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Activation of multiple sites by adenosine analogues in the rat isolated aorta

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1 The presence of A_2 receptors mediating relaxation in the rat isolated aorta has been previously demonstrated. However, agonist dependency of the degree of rightward shift elicited by 8-sulphophenyltheophylline (8-SPT) led to the suggestion that the population of receptors in this tissue is not a homogeneous one. In this study we have re-examined the effects of 8-SPT in the absence and presence of the NO synthase inhibitor L-NAME (N^G-nitro-L-arginine methyl ester) and investigated antagonism of responses by the potent A_{2a} receptor ligands PD 115,199 (N-[2-dimethylamino) ethyl] - N-methyl-4-(2, 3, 6, 7-tetrahydro-2, 6-dioxo-1,3 dipropyl-1H-purin-8-yl)) benzene sulphonamidexanthine), ZM 241385 (4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol), and CGS 21680 (2-[p-(2-carboxyethyl)phenylamino]-5'-N-ethylcarboxamidoadenosine). We have also investigated the antagonist effects of BWA1433 (1,3-dipropyl-8-(4-acrylate)phenylxanthine) which has been shown to have affinity at rat A_3 receptors.

2 Adenosine, **R**-PIA (N^6-**R**-phenylisopropyl adenosine), CPA (N^6-cyclopentyladenosine) and NECA (5'-N-ethylcarboxamidoadenosine) all elicited relaxant responses in the phenylephrine pre-contracted rat isolated aorta with the following potency order ($p[A_{50}]$ values in parentheses): NECA (7.07 ± 0.11) > **R**-PIA (5.65 ± 0.10) > CPA (5.05 ± 0.12) > adenosine (4.44 ± 0.12).

3 8-SPT (10-100 μM) caused parallel rightward shifts of the E/[A] curves to NECA ($pK_B = 5.23 \pm 0.16$). A smaller rightward shift of E/[A] curves to CPA was observed ($pA_2 = 4.85 \pm 0.17$). However, no significant shifts of E/[A] curves to either adenosine or **R**-PIA were observed.

4 In the absence of endothelium E/[A] curves to NECA and CPA were right-shifted compared to controls. However, removal of the endothelium did not produce a substantial shift of adenosine E/[A] curves, and E/[A] curves to **R**-PIA were unaffected by removal of the endothelium.

5 In the presence of L-NAME (100 μM) E/[A] curves to NECA and CPA were right-shifted. However, no further shift of the CPA E/[A] curve was obtained when 8-SPT (50 μM) was administered concomitantly. The locations of curves to **R**-PIA and adenosine were unaffected by L-NAME (100 μM).

6 In the presence of PD 115,199 (0.1 μM) a parallel rightward shift of NECA E/[A] curves was observed ($pA_2 = 7.50 \pm 0.19$). PD 115,199 (0.1 and 1 μM) gave smaller rightward shifts of E/[A] curves to **R**-PIA and CPA, but E/[A] curves to adenosine were not significantly shifted in the presence of PD 115,199 (0.1 or 1 μM).

7 The presence of ZM 241385 (3 nM-0.3 μM) caused parallel rightward shifts of NECA E/[A] curves ($pK_B = 8.73 \pm 0.11$). No significant shifts of E/[A] curves to adenosine, CPA or **R**-PIA were observed in the presence of 0.1 μM ZM 241385.

8 CGS 21680 (1 μM) elicited a relaxant response equivalent to approximately 40% of the NECA maximum response. In the presence of this concentration of CGS 21680, E/[A] curves to NECA were right-shifted in excess of 2-log units, whereas E/[A] curves to **R**-PIA were not significantly shifted.

9 BWA1433 (100 μM) caused a small but significant right-shift of the E/[A] curve to **R**-PIA yielding a pA_2 estimate of 4.1. IB-MECA (N^6-(3-iodo-benzyl)adenosine-5^1-*N*-methyl uronamide) elicited relaxant responses which were resistant to blockade by 8-SPT ($p[A]_{50} = 5.26 \pm 0.13$).

10 The results suggest that whereas relaxations to NECA (10 nM-1 μM) are mediated via adenosine A_{2a} receptors, which are located at least in part on the endothelium, **R**-PIA and CPA may activate A_{2b} receptors on the endothelium and an additional, as yet undefined site, which is likely to be located on the smooth muscle and which is not susceptible to blockade by 8-SPT, PD 115,199 or ZM 241385. This site is unlikely to be an A_3 receptor since the very small shift obtained in the presence of BWA1433 (100 μM), and the low potency of IB-MECA is not consistent with the affinities of these compounds at the rat cloned A_3 receptor. It is suggested that adenosine activates both the A_{2a} and the undefined site, being most potent, but behaving as a partial agonist, at the A_{2a} receptor.

Keywords: Adenosine; rat aorta; xanthines; purinoceptors; nitric oxide; endothelium

Introduction

The existence of two distinct P_1 purinoceptor subtypes, namely A_1 and A_2 has long been established (Van Calker *et al.*, 1979; Londos *et al.*, 1980). Subdivision of the A_2 subtype into A_{2a} and A_{2b} was first suggested on the basis of high and low affinity for adenosine respectively by Daly and co-workers (1983). This sub-classification has since been confirmed by extensive structure-activity relationships (Jacobson, 1990) and a number of compounds which are selective for A_{2a} over A_{2b} receptors are now available, for instance CGS 21680 (2-[p-(2-carbo xyethyl) phenylamino] - 5' - N - ethylcarboxamidoadenosine), which activates A_{2a} receptors but is virtually inactive at A_{2b} sites (Jacobson, 1990). There are also antagonists such as PD 115,199 (N-[2-(dimethylamino)ethyl]-N-methyl-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)benzenesulphonamide), a xanthine based antagonist shown to have a K_i of 15.5 nM at A_{2a} receptors and no appreciable affinity for the low

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affinity A_{2b} site in fibroblasts (Bruns *et al.*, 1987). However, an A_{2a}/A_{2b} selectivity ratio of only 5 has been recently found for this compound in a study comparing antagonism of 5'-N-ethylcarboxamidoadenosine (NECA)-elicited stimulation of adenylate cyclase in PC12 (A_{2a}) and NIH 3T3 (A_{2b}) membranes (Brackett & Daly, 1995). PD 115,199 does, however, have a high A_1 receptor affinity ($K_i = 14$ nM, Bruns *et al.*, 1987). The relatively new non-xanthine antagonist, ZM 241385 (4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol) has been found to have a selectivity for A_{2a} over A_{2b} and A_1 (32 and 420 fold, respectively) with a pA_2 at A_{2a} receptors in the guinea-pig Langendorff heart of 8.57 (Poucher *et al.*, 1995).

All three of the P_1 purinoceptors have been cloned (Tucker & Linden, 1993; Linden *et al.*, 1993) as well as a receptor termed the A_3 receptor (Zhou *et al.*, 1992). There are a number of adenosine analogues with relatively high affinities for rat A_3 receptors one of which is IB-MECA (N^6-(3-iodo-benzyl)-adenosine -5'-*N*-methyluronamide, $K_i = 1.1$ nM, Jacobson *et al.*, 1995). Unlike A_1 and A_2 receptors, the rat A_3 receptor is resistant to blockade by all but a few methylxanthines, which include BWA1433 (1,3-dipropyl-8-(4-acrylate)phenylxanthine, $K_i = 15$ μM (Jacobson *et al.*, 1995).

