not possible to fit the first phase data from all the individual preparations because the upper asymptote was not always sufficiently well-defined. Nevertheless, the $p[A]_{50}$ value of the first phase in the presence of Dip (3 μM) was estimated, by eye, to be around 5.5. Higher concentrations of Dip produced pronounced relaxations of the taenia caecum (data not shown) and so it was not possible to determine the concentration of Dip which would produce no further leftward shift of the adenosine curve and hence, presumably, saturate the uptake process. However, 3 μM Dip may be close to a saturating concentration because it is approximately 190 fold higher than its reported K_I value (16 nM) for the uptake process in guinea-pig left atrium (Kenakin, 1982).

In the presence of Dip (3 μM), 8-PT (0.3–30 μM) produced concentration-dependent rightward shift of the first phase of the adenosine curve although the second phase did not appear to be inhibited (Figure 4a). It was not possible to fit the first phase data to the Hill equation and hence provide an objective estimate of the pK_B value for 8-PT. However, it was possible to obtain an approximate pA_2 value by estimating a dose-ratio by linear interpolation of the mid-region of the adenosine concentration-effect curves obtained in the absence and presence of 3 μM 8-PT. The value obtained (~6.7) is consistent with the value reported for competitive antagonism of P_1-purinoceptors (Griffith *et al.*, 1981).

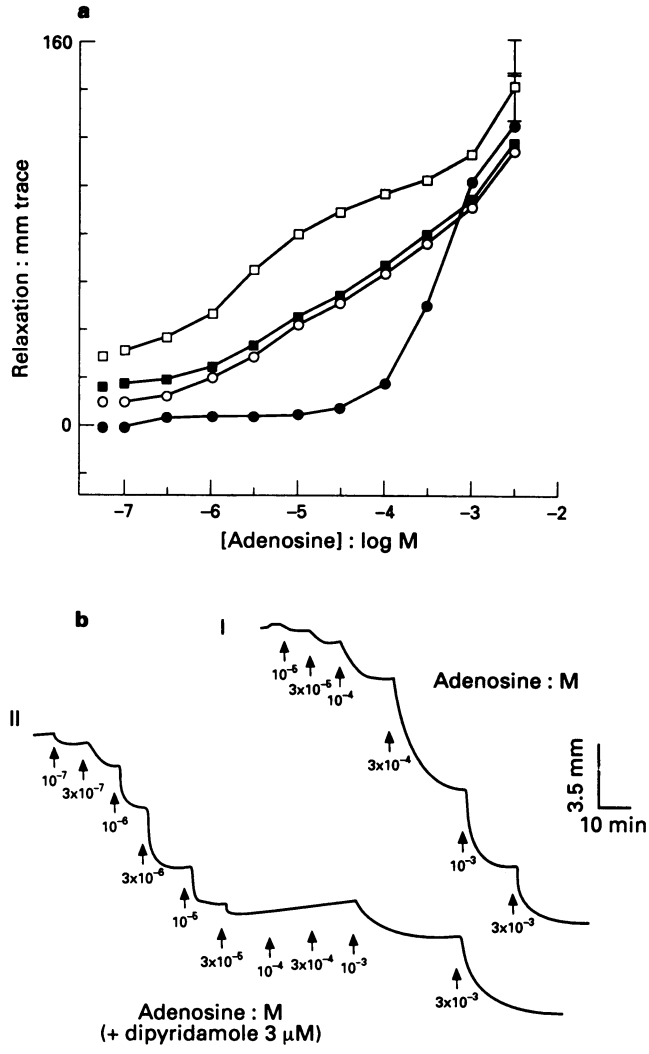

Figure 2 (a) Adenosine concentration-effect curves obtained in the absence (●) and presence of 0.3 (○), 1 (■) and 3 (□) μM dipyridamole on the guinea-pig taenia caecum assay ($n = 6 \pm$ s.e.mean). (b) Examples of experimental traces showing adenosine concentration-effect curves obtained by cumulative dosing in the absence (I) and presence (II) of 3 μM dipyridamole. The scale shown refers to absolute changes in tissue length where 10 mm experimental trace was equivalent to 1.3 mm tissue length.

