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Activation of two sites by adenosine receptor agonists to cause relaxation in rat isolated mesenteric artery

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1 In this study we have characterized the receptor(s) in the rat mesenteric artery mediating relaxant responses to adenosine and a number of adenosine analogues, N^6 -**R**-phenylisopropyladenosine (**R**-PIA), N^6-cyclopentyladenosine (CPA), N^6-(3-iodo-benzyl)-adenosine-5′-N-methyluronamide (IB-MECA) and 5′-N-ethylcarboxamidoadenosine (NECA), by use of the non-selective antagonist 8-sulphophenyltheophylline (8-SPT) and the A_{2A} selective ligands 2-[p-(2-carbonylethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680) and 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]-triazin-5-ylamino]ethyl) phenol (ZM 241385). We have also studied the effects of endothelial removal and uptake inhibition by nitrobenzylthioinosine (NBTI) and the effects of the A_3 receptor antagonist 1,3-dipropyl-8-(4-acrylate)phenylxanthine (BWA1433).

2 Adenosine, NECA, CPA and **R**-PIA all elicited relaxant responses in tissues precontracted with phenylephrine (1 μM) with the following potency order: NECA > **R**-PIA > adenosine = CPA. However, E/[A] curves to NECA were biphasic. CGS 21680 was inactive at concentrations up to 30 μM and IB-MECA elicited relaxant responses which were resistant to blockade by 8-SPT and BWA1433 (100 μM).

3 Removal of the endothelium produced a small but significant decrease in the asymptote of the high potency phase of E/[A] curves to NECA with no change in $p[A]_{50}$. E/[A] curves to adenosine were not altered by removal of the endothelium. However, there were small rightward shifts of E/[A] curves to CPA and **R**-PIA in the absence of endothelium.

4 Inhibition of uptake by NBTI (1 μM) had no effect on E/[A] curves to NECA, CPA or **R**-PIA, but E/[A] curves to adenosine were significantly left-shifted in the presence of NBTI.

5 8-SPT (10 – 100 μM) caused significant rightward shifts of the high potency phase of the E/[A] curves to NECA ($pA_2 = 5.63 \pm 0.26$). The second phase of the concentration-response curve to NECA appeared to be resistant to blockade by 8-SPT, as were E/[A] curves for adenosine, CPA or **R**-PIA. However, in the presence of NBTI (1 μM), 8-SPT (100 μM) gave significant rightward shifts of E/[A] curves to adenosine.

6 ZM 241385 (0.1 – 1 μM) produced significant rightward shifts of the high potency phase of NECA E/[A] curves ($pA_2 = 7.65 \pm 0.25$ in the presence and 7.20 ± 0.12 in the absence of endothelium), while curves to **R**-PIA were not significantly shifted by 1 μM ZM 241385. In the presence of NBTI E/[A] curves to adenosine were significantly rightward shifted by ZM 241385 (0.1 μM, $pA_2 = 7.50 \pm 0.16$).

7 In conclusion, the results suggest activation of A_{2B} receptors located primarily on the smooth muscle by low concentrations of NECA and by adenosine under conditions of uptake blockade, and of another, as yet undefined site which may be intracellular, by higher concentrations of NECA, by CPA, **R**-PIA and adenosine under conditions where uptake is operational.

Keywords: Adenosine; rat mesenteric artery; xanthines; purinoceptors; nitric oxide; endothelium

Introduction

The rat perfused mesenteric vascular bed has been studied fairly extensively but there is some disagreement as to the subclass of A_2 receptor mediating relaxant responses in this preparation, since it has been suggested by Rubino *et al*. (1995) to be of the A_{2B} subtype and by Hiley *et al*. (1995) to be of the A_{2A} subtype. Although adenosine has been shown to cause relaxations in rat isolated mesenteric arterial rings (Vuorinen *et al*., 1992) the receptor type involved was not investigated. We have previously demonstrated the presence of a xanthine-resistant site mediating relaxations to adenosine and some of its analogues in the rat isolated aorta (Prentice & Hourani, 1996a). Our reasons for investigating the rat isolated mesenteric artery were therefore two fold: firstly, to characterize the A_2 subtype mediating relaxations and, secondly, to look for an additional xanthine-resistant site which may be analogous to the site in the aorta.

A number of compounds with A_{2A} over A_{2B} selectivity are available, for example 2-[p-(2-carbonylethyl)-phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680), which is an agonist at A_{2A} receptors but is virtually inactive at A_{2B} sites (Jacobson, 1990). The new non-xanthine antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino] ethyl) phenol (ZM 241385) has 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 9.02 (Poucher *et al*., 1995). The advent of such compounds has allowed subclassification of A_2 receptors in functional studies. We therefore used firstly the non-selective adenosine receptor antagonist 8-sulphophenyltheophylline (8-SPT) and then the A_{2A} selective compounds CGS 21680 and ZM 241385 in order to characterize the A_2 receptors in the rat isolated mesenteric artery.