Adenosine and some of its analogues have been previously shown to relax the rat isolated aorta and the presence of an A_2 receptor has been postulated (Rose'Meyer & Hope, 1990; Moritoki *et al.*, 1990; Lewis *et al.*, 1994). Lewis and co-workers (1994) proposed the presence of an A_{2a} receptor on the basis of the relatively high potency of CGS 21680. However, this group found that the degree of blockade of agonist responses by the P_1 receptor antagonist 8-SPT (8-sulphophenyltheophylline) was agonist-dependent. This observation is not consistent with activation of a homogenous population of receptors by the adenosine analogues employed. In order to elucidate further the nature of the receptor populations in this tissue we investigated the interaction between adenosine and three of its analogues, namely NECA, CPA (N^6-cyclopentyladenosine) and **R**-PIA (N^6-R-phenylisopropyladenosine), and the potent A_{2a} antagonists PD 115,199 and ZM 241385. In the rat aorta CGS 21680 has been shown to behave as a partial agonist, only eliciting approximately 66% of the maximal response to NECA with a $p[A]_{30}$ of 6.3 (Lewis *et al.*, 1994). Therefore, in this study we were also able to use CGS 21680 as an antagonist versus NECA and **R**-PIA.

Methods

Male Wistar albino rats (University of Surrey strain, 200–250 g) were killed by cervical dislocation, the thoracic aorta excised and 6 mm rings set up in 3.5 ml organ baths containing Krebs-Henseleit solution (mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, glucose 11, $MgSO_4$, $7H_2O$ 0.45, KH_3PO_4 1.2, $CaCl_2.2H_2O$ 2.5) maintained at 37°C and continuously gassed with 95% O_2/5% CO_2. Preparations were allowed to equilibrate for 60 min under an initial resting tension of 2 g. Responses were subsequently recorded isometrically by Grass FT03 force displacement transducers and displayed on a Grass polygraph (model 79).

E/[A] curves to phenylephrine were performed in the rat aorta (data not shown) and a concentration corresponding to approximately 80% of maximum response (0.1 μM) was chosen to pre-contract tissues before construction of relaxant E/[A] curves. The response to this concentration of phenylephrine was demonstrated to be stable for the period of time taken to construct relaxant E/[A] curves (up to 60 min, data not shown).

After the tissues had been contracted with phenylephrine, the presence of endothelium was confirmed by the addition of acetylcholine (1 μM). Tissues giving less than 25% relaxation of the phenylephrine contraction were discarded. Preparations were then washed with Krebs solution, incubated for a period of 60 min in the absence or presence of a concentration of antagonist, then contracted again with phenylephrine and cumulative relaxant E/[A] curves constructed. In experiments where the effect of N^G-nitro-L-arginine methyl ester (L-NAME) were examined, after the addition of acetylcholine, tissues were washed and then incubated for 60 min with L-NAME. The tissues were then contracted again with phenylephrine and acetycholine readministered to confirm the activity of the L-NAME. Following this, tissues were washed and then incubated for a further 60 min in the absence or presence of antagonist and/or L-NAME. In experiments where the effects of removal of endothelium were studied the endothelium was destroyed in some tissues by a metal rod. Absence of endothelium was confirmed by administration of acetylcholine (1 μM). Only one E/[A] curve was constructed in each preparation. Results are expressed as percentage decrease in the contraction to phenylephrine and, where possible, $p[A]_{50}$ (midpoint location), upper asymptote (α) and midpoint slope parameter estimates (n_H) were obtained by logistic curve fitting (see Prentice *et al.*, 1995). 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. Data are presented as average ± s.e.mean of at least 3 replicates. When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel rightward shifts of agonist E/[A] 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 and Shankley, 1985). When the criteria were not fully testable, a pA_2 value was estimated from the concentration-ratio produced by a single concentration of antagonist.

For display purposes the average logistic fitting parameters were used to generate a curve upon which the average data points were superimposed.

Where the isolated rat colon muscularis mucosae was used this preparation was set up according to the methods of Bailey & Hourani (1992).

CGS 21680, CPA and 8-SPT were obtained from Research Biochemicals Inc., Natick, MA, U.S.A. PD 115,199 was kindly provided by Dr M Collis, Pfizer Central Research, Sandwich, Kent, U.K., ZM 241385 by Dr S. Poucher, ZENECA Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire, U.K., BWA1433 by The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, U.K. and IB-MECA by Dr K. Jacobson, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A. All drugs were made at a stock concentration of 10 mM. CGS 21680 was made up in 7% ethanol, 8-SPT, phenylephrine, adenosine, NECA and CPA in distilled water, **R**-PIA in 0.06 M HCl, IB-MECA in 100% DMSO, ZM 241385 in 20% DMSO, BW A1433 in 25% ethanol plus 30 mM NaOH and PD 115,199 in 50% DMSO. Further dilutions were made in water.

Results

Adenosine, **R**-PIA, CPA and NECA all elicited relaxations in the phenylephrine pre-contracted isolated rat aorta with the following potency order ($p[A]_{50}$ values in parentheses): NECA (7.07 ± 0.11) > **R**-PIA (5.65 ± 0.10) > CPA (5.05 ± 0.12) > adenosine (4.44 ± 0.12). E/[A] curves to adenosine were shallow compared to **R**-PIA and CPA curves (midpoint slope parameters of 0.83 ± 0.06, 1.88 ± 0.18 and 1.57 ± 0.18 respectively, Figure 1).

8-SPT (10–100 μM) caused parallel rightward shifts of E/[A] curves to NECA yielding a Schild plot with a slope not significantly different from unity (0.88 ± 0.29) and a pK_B value of 5.23 ± 0.16 (Figure 2). A small, but significant, rightward shift of E/[A] curves to CPA was observed in the presence of 8-SPT (50 μM) and a pA_2 value of 4.85 ± 0.17 estimated (Figure 3c). However, no significant shifts of E/[A] curves to either

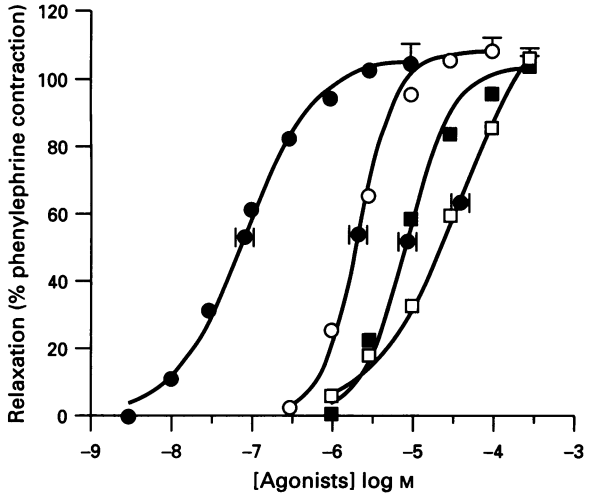

Figure 1 Concentration-response curves to NECA (●), R-PIA (○), CPA (■) and adenosine (□). Data points are average responses (% relaxation of phenylephrine-induced contraction) and the lines through the data were generated by use of the average logistic fitting parameters. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=3-4$).