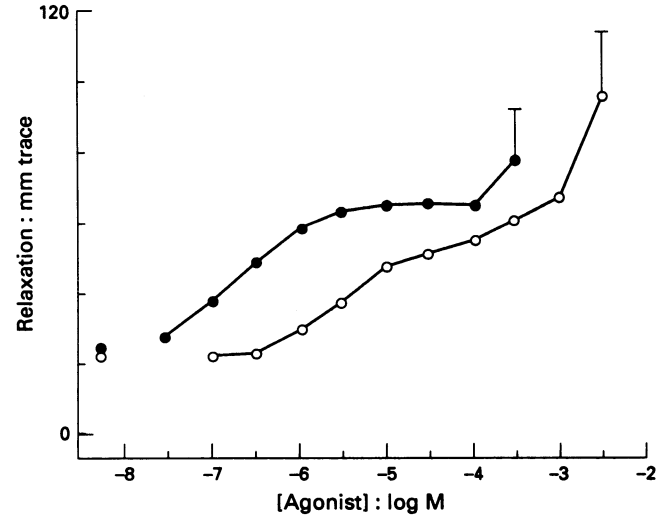

Figure 3 NECA (●) and adenosine (○) concentration-effect curves obtained in the presence of 3 μM dipyridamole on the guinea-pig taenia caecum assay ($n = 3 \pm$ s.e.mean).

Effects of NECA

NECA has been classified as a potent A_2-purinoceptor agonist which is not significantly removed from the receptor compartment by the adenosine uptake process (Collis, 1983; Burnstock *et al.*, 1984). Biphasic concentration-effect curves were obtained with NECA which were similar to those obtained with adenosine in the presence of Dip (3 μM), although both the first and second phase responses took longer than adenosine to reach a plateau. The response to a concentration of NECA (0.1 μM) producing half the maximum first phase response took approximately 15 min to plateau compared to about 5 min for the equivalent adenosine response. The second phase NECA responses took in excess of 60 min to reach a plateau (see Figures 2b and 4d) compared with 15 min estimated for adenosine. Therefore, the responses in both phases of the NECA curve took 3–4 times longer to attain a plateau than the corresponding adenosine responses in the presence of Dip. The presence of 3 μM Dip did not appear to affect the shape or location of