Recently a third receptor termed the A_3 receptor has been cloned (Zhou *et al*., 1992) which is activated by adenosine and some analogues such as N^6-(3-iodo-benzyl)-adenosine-5′-N-methyluronamide (IB-MECA, $K_i = 1.1$ 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, one of which is 1,3-dipropyl-8-(4-acrylate)phenylxanthine (BWA1433, $K_i = 15$ μM, Jacobson *et al*., 1995). Because the underfined site in the aorta shares some characteristics in common with the A_3 receptor we investigated the activity of these A_3 receptor ligands in the present study.

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Methods

Male Wistar albino rats (Bantin and Kingman, Hull) weighing approximately 200–250 g, were killed by cervical dislocation. The abdominal cavity was opened up and the mesenteric artery excised. Tissues were placed directly into Krebs-Henseleit solution (mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, D-glucose 11, $MgSO_4.7H_2O$ 0.45, KH_2PO_4 1.2, $CaCl_2.2H_2O$ 2.5). Excess fat and connective tissue were trimmed from the arteries which were then cut into rings approximately 3 mm long. The rings were carefully mounted between two fine (0.125 mm diameter) tungsten wires and suspended in 3.5 ml organ baths containing Krebs-Henseleit solution maintained at 37°C and continuously gassed with 95% O_2 5% CO_2. Preparations were allowed to equilibrate for approximately 30 min under an initial resting tension of 1 g. Tissue viability was tested with 1 μM phenylephrine, a concentration which should elicit approximately 85% maximum contraction. All tissues were tested for the presence of functional endothelium by use of 1 μM acetylcholine to oppose the vasoconstriction induced by 1 μM phenylephrine. Tissues in which acetylcholine induced less than a 25% decrease in contraction were rejected. Tissues were washed several times with Krebs solution, incubated for a period of 60 min in the presence or absence of a concentration of antagonist or uptake inhibitor, then contracted again with phenylephrine and cumulative relaxant concentration-response (E/[A]) curves constructed. In experiments where the effects of removal of endothelium were studied, the endothelium was destroyed in some tissues by use of a metal rod. Absence of the endothelium was confirmed by administration of acetylcholine (1 μM). Only one E/[A] curve was constructed in each preparation and responses were recorded isometrically with Grass FT03 force displacement transducers and displayed on a Grass polygraph (model 79). Relaxant responses were expressed as a % decrease in the contraction to phenylephrine. In some tissues it was necessary to precontract with the thromboxane A_2 mimetic U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$) and in this case relaxant responses were expressed as % decrease in contraction to U46619. Where possible midpoint location ($[A]_{50}$), upper asymptote (α) and midpoint slope parameter estimates (n_H) were obtained by logistic curve fitting (see Prentice *et al*., 1995) and for display purposes the average logistic fitting parameters were used to generate a line upon which the average data points were superimposed. To allow fitting of NECA E/[A] curves the data were analysed, the 100 and 300 μM responses being omitted. Where it was not possible to fit curves, the concentration giving 40% relaxation of phenylephrine or U46619 contraction (EC_{40}, a level corresponding to approximately half the maximal response of NECA E/[A] curves) was estimated by regression of the linear portion of the curve for each individual tissue. Where curves did not reach a 40% decrease in phenylephrine contraction EC_{20} values were estimated. The effect of drug treatment on the curve fitting parameters or EC_{40} values was assessed by one way analysis of variance or Student's *t* test where appropriate, and *P* values of less than 0.05 were considered to be statistically significant. Data are presented as mean ± s.e.mean of at least three replicates. pA_2 values were estimated from the agonist concentration-ratio produced by a single concentration of antagonist.

Drugs

CGS 21680, N^6-cyclopentyladenosine (CPA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 8-SPT were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). ZM 241 385 was kindly provided 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 other compounds were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). All drugs were made up at stock concentrations of 10 mM except for U46619 which was supplied in methyl acetate solution at 30 mM. CGS 21680 was made up in 7% ethanol, 8-SPT, phenylephrine, acetylcholine, adenosine, 5′-N-ethylcarboxamidoadenosine (NECA), and CPA in distilled water, N^6-**R**-phenylisopropyladenosine (**R**-PIA) in 0.06 M HCl, ZM 241385 in 20% dimethyl sulphoxide (DMSO), IB-MECA and nitrobenzylthioinosine (NBTI) in 100% DMSO, BWA1433 in 25% ethanol plus 30 mM NaOH and DPCPX in 6 mM NaOH containing 6% DMSO. Further dilutions were made up in water except in the case of NBTI which was diluted 1 : 10 in 50% DMSO and then diluted 1 : 10 in distilled water.