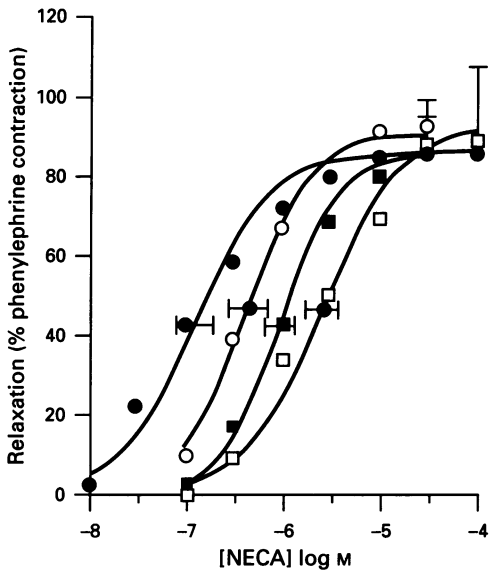

Figure 2 Concentration-response curves to NECA in the absence (●) and presence of 8-SPT 10 μM (○), 50 μM (■) and 100 μM (□). Data points are average responses (% relaxation of phenylephrine-induced contraction) and the lines through the data were generated by use of the average logistic fitting parameters. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=3-4$).

adenosine or **R**-PIA were observed, although the midpoint slope of the E/[A] curve to adenosine in the presence of 8-SPT was significantly increased with respect to the control value ($n_H = 1.44 \pm 0.13$ as compared with 0.86 ± 0.10, $P<0.05$, Figures 3b and d). In the presence of L-NAME (100 μM) E/[A] curves to NECA were right-shifted 10 fold. However, the right-shift produced by a single concentration of 8-SPT (50 μM) in the presence of this concentration of L-NAME was not significantly different from the shift produced in the absence of L-NAME, yielding pA_2 values of 5.26 ± 0.20 and 5.52 ± 0.13, respectively (Figure 3a). E/[A] curves to CPA were likewise right-shifted approximately 10 fold in the presence of L-NAME. However, no further shift was obtained when 8-SPT (50 μM) was administered concomitantly (Figure 3c). Curves to **R**-PIA were unaffected by L-NAME and, in the presence of L-NAME, 8-SPT caused a small right-shift yielding a pA_2 estimate of 4.6 (Figure 3b). There was no appreciable shift of adenosine E/[A] curves in the presence of L-NAME and, as in the absence of L-NAME, concomitant administration of 8-SPT caused no right-shift (Figure 3d). It was not possible to fit all of the adenosine E/[A] curves constructed in the presence of L-NAME since some had no inflexion point. However, it is noteworthy that these curves were apparently steeper than the control E/[A] curves.

Experiments were carried out using endothelium denuded preparations to confirm that administration of L-NAME mimicked the effects of endothelium removal. As in the presence of L-NAME, E/[A] curves to both NECA and CPA were right-shifted in the absence of endothelium by approximately 3 and 10 fold, respectively (Figure 4a and c). There was no substantial right shift of E/[A] curves to adenosine and E/[A] curves to **R**-PIA were not significantly altered in the absence of endothelium (Figure 4b and d).

In the presence of 10 nM PD 115,199 no significant shift of NECA E/[A] curves was observed (control $p[A]_{50} = 7.01 \pm 0.11$ compared with $p[A]_{50}$ in the presence 0.1 μM PD $115,199 = 6.89 \pm 0.23$), but in the presence of PD 115,199 (0.1 μM) a parallel rightward shift of the NECA E/[A] curve was observed (concentration-ratio $= 5.4 \pm 1.6$) which yielded a pA_2 value of 7.50 ± 0.19. However, when the concentration of the antagonist was increased to 1 μM the shift observed was not significantly different from that observed at 0.1 μM (concentration-ratio $= 7.5 \pm 3.3$, Figure 5a). PD 115,199 (0.1 and 1 μM) gave small but significant rightward shifts of E/[A] curves to **R**-PIA and again the shift observed at 1 μM was not significantly different to that at 0.1 μM (concentration-ratios $= 2.5 \pm 0.4$ and 2.6 ± 0.4, respectively, Figure 5b). Similarly, PD 115,199, at both concentrations, elicited small rightward shifts of E/[A] curves to CPA (concentrations-ratios $= 1.7 \pm 0.4$ and 3.0 ± 0.6 at 0.1 and 1 μM, respectively, Figure 5c), which just reached significance in the presence of PD 115,199 (1 μM). E/[A] curves to adenosine were not significantly shifted in the presence of PD 115,199 (0.1 or 1 μM) although, as with 8-SPT (50 μM) the slope of the E/[A] curves to adenosine in the presence of both concentrations of PD 115,199 was increased with respect to the control value ($n_H = 1.76 \pm 0.15$ and 1.91 ± 0.60 at 0.1 and 1 μM respectively as compared with 0.83 ± 0.06, Figure 5d). The possibility that limited solubility of PD 115,199 resulted in the lack of further shift of NECA E/[A] curves when the concentration of this antagonist was increased from 0.1 μM to 1 μM was considered. Although PD 115,199 has been shown to be selective for A_{2a} over A_{2b} receptors, its affinity at A_1 receptors is similar to its A_{2a} receptor affinity (Bruns *et al.*, 1987). We therefore chose to investigate the antagonism by PD 115,199 (0.1 μM and 1 μM) of A_1 receptor-mediated contractile responses to CPA in the rat colon. The concentration-ratios obtained at these two concentrations of the agonist were 2.8 ± 0.7 and 16.0 ± 5.2, respectively, yielding pA_2 values of 7.2 ± 0.2 and 7.1 ± 0.2, respectively.

In the light of the anomolous results obtained with PD 115,199 we decided to investigate the effects of ZM 241385, an antagonist which has been found to be selective for A_{2a} over both A_{2b} and A_1 receptors (Poucher *et al.*, 1995). ZM 241385 (3 nM–0.3 μM) caused parallel rightward shifts of NECA E/[A] curves yielding a Schild plot slope not significantly different from unity (1.16 ± 0.09) and a pK_B value of 8.73 ± 0.11 (Figure 6a). No significant shifts of E/[A] curves to **R**-PIA, CPA and adenosine were observed in the presence of ZM 241385 (0.1 μM, Figures 6b–d). As with 8-SPT (50 μM) and PD 115,199 (0.1 μM), ZM 241385 produced an increase in midpoint slope parameter of E/[A] curves to adenosine (0.91 ± 0.18 as compared to 1.37 ± 0.20) although this did not reach significance at the 5% level.