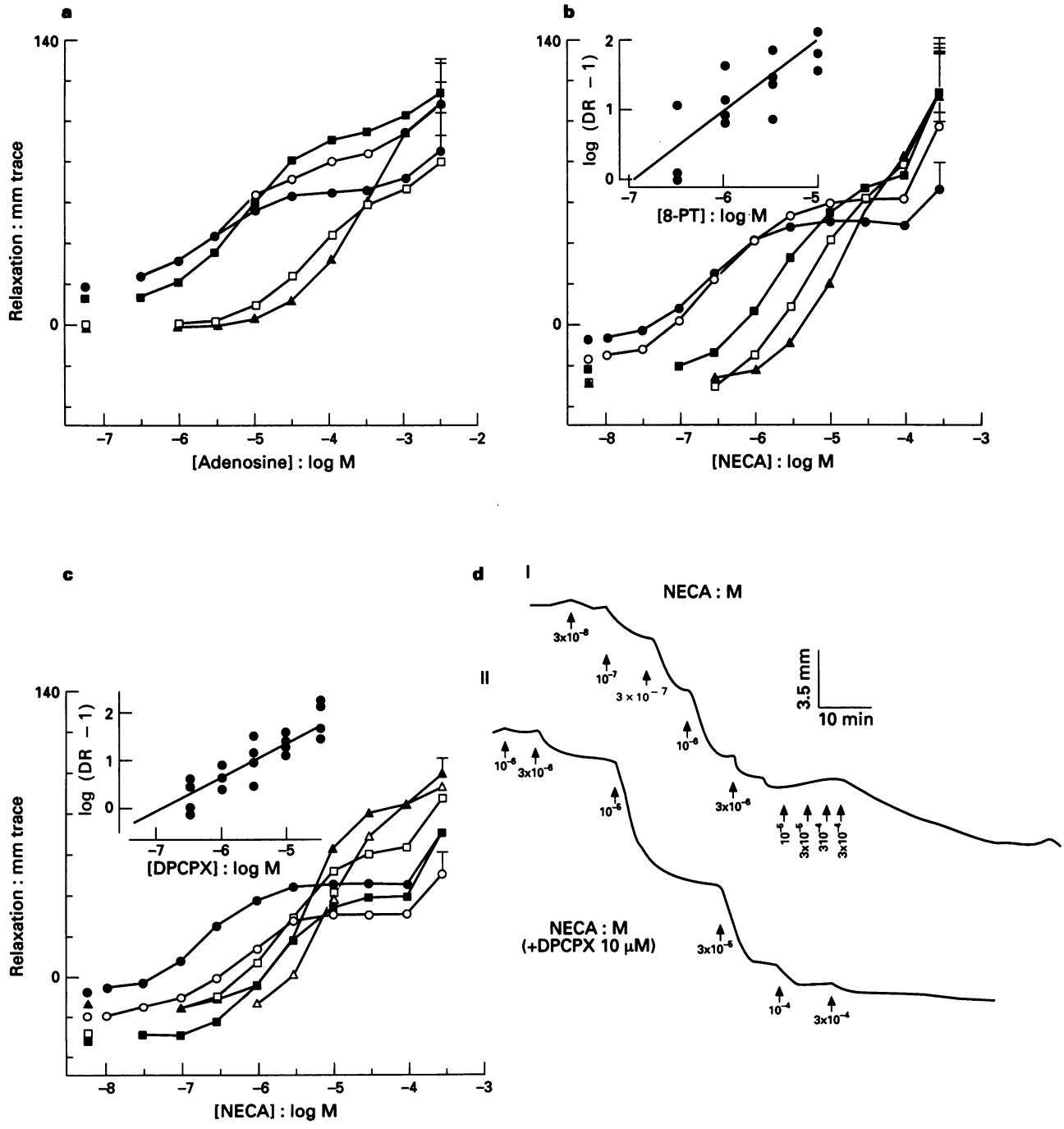

Figure 4 (a) Adenosine concentration-effect curves obtained in the absence (●) and presence of 0.3 (○), 1 (■) 3 (□) and 10 (▲) μM 8-phenyltheophylline with dipyridamole (3 μM) present throughout on the guinea-pig taenia caecum assay ($n = 5/6 \pm$ s.e.mean). (b) NECA concentration-effect curves obtained in the absence (●) and presence of 0.3 (○), 1 (■) 3 (□) and 10 (▲) μM 8-phenyltheophylline (8-PT) on the guinea-pig taenia caecum assay ($n = 4/6 \pm$ s.e.mean). The inset shows the corresponding Schild plot (see text for details). (c) NECA concentration-effect curves obtained in the absence (●) and presence of 0.3 (○), 1 (■) 3 (□) and 10 (▲) and 30 (△) μM DPCPX on the guinea-pig taenia caecum assay ($n = 5/7 \pm$ s.e.mean). The inset shows the corresponding Schild plot (see text for details). (d) Example of experimental trace showing NECA concentration-effect curves obtained by cumulative dosing in the absence (I) and presence (II) of DPCPX (10 μM). The scale shown refers to absolute changes in tissue length where 10 mm experimental trace was equivalent to 1.3 mm tissue length. For abbreviations, see text.

the NECA curve as judged by comparing the NECA curve obtained in the presence of Dip (Figure 3) and the control curves obtained in the absence of Dip (Figures 4b and 4c) albeit in separate experiments.

8-PT and DPCPX (0.3–10 μM) produced concentration-dependent rightward shift of the first phase of the NECA curve without affecting the second phase (Figure 4). Interestingly, in the presence of a higher concentration of DPCPX (30 μM), the curves were monophasic and the concentration-effect profile appeared to change again; all the responses appeared to attain a plateau more rapidly than the first phase control responses. Analysis of the first phase data obtained in the absence and presence of 8-PT (1 μM) gave a pK_B value estimate of 6.97 ± 0.19 (Schild slope parameter, b = 0.99 ± 0.20, d.f. = 19). The Schild slope parameter for DPCPX was significantly different from unity (0.67 ± 0.09, d.f. = 29) but notwithstanding this a pA_2 value of 6.77 ± 0.10 was estimated from the shift obtained in the presence of DPCPX (1 μM). This latter value is possibly consistent with the pK_I value of 7.16 estimated by Martinson et al. (1987) for DPCPX at the A_2-purinoceptors present in human platelets. Therefore, it is likely that the response elicited by low concentrations of NECA and also presumably low concentrations of adenosine in the presence of Dip, are mediated by the A_2-subtype of the P_1-purinoceptor class.