Results

Adenosine, **R**-PIA, CPA and NECA all elicited relaxations in the phenylephrine pre-contracted rat isolated mesenteric artery with the following potency order: NECA ($p[A]_{50}$ = 6.16 ± 0.11) > **R**-PIA ($p[A]_{50}$ = 5.31 ± 0.13) > adenosine (pEC_{40} = 4.58 ± 0.08) = CPA (pEC_{40} = 4.54 ± 0.12). However, E/[A] curves to NECA were overtly biphasic (Figure 1).

DPCPX at an A_1 receptor selective concentration (10 nM) did not significantly affect E/[A] curves to **R**-PIA or NECA (for **R**-PIA $p[A]_{50}$ = 4.96 ± 0.12 in the absence and 5.00 ± 0.10 in the presence of DPCPX and for NECA $p[A]_{50}$ = 6.44 ± 0.17 in the absence and 6.49 ± 0.11 in the presence of DPCPX, data not shown).

A small leftward shift of E/[A] curves to phenylephrine was observed in the absence of endothelium ($p[A]_{50}$ = 7.11 ± 0.10 and 7.51 ± 0.04 in the presence and absence of endothelium respectively, $P < 0.05$) such that 1 μM phenylephrine just elicited 100% maximum response in the absence of endothelium as opposed to approximately 85% in the presence of endothelium (data not shown). However there was no significant difference between the maximum response in mg force in tissues with and without endothelium (725 ± 70 and 824 ± 57 mg, respectively). Removal of the endothelium produced a small but significant decrease in the asymptote (α, % phenylephrine contraction) of the high potency phase of E/[A] curves to NECA (Figure 2a, α = 75.3 ± 0.8 in the presence and 52.5 ± 6.0 in the absence of endothelium, $P < 0.05$). The low potency phase of E/[A] curves to NECA was apparently unaltered by removal of the endothelium. E/[A] curves to adenosine were not affected by removal of the endothelium (pEC_{40} = 4.25 ± 0.23 and 4.20 ± 0.07 in the presence and absence of endothelium respectively, Figure 2b). E/[A] curves to CPA were signifi-

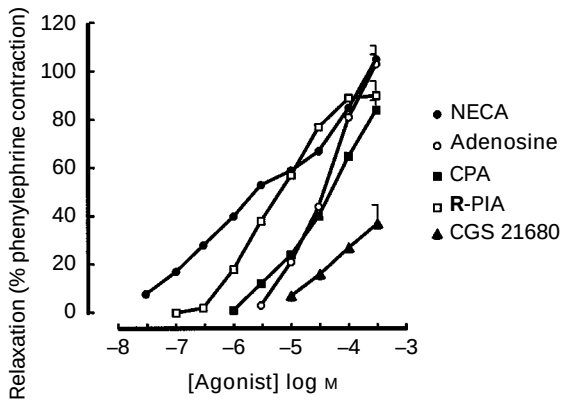

Figure 1 Concentration-response curves to NECA, adenosine, CPA, **R**-PIA and CGS 21680. Data points are average responses (% relaxation of phenylephrine-induced contraction). The s.e.mean for the maximum relaxation obtained is marked for each concentration-response curve, n = 5–7. For abbreviations see text.

cantly shifted to the right, approximately 8 fold, in the absence of endothelium ($pEC_{40} = 5.28 \pm 0.13$ and 4.38 ± 0.22 in the presence and absence of endothelium respectively, $P < 0.05$, Figure 2c). There was a small but significant rightward shift of E/[A] curves to **R**-PIA in the absence of endothelium (Figure 2d, $p[A]_{50} = 5.45 \pm 0.21$ and 4.78 ± 0.09 in the presence and absence of endothelium, respectively, $P < 0.05$).

Inhibition of adenosine uptake by NBTI had no effect on E/[A] curves to CPA, NECA or **R**-PIA either in the presence or absence of endothelium (Figures 2a,c and d). However E/[A] curves to adenosine were significantly shifted to the left in the presence of NBTI both in tissues with ($pEC_{40} = 4.25 \pm 0.23$ in the absence and $p[A]_{50} = 5.32 \pm 0.09$ in the presence of NBTI, $P < 0.05$) and without endothelium ($pEC_{40} = 4.20 \pm 0.01$ in the absence and $p[A]_{50} = 5.56 \pm 0.10$ in the presence of NBTI, $P < 0.05$). Blockade of the adenosine uptake process rendered the E/[A] curves to adenosine hyperbolic (Figure 2b).