It was not possible to obtain reproducible E/[A] curves to CGS 21680 since in some tissues little or no response was observed, whereas in others near maximal responses were ob-

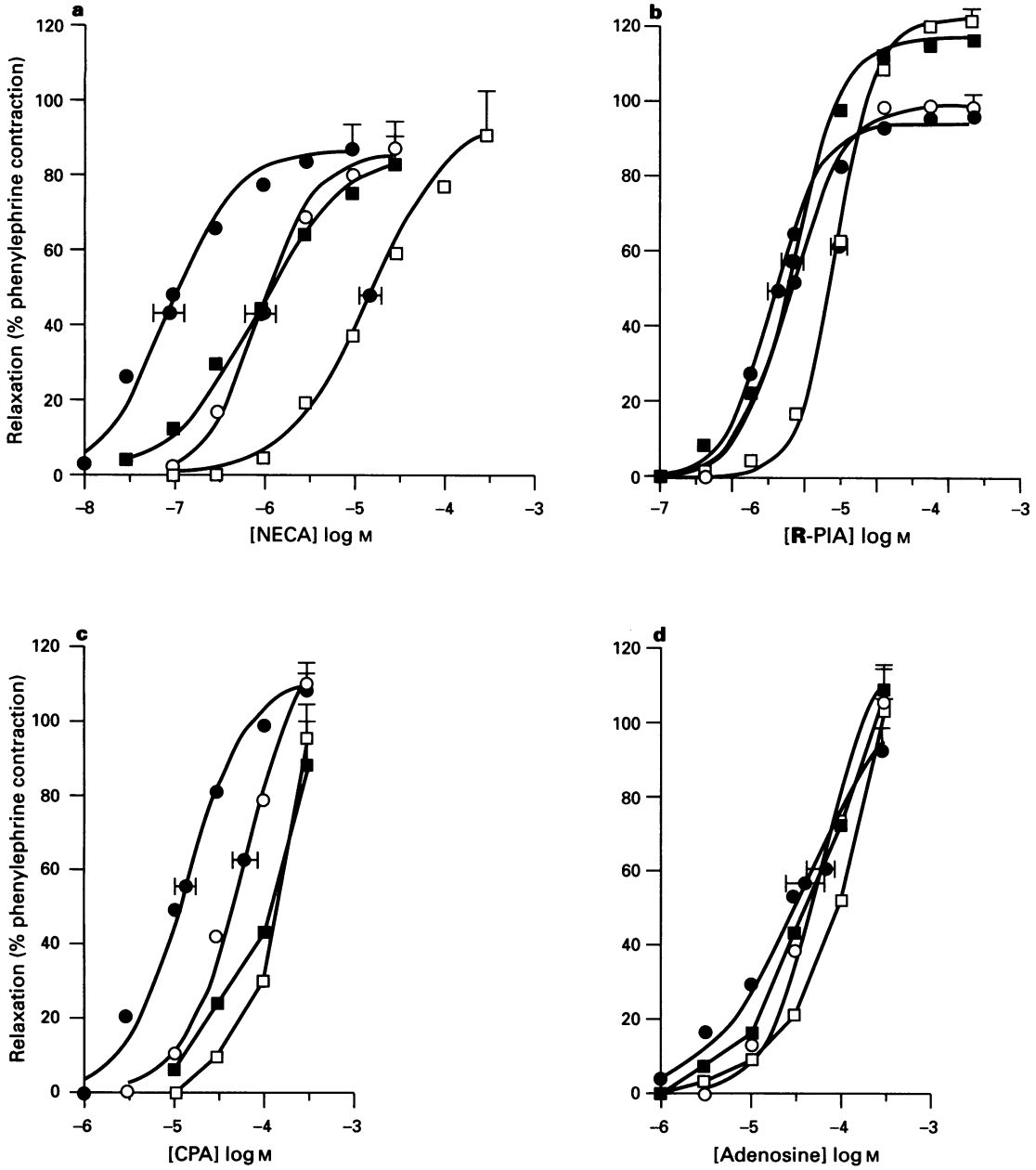

Figure 3 Concentration-response curves to (a) NECA, (b) R-PIA, (c) CPA and (d) adenosine in the rat isolated aorta in the absence (●) and presence of 50 μM 8-SPT (○) and in the presence of 100 μM L-NAME (■) and 50 μM 8-SPT and 100 μM L-NAME (□). Data points are average responses (% relaxation of phenylephrine-induced contraction) and where possible the lines through the data were generated by use of the average logistic fitting parameters. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=3-6$).

tained. On average, CGS 21680 (1 μM) elicited a relaxant response equivalent to approximately 40% of the NECA maximum response. In the presence of this concentration of CGS 21680, E/[A] curves to NECA were right-shifted in excess of 2-log units, whereas E/[A] curves to R-PIA were not significantly shifted ($p[A]_{50}$ values for R-PIA in the absence and presence of CGS 21680 being 5.45 ± 0.15 and 5.25 ± 0.15, respectively, Figure 7).

The possibility that responses to agonists such as R-PIA, which were essentially resistant to blockade by the antagonists used in these studies, were mediated by A_3 receptors was considered. We studied the effects of the antagonist BWA1433, but the use of this compound proved difficult since at the concentration of BWA1433 under study (0.1 mM) E/[A] curves to phenylephrine were both right shifted and reduced in maximum (data not shown). It was therefore necessary to use different concentrations of phenylephrine in the presence of vehicle or BWA1433 (0.3 μM or 1 μM phenylephrine, respectively) in order to achieve approximately equivalent levels of tissue contraction prior to construction of agonist E/[A] curves. Under these conditions BWA1433 (0.1 mM) caused a small but significant right shift of E/[A] curves to R-PIA ($p[A]_{50}$ values for R-PIA in the presence of vehicle or BWA1433 being 4.87 ± 0.12 and 4.50 ± 0.08, respectively, Figure 8) and a pA_2 of 4.1 was estimated from this shift. We also studied the effects of the agonist IB-MECA, but although it elicited relaxant responses which were resistant to blockade by 8-SPT, its potency was relatively low ($p[A]_{50}=5.26\pm0.13$, Figure 9).

Discussion

In this study, cumulative relaxant E/[A] curves to adenosine and some of its analogues were obtained in the rat isolated aorta. Despite the different methodology employed in our study, the locations of E/[A] curves to adenosine and its analogues obtained here are consistent with the data obtained by

