ELSEVIER

European Journal of Pharmacology 415 (2001) 251–255

ejp

www.elsevier.nl/locate/ejphar

Short communication

Relaxation of mouse isolated aorta to adenosine and its analogues does not involve adenosine A_1, A_2 or A_3 receptors

Deborah Prentice *, Katherine Boon, Susanna Hourani

School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, UK

Received in revised form 31 January 2001; accepted 13 February 2001

Abstract

Relaxations to adenosine and analogues were investigated in the mouse aorta in the presence of the adenosine A_1 receptor-selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 30 nM), which did not affect relaxations to adenosine or its analogue N^6-*R*-phenylisopropyladenosine (*R*-PIA) but abolished contractile adenosine A_1 receptor-mediated responses to these agonists. Relaxations to adenosine, 5′-*N*-ethylcarboxamidoadenosine, *R*-PIA, 2-[*p*-(2-carbonylethyl)-phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), and N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA) were unaffected by the adenosine A_1/A_2 receptor antagonist 8-sulphophenyltheophylline (100 μM). IB-MECA relaxations were unaffected by the adenosine A_3 receptor-selective antagonist 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191, 30 μM) and *R*-PIA relaxations were unaffected by N^G-nitro-L-arginine methyl ester (100 μM) and endothelium removal. In conclusion, relaxant responses to adenosine and analogues do not involve adenosine A_1, A_2 or A_3 receptors and are endothelium- and nitric oxide-independent.

Keywords: Adenosine; Aorta, mouse; Xanthine; Purinoceptor; Endothelium

1. Introduction

Adenosine has important effects on the vasculature, and in general, adenosine A_1 receptors mediate constriction, adenosine A_{2A} and A_{2B} receptors mediate relaxation and adenosine A_3 receptors may mediate indirect effects via mast cell degranulation (Ralevic and Burnstock, 1998). However, increasingly, there is evidence for an additional mechanism(s), distinct from the four currently cloned adenosine receptors, by which adenosine and some of its analogues induce relaxations (rat aorta, Lewis et al., 1994; Prentice and Hourani, 1996; rat mesenteric artery, Prentice et al., 1997; frog aorta, Knight and Burnstock, 1996; guinea pig aorta, Collis and Brown, 1983; hamster aorta, Prentice and Hourani, 2000; rat renal artery, Martin and Potts, 1994).

The adenosine A_{2A} receptor is regarded as playing a key role in the vasodilator responses in many tissues and adenosine A_{2A} receptor knockout mice have been produced, which have elevated blood pressure, although it is not clear if this is a central or a peripheral effect (Ledent et al., 1997). Our intention, ultimately, is to compare responses to adenosine and its analogues in isolated blood vessels from normal and adenosine A_{2A} receptor knockout mice to investigate the role of peripheral adenosine A_{2A} receptors in the control of blood pressure. Since adenosine receptors mediating relaxations in aortic rings isolated from both rats and hamsters have been characterised as A_{2A} (Prentice and Hourani, 1996, 2000), we chose to look initially at isolated murine aorta. We studied the effects of adenosine, 5′-*N*-ethylcarboxamidoadenosine (NECA), the adenosine A_1 receptor-selective agonist N^6-*R*-phenylisopropyladenosine (*R*-PIA), the adenosine A_3 receptor-selective agonist N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (IB-MECA, Jacobson et al., 1995) and the adenosine A_{2A} receptor-selective agonist 2-[*p*-(2-carbonylethyl)-phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680, Fredholm et al., 1994), as well as the adenosine A_1/A_2 receptor antagonist 8-sulphophenyltheophylline (Fredholm et al., 1994) and the adenosine A_3 receptor-selective antagonist 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydro-

* Corresponding author. Tel.: +44-1483-259710; fax: +44-1483-576978.

E-mail address: D.Prentice@Surrey.ac.uk (D. Prentice).

0014-2999/01/$ - see front matter
PII: S0014-2999(01)00841-X

pyridine-3,5-dicarboxylate (MRS1191, Jiang et al., 1997) in isolated aorta from normal mice and we report here some unexpected results suggesting the absence of any relaxant adenosine A_1, A_2 or A_3 receptor.

2. Materials and methods

Adult male CD1 mice (Charles River, UK, 30–35 g) were killed by cervical dislocation, the thoracic aorta excised and one or two rings approximately 3 mm long were taken per animal. These were mounted between two fine (0.125 mm diameter) tungsten wires in 3.5 ml organ baths containing Krebs–Henseleit solution (mM: NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; D-glucose, 11; $MgSO_4 \cdot 7H_2O$, 0.45; K_3PO_4, 1.2; $CaCl_2 \cdot 2H_2O$, 2.5), maintained at 37°C and gassed with 95% O_2/CO_2. Preparations were allowed to equilibrate for 30 mm under an initial resting tension of 1 g. Tissue viability was tested using 0.1 μM phenylephrine, a concentration that elicits approximately 85% maximum contraction. All tissues were tested for the presence of functional endothelium using 1 μM acetylcholine. Tissues in which acetylcholine did not induce a relaxation were rejected. Tissues were then washed, incubated for a period of 60 min, either in the presence of one of the antagonists or with an appropriate solvent control, then contracted again with phenylephrine, and cumulative relaxant concentration–response curves to adenosine analogues were constructed. In preliminary experiments, *R*-PIA and adenosine caused contractions at relatively low concentrations (0.1 μM, $29 \pm 5\%$ phenylephrine maximum) and relaxations at higher concentrations. The contractions were abolished by both 8-sulphophenyltheophylline (100 μM) and the selective adenosine A_1 receptor-selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (30 nM), though the relaxations were unaffected by these antagonists. Because in these experiments we were interested in characterising the receptors mediating relaxation, all further experiments were conducted in the presence of 30 nM DPCPX to eliminate the confounding influence of adenosine A_1 receptors mediating contractions. DPCPX was administered to all tissues at the beginning of the 60-min incubation period.