8-SPT ($10 - 100\,\mu$M) caused significant rightward shifts of the high potency phase of the E/[A] curves to NECA (Figure 3a). The concentration-ratio obtained in the presence of $10\,\mu$M 8-SPT yielded a pA_2 value of 5.63 ± 0.26. The second phase of the response curve to NECA appeared to be resistant to blockade by 8-SPT. In the presence of 100 μM 8-SPT no significant shifts of E/[A] curves were observed for either

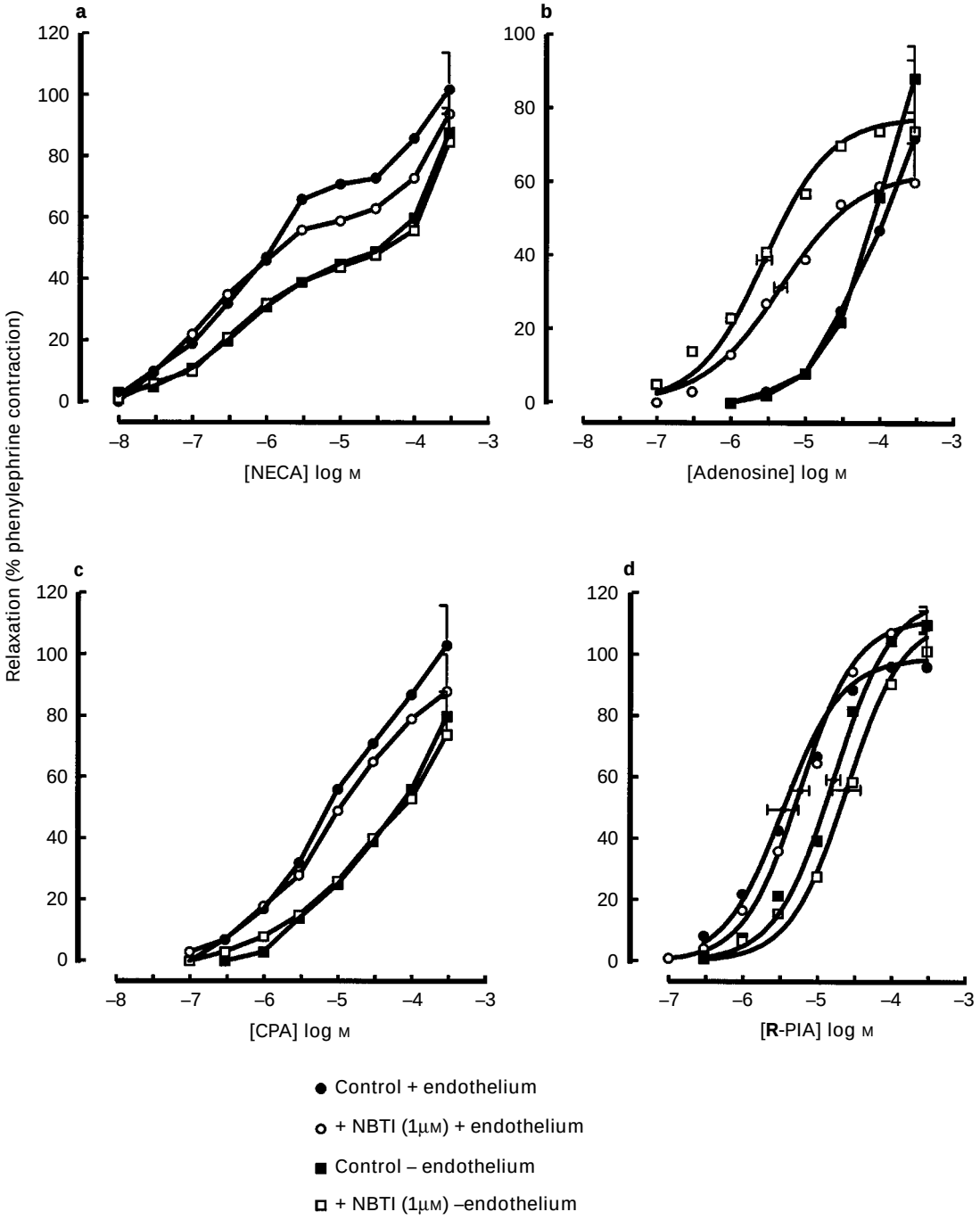

Figure 2 Concentration-response curves to (a) NECA, (b) adenosine, (c) CPA and (d) **R**-PIA in the presence and absence of endothelium and in the absence and presence of NBTI (1 μM). Data points are average responses (% relaxation of phenylephrine-induced contraction) and, where possible, the lines through the data were generated by use of the average logistic fitting parameters. Either average $p[A]_{50}$ and α values are marked together with their associated s.e.mean, or the s.e.mean for the maximum relaxation obtained is marked; $n = 3 - 7$. For abbreviations see text.

adenosine (Figure 3b, control $pEC_{40} = 4.48 \pm 0.13$ compared with 3.95 ± 0.15 in the presence of 100 μM 8-SPT), CPA (Figure 3c, control $pEC_{40} = 4.54 \pm 0.12$ compared with 4.29 ± 0.09 in the presence of 100 μM 8-SPT) or **R**-PIA (Figure 3d, $p[A]_{50} = 5.31 \pm 0.13$ compared with 4.98 ± 0.10 in the presence of 100 μM 8-SPT). However, in the presence of NBTI (1 μM), 8-SPT (100 μM) gave rightward shifts of E/[A] curves to adenosine in tissues with ($p[A]_{50} = 5.37 \pm 0.06$ in the absence and $pEC_{20} = 4.55 \pm 0.08$ in the presence of 8-SPT, Figure 3e) and without endothelium ($p[A]_{50} = 5.52 \pm 0.07$ in the absence and $pEC_{20} = 3.93 \pm 0.26$ in the presence of 8-SPT, Figure 3f). Approximate dose-ratios estimated at the 20% relaxation of phenylephrine contraction level yielded pA_2 estimates for 8-SPT against adenosine of 5.2 and 6.0 in the presence and absence of endothelium, respectively.