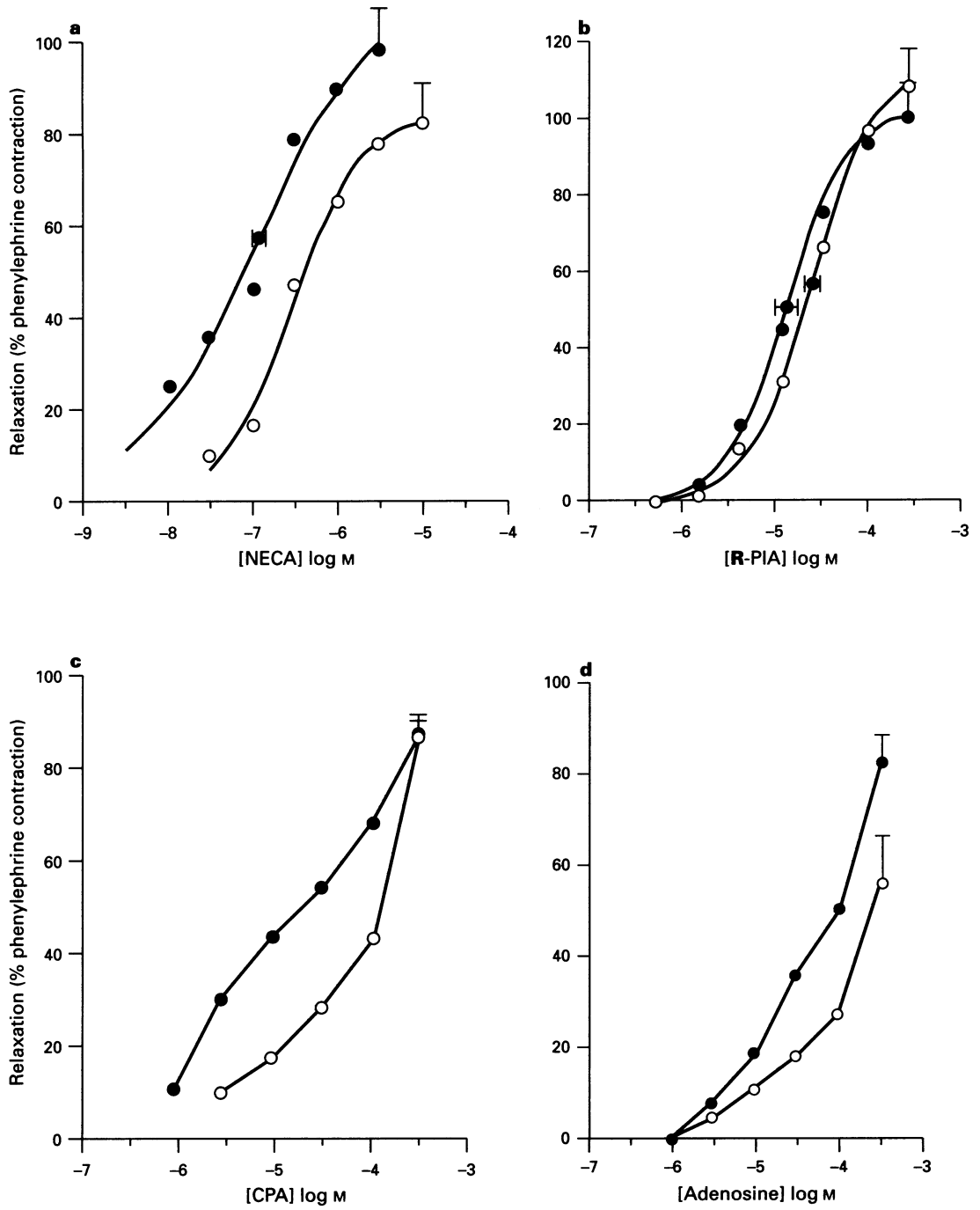

Figure 4 Concentration-response curves to (a) NECA, (b) **R**-PIA, (c) CPA and (d) adenosine in the rat isolated aorta in the presence (●) and absence of endothelium (○). Data points are average responses (% relaxation of phenylephrine-induced contraction) and, where possible, the lines through the data were generated by use of the average logistic fitting parameters. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=3-4$).

Lewis and co-workers ($pEC_{30}=6.7$ for NECA, 4.7 for adenosine and 4.5 for CPA). In the present study it was not possible to obtain reproducible E/[A] curves to CGS 21680 due to a variation in response level between tissues, but a concentration of 1 μM CGS 21680 was selected which according to Lewis *et al.* (1994) should elicit a maximal response, and this concentration was then used to antagonize responses to NECA and **R**-PIA. The average response obtained to 1 μM CGS 21680 in the present study (approximately 40% of NECA maximum) was considerably lower than the 66% obtained by Lewis *et al.* (1994), therefore, it may be that the difficulties encountered in constructing an E/[A] curve to CGS 21680 can be explained by the apparently lower efficacy of this agonist. This variation in response level may be related to variations in receptor density or receptor-effector coupling in different tissues. The variation may also be due to differences in integrity of the endothelium between tissues, although there was no correlation between percentage relaxation to acetylcholine and to CGS 21680 as might be predicted if this were the case (data not shown).

The potency order obtained in the present study (NECA > **R**-PIA > CPA = adenosine), together with the high potency of CGS 21680 observed by Lewis *et al.* (1994) is consistent with activation of A_{2a} receptors by these agonists. However, the agonist dependency of blockade by the P_1 purinoceptor antagonist, 8-SPT noted here and in the previous study suggests that the population of receptors in the rat aorta is not a homogenous one. 8-SPT (10–100 μM) right-shifted NECA E/[A] curves in a manner consistent with simple competition and the pK_B value estimated for 8-SPT (5.23 ± 0.16) is

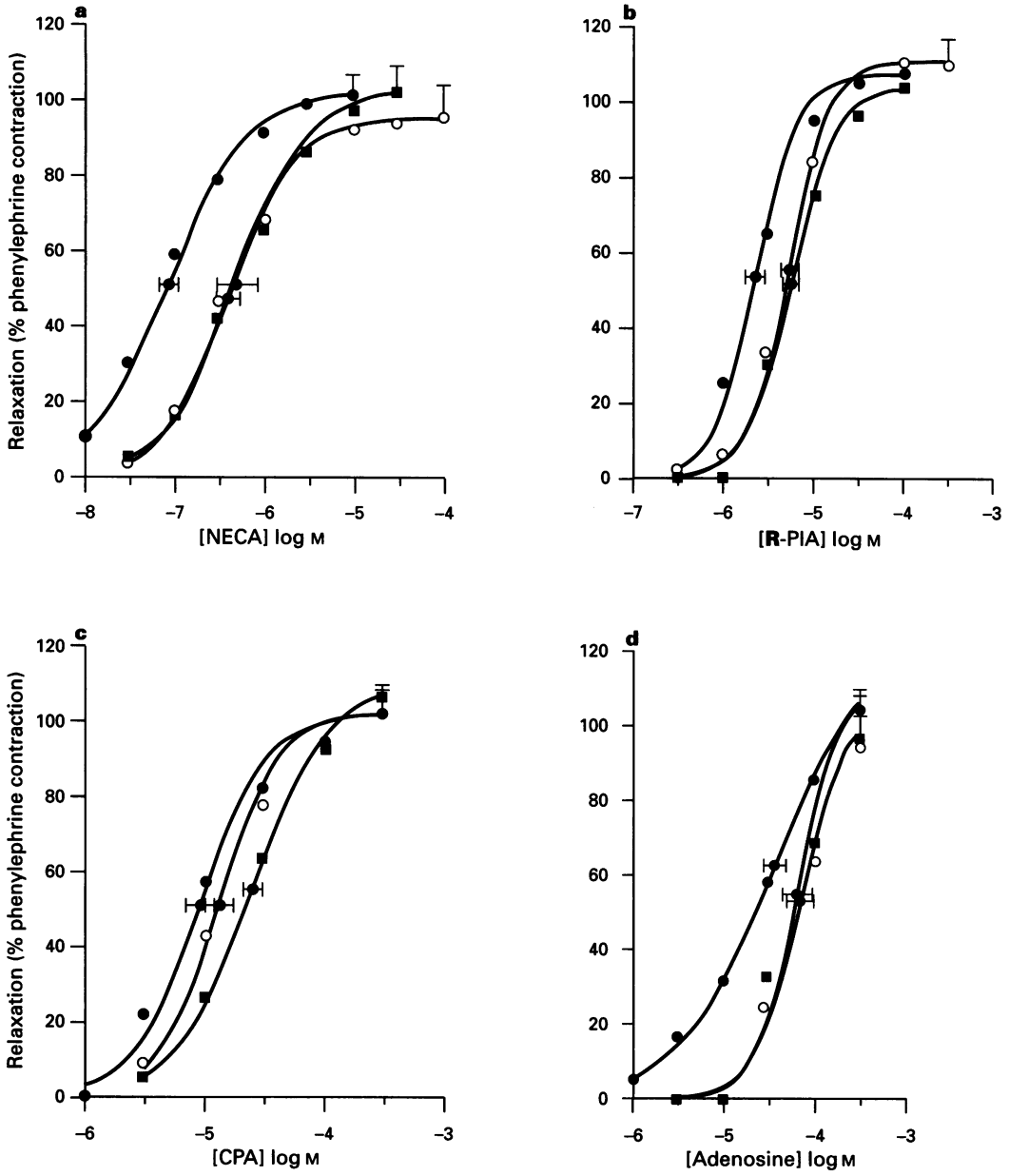

Figure 5 Concentration-response curves to (a) NECA, (b) R-PIA, (c) CPA and (d) adenosine in the rat isolated aorta in the absence (●) and presence of PD 115,199 0.1 μM (○) and 1 μM (■). Data points are average responses (% relaxation of phenylephrine-induced contraction) and the lines through the data were generated by use of the average logistic fitting parameters. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=3-6$).