The rightward shifts of the first phase of the NECA curves by DPCPX and 8-PT were associated with other complex changes. The rightward shift obtained with 8-PT was accompanied by a trend towards an increase in the upper asymptote of the first phase of the curve, although the differences in asymptote values were not significant as tested by analysis of variance (Table 1). However, analysis of variance did reveal a significant difference between the DPCPX treatment groups of the NECA curve asymptote values ($F_{(4,20)} = 3.4132$, $P < 0.05$). Subsequent analysis using the Bonferroni modified t test for multiple comparisons indicated that the value in the presence of 10 μM DPCPX was significantly increased from the control value ($t = 2.54$, $P < 0.05$). Furthermore, DPCPX, but not 8-PT, showed significant heterogeneity on analysis of variance in the midpoint slopes of the first phase of the NECA curves (Table 1). However, the steeper slopes at the two highest concentrations of DPCPX were not significantly different from the control as judged by the Bonferroni modified t test.

The finding that an A_1-purinoceptor selective antagonist, DPCPX, increased the upper asymptote of the first phase of the NECA curve suggested that the amplification could be due to NECA activating A_1-purinoceptors coupled to contraction of the guinea-pig taenia caecum. This hypothesis was investigated further with a series of 9-methyl adenine analogues which were shown, by competitive analysis, to exhibit a range of affinity values on the guinea-pig left atrium A_1-purinoceptor assay (Table 2). There was no relationship between the compounds' A_1-purinoceptor affinity and their ability to increase the upper asymptote of the first phase of the NECA curve. Indeed, N-0838 (100 μM), the unsubstituted parent compound, 9-methyl adenine, did not produce a significant shift of the NECA curve on the guinea-pig left atrium or taenia caecum assays although it increased the upper asymptote 2.3 fold in the guinea-pig taenia caecum.

Effects of phosphodiesterase inhibition

Several xanthine based adenosine ligands are also recognised as being inhibitors of phosphodiesterase. Such an action of the antagonists used in this study could, in principle at least, produce the increase in upper asymptote of the first phase of the NECA and adenosine concentration-effect curves. This was investigated by comparing the effects of the antagonists and the selective phosphodiesterase inhibitor, Ro 20-1724 (Bergstrand et al., 1977) on the curves obtained on the 5-Mef pre-contracted taenia caecum assay with NECA and the selective β-adrenoceptor agonist, isoprenaline. Ro 20-1724 (30 μM) produced a significant relaxation of the taenia caecum, equivalent to approximately 20% of the maximum response obtained with NECA under control conditions, and

Table 2 pK_B estimates for a series of 9-substituted adenine derivatives at A_1-purinoceptors on the guinea-pig isolated left atrium assay and their effect, at the concentration shown in parentheses, on the maximum response of the first phase of the adenosine concentration-effect curve obtained on the guinea-pig taenia caecum assay

Ligand	*Guinea-pig left atrium* (pK_B ± s.e.)	α_B/α ± s.e.mean	[*Ligand*]
N-0838	<4	2.32 ± 0.24*	(100 μM)
N-0861	6.28 ± 0.09	2.19 ± 0.22*	(10 μM)
N-0840	6.17 ± 0.11	1.35 ± 0.27	(30 μM)
N-0837	5.26 ± 0.10	2.32 ± 0.23*	(100 μM)
N-0946	5.84 ± 0.06	1.66 ± 0.48	(30 μM)

The pK_B values were estimated according to the methods described in the text using NECA as agonist. In each case the corresponding Schild plot slope parameter was not significantly different from unity. N-0838 (100 μM) did not produce a significant shift of the NECA curve. The effect on the maximum response in the taenia caecum assay (α_B/α) is expressed as the ratio of the first phase maximum responses estimated in the presence and absence of the antagonist at the concentration shown in parentheses. *Significantly greater than unity ($P < 0.05$).