In order to characterize the A_2 receptor subtype mediating those relaxant responses that were susceptible to blockade by 8-SPT the A_{2A} selective compounds CGS 21680 and ZM 241385 were used. Relaxant responses to CGS 21680 were only observed at concentrations above 10 μM (Figure 1). However, these responses were not susceptible to blockade by 8-SPT (50 μM, data not shown).

ZM 241385 (0.1 – 1 μM) produced significant rightward shifts of the high potency phase of NECA E/[A] curves in the

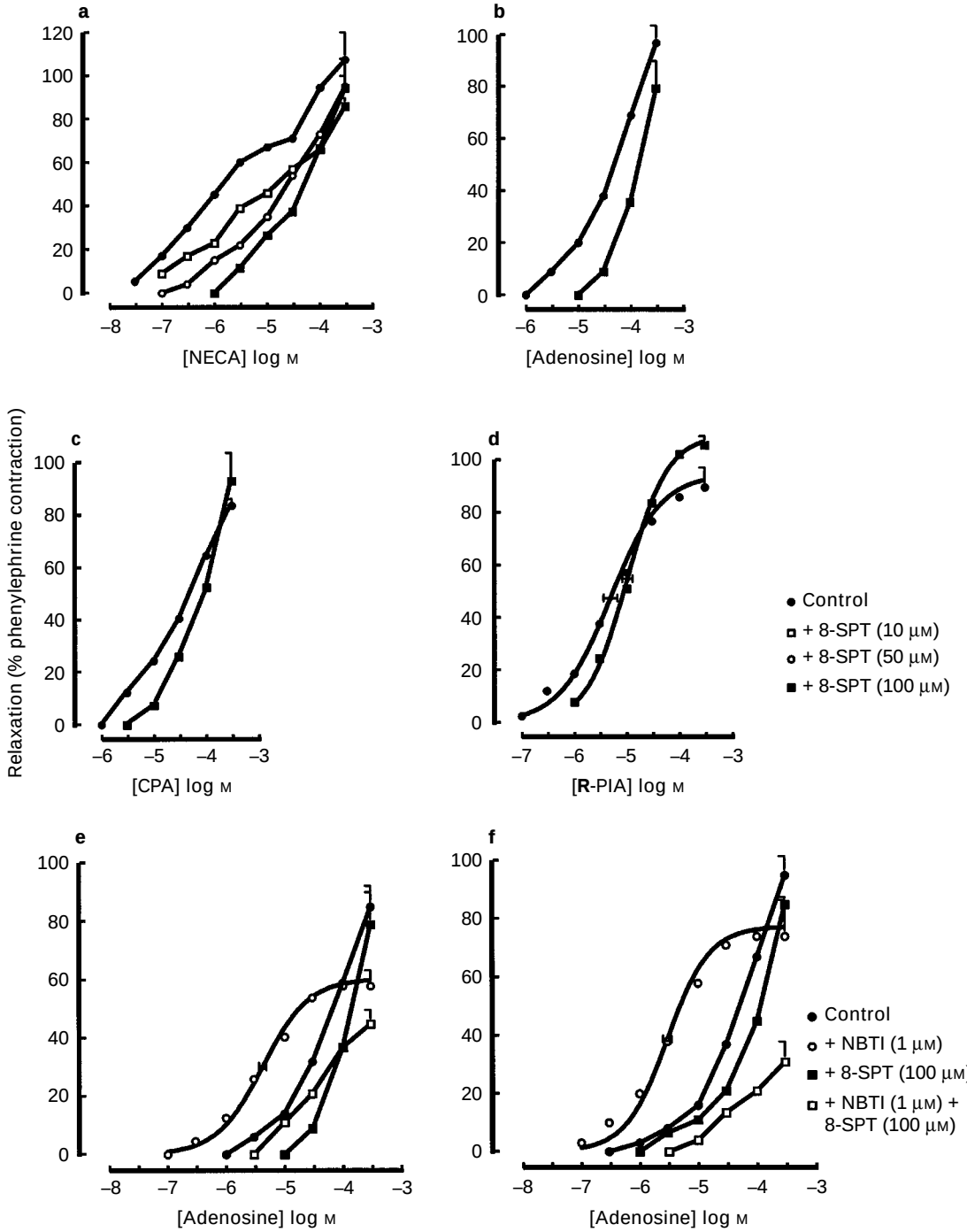

Figure 3 Concentration-response curves to (a) NECA, (b) adenosine, (c) CPA and (d) **R**-PIA in the absence and presence of 8-SPT (10 μM, 50 μM and 100 μM) and concentration-response curves to adenosine in the presence (e) and absence (f) of endothelium, in the absence and presence of NBTI (1 μM) and in the absence and presence of 8-SPT (100 μM). 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. Either average $p[A]_{50}$ and α values are marked together with their associated s.e.mean, or the s.e.mean for the maximum relaxation obtained is marked; $n = 3-7$. For abbreviations see text.