consistent with its affinity at rat striatal A_{2a} receptors (4.8, Bruns *et al.*, 1986). Similarly, the A_{2a} selective antagonist PD 115,199 (0.1 μM) right-shifted E/[A] curves to NECA giving a pA_2 of 7.50 ± 0.19 which is consistent with its K_i at the high affinity A_2 receptor in rat striatal membranes (15.5 nM, Bruns *et al.*, 1987). The reason for the lack of further shift of NECA E/[A] curves in the presence of a 10 fold higher concentration of PD 115,199, is unclear, but is unlikely to be due to limited solubility at this concentration since, in the rat colon, contractile responses to NECA (mediated via A_1 receptors) were antagonized by both 0.1 and 1 μM and the right shifts obtained at these concentrations yielded pA_2 estimated which were not significantly different. In the light of the anomolous results obtained with PD 115,199 in the rat aorta, we chose to investigate the effects of a relatively new A_{2a} selective antagonist ZM 241385. This compound over a wide concentration range (3 nM – 0.3 μM) antagonized responses to NECA in the rat aorta in a manner consistent with simple competition, yielding a pK_B value of 8.73 ± 0.11 which is close to the pA_2 value obtained for this compound at A_{2a} receptors in the guinea-pig Langendorff heart (8.57, Poucher *et al.*, 1995). The demonstration that the A_{2a} selective agonist CGS 21680 behaves as a partial agonist in this tissue (Lewis *et al.*, 1994) allowed the use of this compound as an antagonist of A_{2a} receptors. The large rightward shift (dose-ratio > 100) of NECA E/[A] curves afforded by CGS 21680 is consistent with its binding affinity at the A_{2a} receptor in rat striatum (15 nM, Jacobson *et al.*, 1995). These data confirm the conclusions of Lewis *et al.* (1994) that there are A_{2a} receptors, which are activated by NECA, mediating relaxations in the rat isolated aorta.

Responses to R-PIA were resistant to blockade by CGS 21680 and responses to both CPA and R-PIA were resistant to blockade by ZM 241385 suggesting that these agonists activate an additional site distinct from the A_{2a} receptor. There were, however, small rightward shifts of E/[A] curves to these two agonists in the presence of 8-SPT and PD 115,199. Although 8-SPT is generally regarded as non-selective, Bruns *et al.* (1986) obtained a 12 fold selectivity for A_{2b} over A_{2a} receptors for this

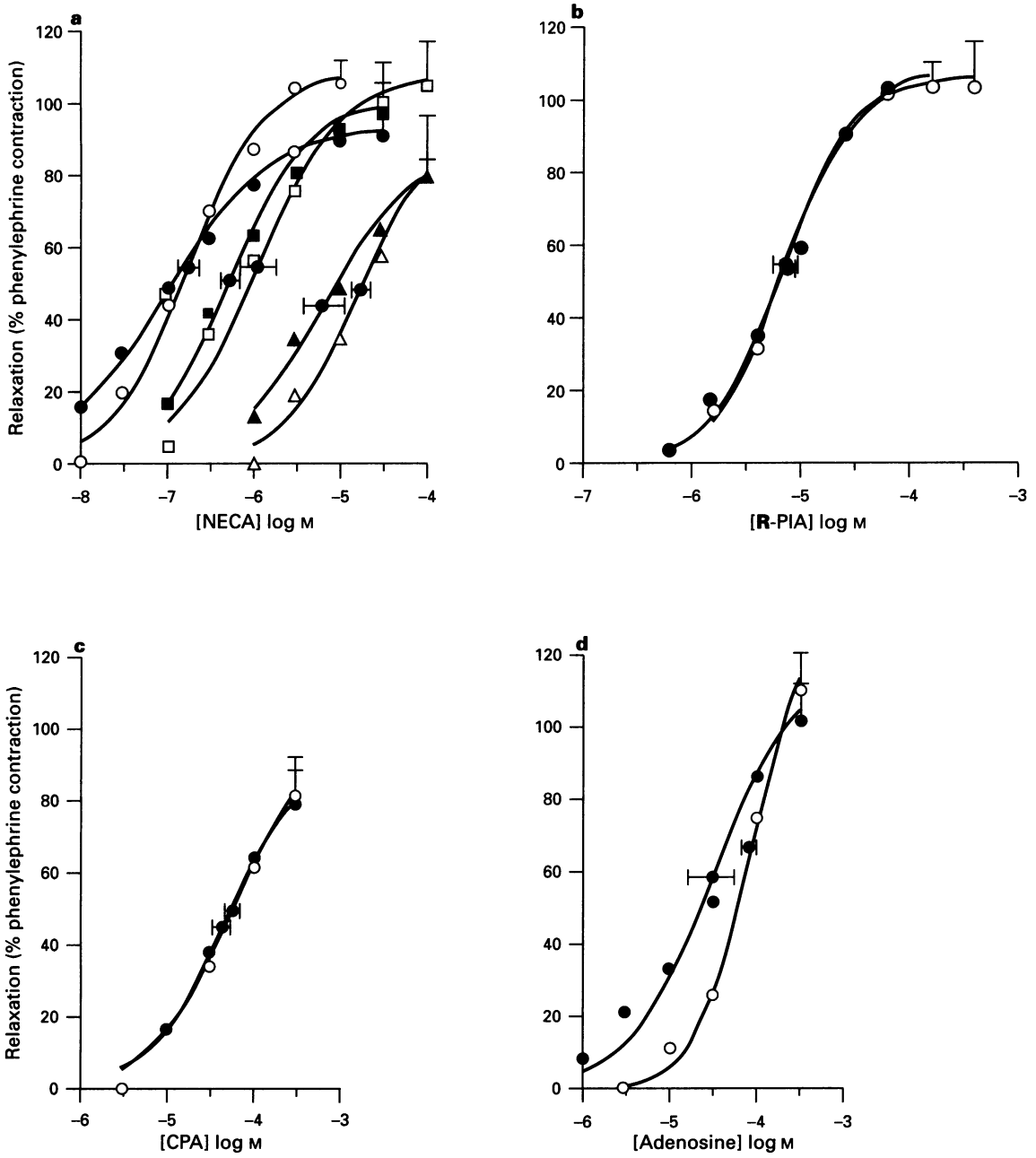

Figure 6 Concentration-response curves in the rat isolated aorta to (a) NECA in the absence (●) and presence of ZM 241385 3 nM (○), 10 nM (■), 30 nM (□), 0.1 μM (▲) and 0.3 μM (△) and (b) **R**-PIA, (c) CPA and (d) adenosine in the absence (●) and presence of ZM 241385 0.1 μM (○). Data points are average responses (% relaxation of phenylephrine-induced contraction) and the lines through the data were generated by use of the average logistic fitting parameters. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=3-6$).

compound and, according to Brackett & Daly (1995), PD 115,199 is only 5 fold selective for A_{2a} over A_{2b} receptors. At the concentrations used in the present study, both antagonists are, therefore, likely to afford A_{2b} receptor blockade. It may be that the small shifts of E/[A] curves to CPA and **R**-PIA are indicative of activation of A_{2b} receptors by these agonists. Since NECA has an affinity at A_{2a} receptors some 250 fold higher than at A_{2b} receptors (Bruns *et al.*, 1986), antagonism of A_{2a} receptor-mediated NECA responses would probably not be compromised by the presence of a small A_{2b} receptor population. If the responses to CPA and **R**-PIA were entirely A_{2b} receptor mediated then the shifts of E/[A] curves to these agonists afforded by 8-SPT would be at least as large as the NECA E/[A] curve shifts obtained in the presence of this antagonist. This was clearly not the case. The results may instead by explained by activation of A_{2a} receptors alone by NECA and of A_{2b} receptors and an additional site by **R**-PIA and CPA.