Table 1 The effect of 8-phenyltheophylline (8-PT) and 1,3-dipropyl, 8-cyclopentylxanthine (DPCPX) on estimates of the location ($p[A]_{50}$), midpoint slope and upper asymptote (mm trace) of the first phase of the NECA concentration-effect curves obtained on the guinea-pig taenia caecum assay as shown in Figures 4b and 4c

[8-PT]: μM	0	0.3	1	3	10	
$p[A]_{50}$	6.53	6.26	5.50	5.25	4.81	
(s.e.mean)	(0.16)	(0.22)	(0.15)	(0.13)	(0.06)	
asymptote	49.3	59.8	72.8	84.2	97.2	
(s.e.mean)	(14.0)	(34.6)	(28.5)	(20.3)	(23.6)	
slope	1.32	1.25	1.05	0.98	1.01	
(s.e.mean)	(0.02)	(0.04)	(0.10)	(0.06)	(0.07)	
[DPCPX]: μM	0	0.3	1	3	10	30
$p[A]_{50}$*	6.70	6.20	5.86	5.64	5.34	4.97
(s.e.mean)	(0.10)	(0.10)	(0.07)	(0.14)	(0.07)	(0.14)
asymptote*	45.2	32.3	38.4	64.0	82.0†	92.2
(s.e.mean)	(6.3)	(9.1)	(14.3)	(14.3)	(9.1)	(10.2)
slope*	1.30	1.09	1.28	1.16	1.77	1.45
(s.e.mean)	(0.08)	(0.09)	(0.05)	(0.07)	(0.11)	(0.11)

$n = 4/7$ ± s.e.mean.
*Significant differences between treatment group values ($P < 0.05$) as tested by one-way analysis of variance. †Significant ($P < 0.05$) difference from control value as tested by Bonferroni modified t test.

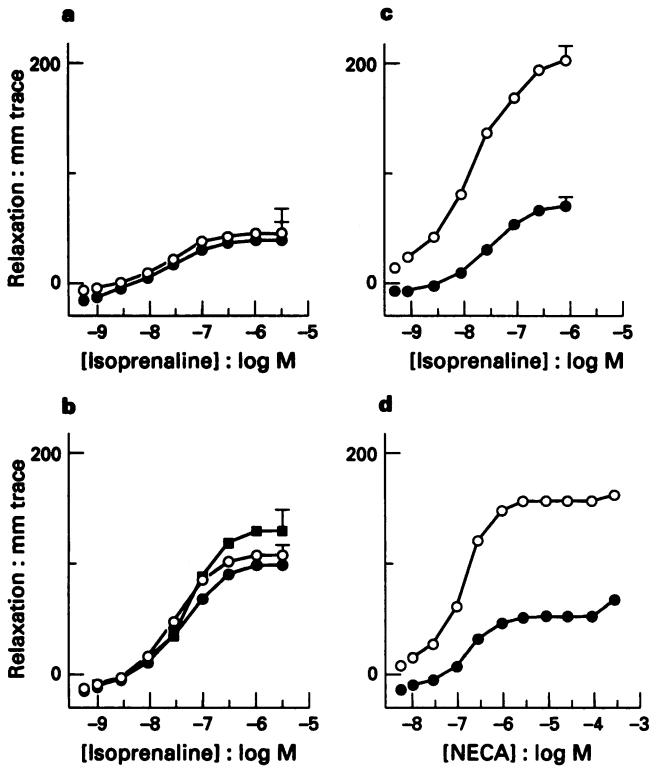

Figure 5 Isoprenaline concentration-effect curves obtained in the absence (●) and presence of (a) 10 μM 8-phenyltheophylline (○); (b) 10 (○) and 30 (■) μM DPCPX and (c) 30 μM of the phosphodiesterase inhibitor Ro 20-1724 (○) on the guinea-pig taenia caecum assay. Panel (d) shows NECA concentration-effect curves obtained in the absence (●) and presence (○) of 30 μM Ro 20-1724. For abbreviations, see text.

produced a significant 250% increase in the upper asymptote of the first phase of the NECA curve (Figure 5a). Similarly, Ro 20-1724 (30 μM) produced a relaxation equivalent to 23% of the upper asymptote of a control concentration-effect curve obtained with isoprenaline and produced a 250% increase in the upper asymptote of the isoprenaline curve (Figure 5b). In contrast, 10 μM 8-PT and 30 μM DPCPX, did not have a significant effect on the upper asymptote of the isoprenaline curves.