presence of endothelium with the concentration-ratio obtained in the presence of 1 μM ZM 241385 yielding a pA_2 value of 7.65 ± 0.25 (Figure 4a). In the absence of endothelium this concentration of ZM 241385 yielded a pA_2 value of 7.20 ± 0.12 (data not shown), which was not significantly different from the value obtained in the presence of endothelium. Curves to **R**-PIA were not significantly shifted in the presence of 1 μM ZM 241385 either in the presence (Figure 4b, $p[A]_{50} = 5.11 \pm 0.02$ and 4.86 ± 0.16 in the absence and presence of ZM 241385, respectively) or absence of endothelium (data not shown, $p[A]_{50} = 5.02 \pm 0.18$ and 4.89 ± 0.12 in the absence and presence of ZM 241385, respectively).

E/[A] curves to adenosine in the presence of NBTI to block adenosine uptake were shifted to the right by 0.1 μM ZM 241385 ($p[A]_{50} = 5.58 \pm 0.17$ and 4.94 ± 0.12 in the absence and presence of ZM 241385, respectively) yielding a pA_2 value of 7.50 ± 0.16 (Figure 4c).

Since responses to **R**-PIA were essentially resistant to blockade by both 8-SPT and ZM 241385, the possibility that they were mediated by A_3 receptors was considered. We therefore investigated the effects of A_3 receptor agonist IB-MECA. Relaxant E/[A] curves to IB-MECA were obtained which were not susceptible to blockade by 8-SPT (100 μM, data not shown, control $p[A]_{50} = 5.38 \pm 0.06$ compared with 5.37 ± 0.10 in the presence of 8-SPT). We also studied the effects of the antagonist BWA1433, but the use of this compound proved difficult because at the concentration of BWA1433 under study (100 μM) E/[A] curves to phenylephrine were both rightward shifted and reduced in maximum (data not shown) such that different concentrations of phenylephrine were required in the presence of vehicle or BWA1433 (1 μM or 3 μM phenylephrine, respectively) in order to achieve approximately 85% maximum phenylephrine contraction. However, these concentrations elicited significantly different absolute tensions (mg, 1068.8 ± 168.1 and 490.0 ± 106.9 in the presence of vehicle and BWA1433, respectively). We therefore used the thromboxane A_2 mimetic U46619 to contract the tissues instead of phenylephrine, as E/[A] curves to U46619 were rightward shifted to a lesser extent than those to phenylephrine (control $p[A]_{50} = 7.39 \pm 0.11$ compared with 7.04 ± 0.05 in the presence of BWA1433) with no effect on the maximum contraction. There was no significant difference between relaxant E/[A] curves to IB-MECA constructed after contraction with U46619 (0.3 μM) in the absence and presence of BWA1433 (100 μM, data not shown, control $pEC_{40} = 5.3 \pm 0.2$ compared with 5.2 ± 0.2 in the presence of BWA1433).

Discussion

In this study, cumulative relaxant E/[A] curves to adenosine and three of its analogues, **R**-PIA, CPA and NECA were obtained in the rat isolated mesenteric artery. However, the maxima of the adenosine and CPA curves could not be reached because the 100 μM concentration represents the limit of their solubilities. The potency order obtained (NECA > **R**-PIA > adenosine = CPA) is consistent with activation of A_2 receptors by these agonists. However, the E/[A] curves to NECA were biphasic suggesting that NECA activates two receptor sites to cause relaxation in this tissue.

The absence of any effect of an A_1-selective concentration of DPCPX (10 nM) upon E/[A] curves to NECA and **R**-PIA indicates that relaxations to these agonists are not A_1 receptor-mediated nor are they compromised by an A_1 receptor-mediated contractile component.

Removal of the endothelium caused depression of the high potency phase of the NECA E/[A] curve and small rightward shifts of E/[A] curves to CPA and **R**-PIA but not to adenosine, suggesting that responses are largely mediated via a non-endothelial site. The small shifts of E/[A] curves to CPA and **R**-PIA and the decrease in asymptote of NECA E/[A] curves observed in the absence of endothelium may be accounted for by some functional antagonism afforded by the small leftward shift of E/[A] curves to phenylephrine. However, it is unclear why there should be no such effect on the E/[A] curves to adenosine.