E/[A] curves to adenosine were not significantly right-shifted by 8-SPT, PD 115,199 or ZM 241385, although there was an increase in the midpoint slope parameter in the presence of these antagonists. These results may be accounted for by activation of A_{2a} receptors at the lower concentrations of adenosine with this compound behaving as a partial agonist, and of the postulated undefined site at the higher concentrations of adenosine used. This combination of receptor activation would result in shallow E/[A] curves which may or may not be overtly biphasic. In this study E/[A] curves to adenosine, though not obviously biphasic, were indeed shallow with respect to **R**-PIA and CPA E/[A] curves. The undefined site is apparently resistant to blockade by 8-SPT, PD 115,199 and ZM 241385, so that in the presence of any of these antagonists the A_{2a} receptor mediated component of the adenosine E/[A] curve will be antagonized leaving E/[A] curves mediated via the undefined site alone and the midpoint slope parameter of E/[A] will be in-

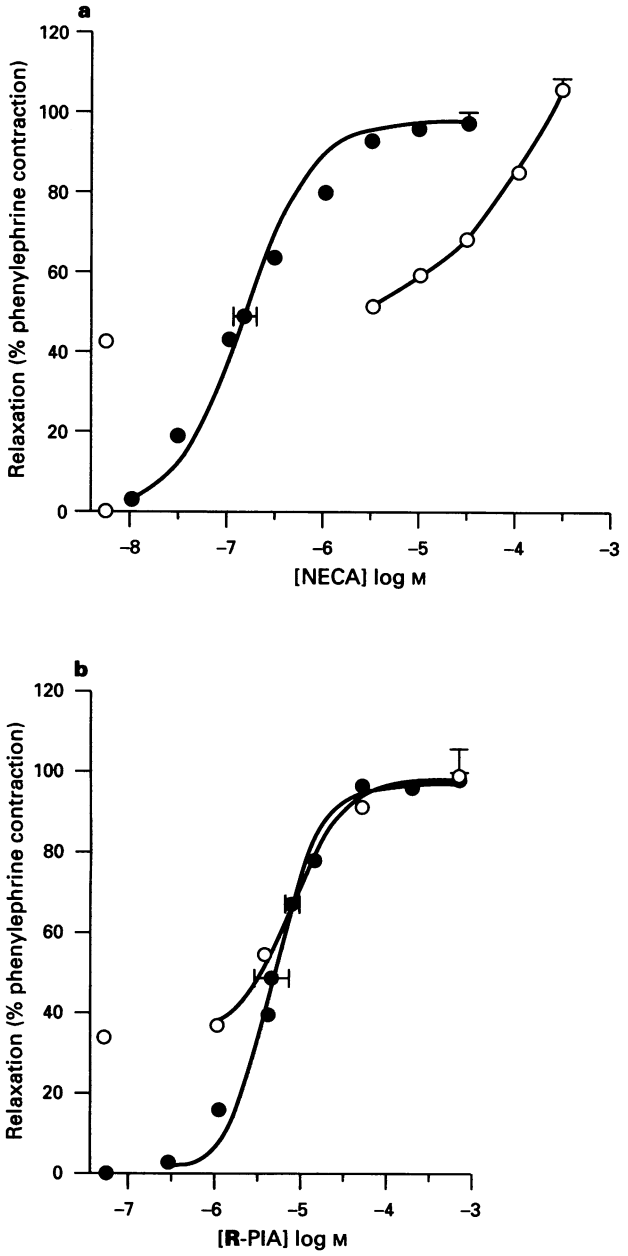

Figure 7 Concentration-response curves to (a) NECA and (b) R-PIA in the rat isolated aorta in the absence (●) and presence (○) of 1 μM CGS 21680. Data points are average responses (% relaxation of phenylephrine-induced contraction) and, where possible, the lines through the data were generated by use of the average logistic fitting parameters except in the case of NECA in the presence of CGS 21680. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=5-7$).

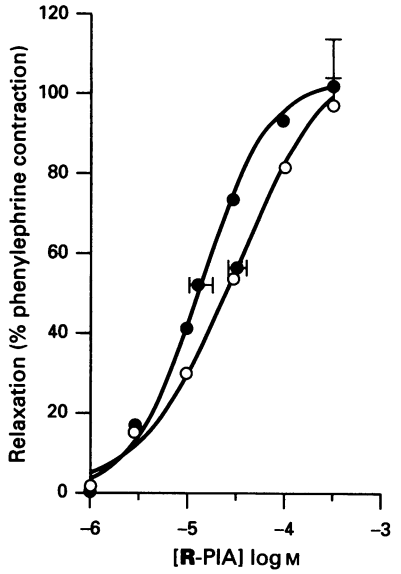

Figure 8 Concentration-response curves to R-PIA in the absence (●) and presence of BWA1433 100 μM (○). Data points are average responses (% relaxation of phenylephrine contraction) and the lines through the data were generated by use of the average logistic fitting parameters. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=4-7$).

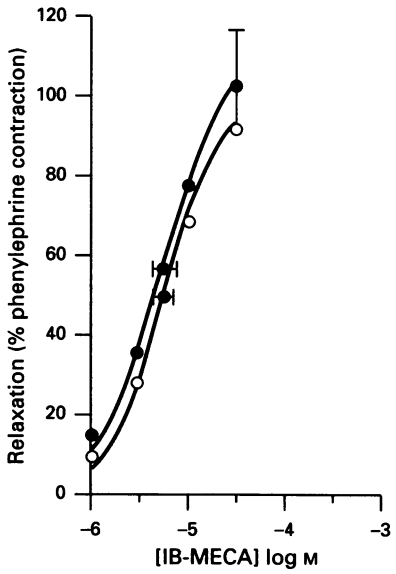

Figure 9 Concentration-response curves to IB-MECA in the absence (●) and presence of 8-SPT 50 μM (○). Data points are average responses (% relaxation of phenylephrine-induced contraction) and the lines through the data were generated by use of the average logistic fitting parameters. Average $p[A]_{50}$ and α values are marked, together with their associated s.e.mean ($n=4$).

creased accordingly. The suggestion that the natural ligand, adenosine, may behave as a partial agonist at the A_{2a} receptor is not without precedent, since Cristalli *et al.* (1994) found that adenosine behaved as a partial agonist with respect to NECA at A_{2a} receptors on human platelets.