Discussion

The finding that adenosine was relatively impotent in the absence of agonist uptake blockade was not surprising because agonist uptake systems are known to be able to lower significantly the concentration of agonists in the receptor compartment of isolated tissue bioassays. However, the difference in the behaviour of adenosine in the absence and presence of uptake blockade could not be accounted for simply by changes in the concentration of adenosine at a homogeneous population of receptors. First, in the presence of adenosine uptake blockade (achieved with Dip), the adenosine concentration-effect curve was biphasic. Second, the selective P_1-purinoceptor antagonist, 8-PT was only effective against the first phase responses exposed by the uptake blockade. Although the criteria for competitive antagonism could not be tested objectively because of the complicating presence of the second phase, the pA_2 value estimated from the dose-ratios obtained in the presence of low concentrations of this antagonist suggested that the first phase was mediated by P_1-purinoceptors. The idea that the second phase responses obtained in the presence of Dip and all of the responses obtained in the absence of Dip were mediated by the same non-P_1-purinoceptor mechanism of action of adenosine was supported by the observation that the time course of the individual responses were similar and significantly slower than those mediated by P_1-purinoceptors.

If the resistant action involved the stimulation of additional purinoceptors present in the same compartment as the P_1-receptors, then uptake blockade, which is assumed to increase the agonist concentration in the receptor compartment, might have been expected to increase the potency of adenosine at both sites to an equal extent, which was not the case. However, this expectation is based on the assumption that the activation of the putative additional receptors occurs over an adenosine concentration-range which can be significantly lowered by the uptake process. In fact, the concentration-range of adenosine over which the second phase responses were obtained is in the region usually considered to saturate the uptake process (~10 μM, Kenakin, 1982; Schrader *et al.*, 1972). Therefore, although at first sight the data might suggest an intracellular rather than an extracellular site of action, it is not possible to draw any unequivocal conclusions regarding the location of the site mediating the resistant action. An intracellular site of action may have been implicated if the second phase responses were inhibited by Dip. In the event, as far as we could ascertain (Figure 1), the second phase responses appeared unchanged in the presence of Dip. This observation, that the second phase responses were independent of the Dip-sensitive adenosine uptake process, was supported by the finding that the agonist NECA, which is reported not to be taken up, also produced, at high concentrations, relatively slow time course responses which were not blocked by P_1-purinoceptor antagonists. Therefore, an explanatory model to account for these data requires that the second phase responses are mediated by high concentrations of adenosine and NECA acting either at an intracellular site which is accessed independently from the Dip-sensitive uptake site or an extracellular site which is activated at concentrations of adenosine above those which saturate the uptake process. If the site of action is intracellular, the high concentration of adenosine and NECA (above 10 μM) required to produce the second phase responses suggest that access to the intracellular compartment could be gained by simple diffusion. Indeed, Schrader *et al.* (1972) found that the Dip-sensitive component of uptake in human erythrocytes was saturated at 10 μM adenosine. At concentrations higher than this adenosine was able to gain access by simple diffusion.

The refractoriness of the adenosine-alone responses to blockade by 8-PT and DPCPX suggests that A_2-receptors are not involved. Burnstock *et al.* (1984) also found that adenosine's relaxation of the taenia caecum was not blocked by 8-PT. There are other reports that adenosine and some of its analogues are able to produce non A_2-purinoceptor-mediated responses in other smooth muscle preparations: Brackett & Daly (1991) noted xanthine P_1-purinoceptor antagonist-resistant relaxations to NECA in the guinea-pig isolated trachea; Martin (1992) and Collis & Brown (1983) noted relaxations to high concentrations of adenosine and analogues in guinea-pig aorta that were refractory to 8-PT: although none of these authors drew attention to the changes in the time courses of the responses. However, the latter group found that these responses were depressed by Dip and thus concluded that they were mediated by an intracellular site. In contrast, we found that the P_1-purinoceptor antagonist resistant responses were neither depressed nor potentiated by Dip and therefore require another mechanism of access to the putative intracellular site such as diffusion. Collis & Brown (1983) postulated that adenosine caused the relaxation in the guinea-pig aorta by inhibiting the enzyme, 5-adenosylhomocysteine hydrolase leading to the accumulation of substrates which in turn caused inhibition of cyclic nucleotide PDE.