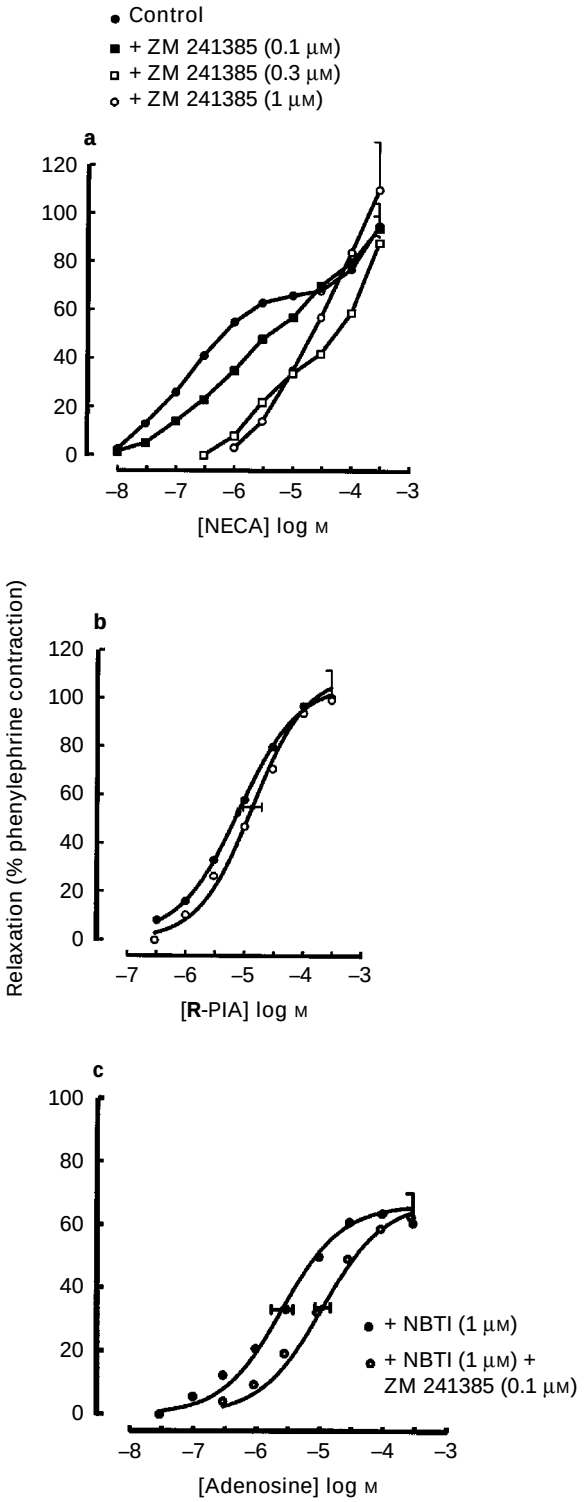

Figure 4 Concentration-response curves to (a) NECA and (b) **R**-PIA in the absence and presence of ZM241385 (0.1 μM, 0.3 μM and 1 μM), (c) Concentration-response curves to adenosine in the presence of 1 μM NBTI and in the absence and presence of ZM 241383 (0.1 μM). Data points are average responses (% relaxation of phenylephrine-induced contraction), and, where possible, the lines through the data were generated by use of the average logistic fitting parameters. Either average $p[A]_{50}$ and α values are marked together with their associated s.e.mean, or the s.e.mean for the maximum relaxation obtained is marked; $n = 3-6$. For abbreviations see text.

The lack of effect of the uptake inhibitor NBTI on E/[A] curves to NECA, **R**-PIA and CPA was not surprising, since these analogues are widely accepted not to be substrates for the uptake process. However, E/[A] curves to adenosine were shifted to the left and rendered hyperbolic in the presence of 1 μM NBTI. This curve shape change suggests that responses to adenosine in the absence and presence of uptake blocker are mediated via different sites one of which may be intracellular. There was no difference between the effects of NBTI in tissues with and without endothelium, suggesting that the sites involved are not located on the endothelium.

The higher potency phase of the E/[A] curve to NECA was rightward shifted by the non-selective adenosine receptor antagonist 8-SPT with a pA_2 value which is consistent with its affinity range at A_2 receptors (0.5–10 μM, Fredholm *et al*., 1994), suggesting that it is mediated by an A_2 receptor. The second phase of the NECA dose-response curve was apparently resistant to blockade by 8-SPT. The lack of significant shifts of CPA, **R**-PIA and adenosine E/[A] curves in the presence of 100 μM 8-SPT suggests that relaxant responses to these agonists are primarily mediated via a xanthine-resistant site, which is likely to be the same as that mediating the low potency phase of the NECA E/[A] curve. There are marked differences between the effects of 8-SPT (10 μM) upon E/[A] curves to adenosine in the absence and presence of NBTI (see Figure 3), namely no significant shift in the absence of NBTI but substantial shifts (approximately 10–30 fold) in the presence of NBTI, which were consistent with the affinity range for 8-SPT at A_2 receptors. Therefore under normal physiological conditions, i.e. when the adenosine uptake process is operational, adenosine responses are mediated via a non-A_2 site. It is only when uptake is inhibited that responses which can be blocked by 8-SPT are revealed. Indeed, the magnitude of the rightward shift in the presence of NBTI indicates that adenosine responses mediated via the non-A_2 site are abolished, suggesting that this site is intracellular. Although E/[A] curves to NECA, CPA and **R**-PIA were unaffected by the uptake inhibitor, these agonists may gain access to an intracellular site via simple diffusion since they are more lipophilic than adenosine itself.