The right-shift of NECA E/[A] curves obtained both in the absence of endothelium and in the presence of L-NAME clearly suggests that the A_{2a} receptors are located, at least in part, on the endothelium. The fact that the pA_2 estimates for 8-SPT obtained in the absence or presence of L-NAME were not significantly different suggests that the residual NECA response in the presence of L-NAME is also mediated by A_{2a} receptors but it is not possible to say whether they are located on the endothelium or the smooth muscle. The absence of any right-shift of E/[A] curves to *R*-PIA either in the absence of endothelium or in the presence of L-NAME and the lack of any appreciable further shift of either CPA or R-PIA E/[A] curves when 8-SPT was co-incubated with L-NAME substantiates the suggestion that these compounds activate an undefined site, and suggests that it is not located on the endothelium. The apparent steepening of the E/[A] curves to adenosine in the presence of L-NAME adds credence to the suggestion that the A_{2a} receptors are located on the endothelium whereas the undefined site is not.

Assuming adenosine, R-PIA and CPA do activate the second site in the presence of A_{2a}/A_{2b} receptor blockade (50 μM 8-SPT, or 1 μM CGS 21680), and assuming that the $p[A]_{50}$ values estimated for these agonists under these conditions reflect the potency of these agonists at the second site, then an agonist potency order at the postulated site can be surmised (ap-

proximate $p[A]_{50}$ in parentheses): **R**-PIA (5.6) > CPA (4.3) > adenosine (4.2) > NECA (< 4). The relatively high potency of the N^6-substituted compounds coupled with the resistance to blockade by 8-SPT, PD 115,199 and ZM 241385 could imply that A_3 receptors are involved, since N^6 substitution of adenosine analogues tends to improve affinity at A_3 receptors and rat A_3 receptors are resistant to blockade to these by all but a few xanthine based antagonists (Jacobson *et al.*, 1995). However, the small shift of **R**-PIA E/[A] curves obtained in the presence of BWA1433 (0.1 mM) was not consistent with the affinity of this antagonist obtained at rat A_3 receptors. Also IB-MECA elicited relaxant responses in the rat aorta which were not susceptible to blockade by 8-SPT (50 μM), but the potency of this agonist was low ($p[A]_{50} = 5.26$). Although the $p[A]_{50}$ value for an agonist may be greater than its pK_A value (ie a high efficacy agonist), in theory the $p[A]_{50}$ value may be approximately equal to (ie a low efficacy agonist) but not substantially less than the pK_A value. The observed potency of IB-MECA is, therefore, not consistent with its affinity of 1.1 nM obtained at rat A_3 receptors (Jacobson *et al.*, 1995). In fact, whereas in the present study CPA and IB-MECA were approximately equipotent, at rat A_3 receptors IB-MECA has an affinity approximately 100 fold greater than that of CPA (Jacobson *et al.*, 1995). Although we were unable to obtain an absolute potency value for NECA at the undefined site, it is clear from the shift of the NECA E/[A] curve in the presence of CGS 21680 (1 μM) that the $p[A]_{50}$ value for NECA at this site must be in excess of 4. NECA has been found to have an affinity at A_3 receptors of 0.11 μM (Jacobson *et al.*, 1995) which, again, is hard to reconcile with such a low potency at the undefined site. It is therefore unlikely that the additional site in the rat aorta mediating relaxations is the A_3 receptor.

It has been found that adenosine and some of its analogues are able to produce xanthine-resistant relaxations in other smooth muscle preparations: Brackett and Daly (1991) noted xanthine-resistant relaxations to NECA in the guinea-pig isolated trachea; Collis & Brown (1983) and Martin (1992) demonstrated relaxations of guinea-pig aorta to high concentrations of adenosine and analogues that were refractory to 8-phenyltheophylline (8-PT); Prentice *et al.* (1995) noted that adenosine and NECA induced relaxations of guinea-pig taenia caecum which were resistant to blockade by both 8-PT and DPCPX. It may be that all of these relaxant responses are mediated by a site similar to the, as yet, undefined site in the rat isolated aorta.

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References

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.

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.

BRACKETT, L.E. & DALY, J.W. (1995). Functional characterisation of the A_{2b}adenosine receptor in NIH 3T3 fibroblasts. *Biochem. Pharmacol.*, **47**, 801–814.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A. & HAYS, S.J. (1987). PD 115,199: An antagonist ligand for adenosine A_2 receptors. *Naunyn Schmied. Arch. Pharmacol.*, **335**, 64–69.

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.

CRISTALLI G, VITTORI, S., THOMPSON, R.D., PADGETT, W.L., SHI, D., DALY, J.W. & OLSSON, R.A. (1994). Inhibition of platelet aggregation by adenosine receptor agonists. *Naunyn Schmied. Arch. Pharmacol.*, **349**, 644–650.

DALY, J.W., BUTTS-LAMB, P. & PADGETT, W. (1983). Subclasses of adenosine receptors in the central nervous system: Interaction with caffeine and related methylxanthines. *Cellular Molecular Neurobiology*, **3**, 69–80.

JACOBSON, K.A. (1990). Adenosine (P_1) and ATP (P_2) receptors. In *Comprehensive Medicinal Chemistry. Membranes and Receptors.* ed. Emmett, J.C. Oxford: Pergamon Press.

JACOBSON, K.A., KIM, H.O., SIDDIQI, S.M., OLAH, M.E., STILES, G.L. & VON LUBITZ, D.K.J.E. (1995). A_3-adenosine receptors: design of selective ligands and therapeutic prospects. *Drugs of the Future*, **20**, 689–699.

LEWIS, C.D., HOURANI, S.M.O., LONG, C.J. & COLLIS, M.G. (1994). Characterisation of adenosine receptors in the rat isolated aorta. *Gen. Pharmacol.*, **25**, 1381–1387.

LINDEN, J., TUCKER, T.L., ROBEVA, A.S., GRABER, S.G. & MUNISHI, T. (1993). Properties of recombinant adenosine receptors. *Drug. Devel. Res.*, **28**, 232–236.

LONDOS, C., COOPER, D.M.F. & WOLFF, J. (1980). Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2551–2554.

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.

MORITOKI, H., MATSUGI, T., TAKASE, H., UEEDA, H. & TANIOKA, A. (1990). Evidence for the involvement of cyclic GMP in adenosine-induced, age-dependent vasodilation. *Br. J. Pharmacol.*, **100**, 569–575.

POUCHER, S.M., KEDDIE, J.R., SINGH, P., STOGGALL, S.M., CAULKETT, P.W.R., JONES, G. & COLLIS, M.G. (1995). The *in vitro* pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist. *Br. J. Pharmacol.*, **115**, 1096–1102.

PRENTICE, D.J., SHANKLEY, N.P. & BLACK, J.W. (1995). Pharmacological analysis of the interaction between purinoceptor agonists and antagonists in the guinea-pig taenia caecum. *Br. J. Pharmacol.*, **115**, 549–556.

ROSE'MEYER, R.B. & HOPE, W. (1990). Evidence that A_2 purinoceptors are involved in endothelium-dependent relaxation of the rat thoracic aorta. *Br. J. Pharmacol.*, **100**, 576–580.

TUCKER, A.L. & LINDEN, J. (1993). Cloned receptors and cardiovascular responses to adenosine. *Cardiovasc. Res.*, **27**, 62–67.

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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