The presence in the guinea-pig taenia caecum of the A_3-

receptor which has been reported to mediate responses to adenosine and analogues which are not blocked by 8-PT and DPCPX (Zhou *et al.*, 1992) cannot be ruled out. However, this receptor subtype is possibly not responsible for the second phase relaxant responses observed in this study because it is claimed to be coupled to the inhibition of adenylate cyclase. Inhibition of adenylate cyclase would be expected to produce contraction rather than relaxation of the taenia caecum.

In contrast to our results, Hourani *et al.* (1991) have reported simple competitive blockade of responses to adenosine (in the absence of Dip) by 8-(*p*-sulphophenyl) theophylline in the taenia caecum. However responses were obtained at lower concentrations of adenosine than were required in this study. The reason for this discrepancy is unclear, however, it is possible that there were differences in the activity of the tissue adenosine uptake systems between the two studies. Differences in experimental design might also contribute to the difference in results, for instance, in this study a higher contractile response level was elicited (80% max cf. 50–70% max) which might afford more functional antagonism of the A_2 receptor-mediated relaxant responses. In any event Hourani *et al.* (1991) did not achieve the same high organ bath concentrations of adenosine required to elicit marked second phase responses.

The affinity values estimated for 8-PT and DPCPX suggest that responses to low concentrations of NECA and adenosine (+Dip) are likely to be mediated by the A_2-purinoceptor as originally concluded by Burnstock *et al.* (1984). However, it is apparent that both 8-PT and DPCPX have complex pharmacological profiles in the guinea-pig taenia caecum preparation and are able to amplify NECA concentration-effect curves at the same concentrations as those used to produce blockade of the A_2-purinoceptor. The suggestion that the amplification elicited by the antagonists might be due to the inhibition of a contractile A_1-purinoceptor-mediated response was considered. Although some of the 9-methyl adenosine compounds did produce a significant increase in the upper asymptote of the NECA curves, the data indicate that there was no correlation between their A_1-purinoceptor affinity and propensity to cause amplification within the series of analogues (Table 2). If it can be assumed that this family of antagonists cause amplification by the same mechanism as the two xanthine compounds, then it is unlikely that the amplification observed is due to removal of an A_1-purinoceptor mediated contractile response in the taenia caecum.

If the amplification of the NECA concentration-effect curves were due to phosphodiesterase inhibition by the antagonists, an action previously recognised for some xanthine derivatives, then effects of NECA curves should be mirrored by effects upon the curves obtained with other agonist-receptor systems which are mediated by the production of cyclic AMP. However, in contrast to the phosphodiesterase inhibitor Ro 20-1724, both 8-PT and DPCPX at the highest concentrations tested did not have a significant effect on the upper asymptote of isoprenaline concentration-effect curves. Another possible mechanism was suggested by the work of Ramkumar & Stiles (1988) who reported that the xanthine derivative, 8-(-[[[[(2-amino-ethyl)-aminocarbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine (XAC) interacts with the inhibitory G protein (Gi), possibly through an allosteric site leading to elevation of adenylate cyclase activity. If this is a general property of xanthine derivatives then it may account for the amplification seen, although once again an effect of DPCPX on isoprenaline responses may have been expected.

In conclusion, it is evident that adenosine and its analogue NECA are able to elicit both A_2 and non-A_2-receptor mediated relaxant responses in the guinea-pig taenia caecum and, under conditions where the adenosine uptake process is functional, non-A_2-receptor mediated responses predominate. It is also clear that in this tissue both 8-PT and DPCPX exhibit complex profiles of activity. Both the response mechanism heterogeneity and the antagonist effects make interpretation of agonist/antagonist interactions difficult and potentially misleading.