To address the question of which A_2 receptor subtype mediates responses to the lowest concentrations of NECA and to adenosine in the presence of an uptake blocker, the A_{2A} selective agonist CGS 21680 was used. Relaxant responses to CGS 21680 were only observed at concentrations greater than 10 μM and as the affinity of CGS 21680 at A_{2A} receptors is 15 nM (Jacobson *et al*., 1995), these relaxant responses are unlikely to be mediated via A_{2A} receptors. This was confirmed by use of 50 μM 8-SPT, which failed to block the responses to CGS 21680. It is likely, therefore, that the high potency phase of the E/[A] curve to NECA is mediated via A_{2B} receptors. This was confirmed with the A_{2A} selective antagonist ZM 241385, which produced rightward shifts with pA_2 estimates of 7.65 (in the presence of endothelium) and 7.20 (in the absence of endothelium) which are not consistent with the affinity for ZM 241385 at A_{2A} receptors (9.02, Poucher *et al*., 1995) but are closer to the A_{2B} affinity value (7.1, Poucher *et al*., 1995). Likewise, in the presence of NBTI, to block uptake, E/[A] curves to adenosine were shifted to the right by ZM 241385 (0.1 μM) yielding a pA_2 of 7.5, suggesting activation of A_{2B} receptors. This is consistent with the findings of Rubino *et al*. (1995) in the rat mesenteric bed; they suggested that A_{2B} receptors were responsible for vasodilatation. The resistance of responses to **R**-PIA to blockade by ZM 241385 (1 μM) in the present study again indicates activation by this agonist of a site distinct from the A_{2B} receptor.

Assuming that the approximate potency values for agonists under conditions of A_2 receptor blockade (100 μM 8-SPT) reflect the potency of these agonists at the non-A_2 site, then an agonist potency order at this site can be surmised: **R**-PIA ($p[A]_{50} = 4.98 \pm 0.10$) > NECA ($pEC_{40} = 4.47 \pm 0.07$) > CPA ($pEC_{40} = 4.29 \pm 0.09$) = adenosine ($pEC_{40} = 3.95 \pm 0.15$). This potency order is consistent with the binding affinity order obtained in studies with the rat A_3 clone expressed in CHO cells (Van Galen *et al*., 1994), suggesting that A_3 receptors might be involved. We therefore investigated the effects of the potent A_3 receptor agonist IB-MECA. IB-MECA caused relaxations which were resistant to blockade by 8-SPT (100 μM) consistent with the presence of A_3 receptors, and indeed the high potency of IB-MECA ($p[A]_{50} = 5.39 \pm 0.06$) relative to the other adenosine analogues at the undefined site supports activation of A_3 receptors. However, there was no significant shift of E/[A] curves to IB-MECA in U46619-precontracted tissues in the presence of 100 μM BWA1433, suggesting that this agonist is not activating A_3 receptors in the rat isolated mesenteric artery. In addition, although the agonist potency order for the non-A_2 site is not inconsistent with it being an A_3 receptor, for all of the analogues used in the present study the potency values were substantially lower than their affinity values at rat A_3 receptors. Overall the non-A_2 site is, therefore, unlikely to be a functional correlate of the A_3 receptor.

Adenosine and some of its analogues induce both xanthine-sensitive and xanthine-resistant relaxations in other smooth muscle preparations, for example in the rat aorta where, in contrast to this study, the presence of A_{2A} receptors and an undefined site has been established (Lewis *et al*., 1994; Prentice & Hourani, 1996a). Collis & Brown (1983) also noted relaxations in the guinea-pig aorta to adenosine and analogues which were refractory to blockade by 8-phenyltheophylline and which were depressed in the presence of the uptake inhibitor dipyridamole, indicating the presence in this tissue of an intracellular site. Xanthine-resistant sites have also been noted in guinea-pig taenia caecum (Prentice *et al*., 1995; Prentice & Hourani, 1996b), guinea-pig trachea (Brackett & Daly, 1991) and frog aorta (Knight & Burnstock, 1996). It may be that all of these relaxant responses are mediated by the same site. This unknown site mediating relaxations in the mesenteric artery and, indeed, in all of the other smooth muscle preparations previously mentioned is likely to be of physiological significance, since the responses to the endogenous agonist, adenosine, appear to be entirely mediated via this mechanism in the absence of uptake blockade.

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