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References

BERGSTRAND, H., KRISTOFFERSSON, J., LUNDQUIST, B. & SCHURMANN, A. (1977). Effects of antiallergic agents, compound 48/80 and some reference inhibitors on the activity of partially purified human lung tissue adenosine cyclic 3',5'-monophosphate and guanosine cyclic 3',5'-monophosphate phosphodiesterase. *Mol. Pharmacol.*, **13**, 38–43.

BLACK, J.W., LEFF, P. & SHANKLEY, N.P. (1985). Further analysis of anomalous pK_B values for histamine H_2-receptor antagonists in the isolated mouse stomach. *Br. J. Pharmacol.*, **86**, 581–587.

BLACK, J.W. & SHANKLEY, N.P. (1985). The isolated stomach preparation of the mouse: a physiological unit for pharmacological analysis. *Br. J. Pharmacol.*, **86**, 571–579.

BRACKETT, L.E. & DALY, J.W. (1991). Relaxant effects of adenosine analogues on guinea pig trachea *in vitro*: Xanthine sensitive and xanthine-insensitive mechanisms. *J. Pharmacol. Exp. Ther.*, **257**, 205–213.

BROWN, C.M. & BURNSTOCK, G. (1981). Evidence in support of the P_1/P_2 purinoceptor hypothesis in the guinea pig taenia coli. *Br. J. Pharmacol.*, **73**, 617–624.

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. New York: Raven Press.

BURNSTOCK, G., HILLS, J.M. & HOYLE, C.H. (1984). Evidence that the P_1 purinoceptor in the guinea pig taenia coli is an A_2 subtype. *Br. J. Pharmacol.*, **81**, 533–541.

COLLIS, M.G. (1983). Evidence for an A_1-adenosine receptor in the guinea-pig atrium. *Br. J. Pharmacol.*, **78**, 207–212.

COLLIS, M.G. & BROWN, C.M. (1983). Adenosine relaxes the aorta by interacting with an A_2 receptor and an intracellular site. *Eur. J. Pharmacol.*, **96**, 61–69.

GRIFFITH, S.G., MEGHJI, P., MOODY, C.J. & BURNSTOCK, G. (1981). 8-Phenyltheophylline: A potent P_1-purinoceptor antagonist. *Eur. J. Pharmacol.*, **75**, 61–64.

HOURANI, S.M.O., BAILEY, S.J., NICHOLLS, J. & KITCHEN, I. (1991). Direct effects of adenylyl 5'-(β,γ-methylene)diphosphonate, a stable ATP analogue, on relaxant P_1-purinoceptors in smooth muscle. *Br. J. Pharmacol.*, **104**, 685–690.

KENAKIN, T.P. (1982). The potentiation of cardiac responses to adenosine by benzodiazepines. *J. Pharmacol. Exp. Ther.*, **222**, 752–758.

MARTIN, P.L. (1992). Relative agonist potencies of C_2-substituted analogues of adenosine: evidence for adenosine A_{2B} receptors in the guinea pig aorta. *Eur. J. Pharmacol.*, **216**, 235–242.

MARTINSON, E.A., JOHNSON, R.A. & WELLS, J.N. (1987). Potent adenosine receptor antagonists that are selective for the A_1 receptor subtype. *Mol. Pharmacol.*, **31**, 247–252.

RAMKUMAR, V. & STILES, G.L. (1988). A novel site of action of a high affinity A_1 adenosine receptor antagonist. *Biochem. Biophys. Res. Commun.*, **153**, 939–944.

SATCHELL, D.G. & MAGUIRE, M.H. (1982). Evidence for separate receptors for ATP and adenosine in the guinea pig taenia coli. *Eur. J. Pharmacol.*, **81**, 669–672.

SCHRADER, J., BERNE, R.M. & RUBIO, R. (1972). Uptake and metabolism of adenosine by human erythrocyte ghosts. *Am. J. Physiol.*, **223**, 159–166.

SPEDDING, M. & WEETMAN, D.F. (1976). Identification of separate receptors for adenosine and adenosine 5-triphosphate in causing relaxations of the isolated taenia of the guinea pig caecum. *Br. J. Pharmacol.*, **57**, 305–310.

VAN CALKER, D., MULLER, 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.

WALLENSTEIN, S., ZUCKER, C.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

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

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