In experiments where the involvement of endothelium and nitric oxide synthase were investigated, either the endothelium was destroyed using a metal rod or tissues were incubated with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM). Absence of endothelium or blockade of nitric oxide synthase was confirmed by loss of the acetylcholine response.

Where two rings were taken from the same animal, one was used as a control and one as an antagonist-treated preparation. Only one concentration–response curve was constructed in each preparation, except for studies using adenosine where, because the variation between tissues was large, paired curves were constructed in the same tissue ring before and after incubation with either 8-sulphophenyltheophylline (100 μM) or nitrobenzylthioinosine (1 μM). In order to check for reproducibility of concentration effect curves in the same tissue, two sequential control curves were constructed in a single aortic ring.

Responses were recorded isometrically using Grass FTO3 force displacement transducers and displayed on a Grass polygraph (model 79). Results are expressed as % decrease in the contraction to phenylephrine and 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). In the paired curve experiments, IC_{30} (concentration required to achieve 30% relaxation) values were estimated by regression of the linear portion of the curve and ratios of the $IC_{30\ control}/IC_{30\ treated}$ (IC_{30} ratio) were calculated.

The effect of drug treatment was assessed by Student's *t*-test and P values of less than 0.05 were considered significant. Data are presented as mean ± S.E.M. of values obtained from at least three different animals.

All drugs were obtained from Sigma-Aldrich, UK and were made up at stock concentrations of 10 mM. CGS 21680 was made up with 7% ethanol, 8-sulphophenyltheophylline, phenylephrine, acetylcholine, adenosine, NECA and L-NAME in distilled water, *R*-PIA in 0.06 M HCl, DPCPX in 6 mM NaOH containing 6% dimethylsulphoxide (DMSO) and MRS1191, IB-MECA and nitrobenzylthioinosine in 100% DMSO. Further dilutions were made up in water except for MRS1191, which was diluted 100-fold in 33% DMSO and nitrobenzylthioinosine, which was diluted 10-fold in 50% DMSO and then diluted 10-fold in distilled water.

3. Results

In preliminary experiments, *R*-PIA and adenosine caused contractions at relatively low concentrations (0.1 μM, $29 \pm 5\%$ phenylephrine maximum) and relaxations at higher concentrations. The contractions were abolished by both the non-selective adenosine A_1/A_2 receptor antagonist 8-sulphophenyltheophylline (100 μM) and by an adenosine A_1 receptor-selective concentration of DPCPX (30 nM), suggesting that they were adenosine A_1 receptor mediated (data not shown). Relaxations to adenosine and *R*-PIA were unaffected by these antagonists suggesting that they were not adenosine A_1 receptor mediated (data not shown). All further experiments were therefore conducted in the presence of 30 nM DPCPX in order to eliminate the confounding influence of adenosine A_1 receptor-mediated contractions.

NECA and CGS 21680 caused only small relaxations ($< 30\%$) at relatively high concentrations (> 30 and 3 μM, respectively), which were not significantly affected by 8-sulphophenyltheophylline ($P > 0.05$) (100 μM, Fig. 1A and B). Relaxations to adenosine were variable, with

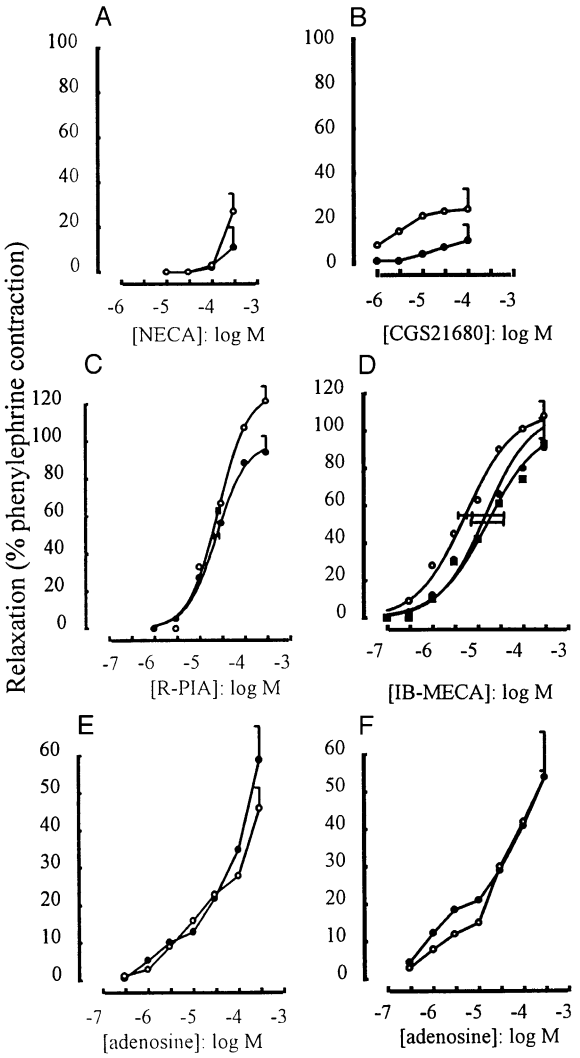

Fig. 1. Concentration–response curves to (A) NECA in the absence (●) or presence (○) of 100 μM 8-sulphophenyltheophylline, (B) CGS 21680 in the absence (●) or presence (○) of 100 μM 8-sulphophenyltheophylline, (C) *R*-PIA in the absence (●) or presence (○) of 100 μM 8-sulphophenyltheophylline, (D) IB-MECA in the absence (●) or presence of 100 μM 8-sulphophenyltheophylline (○) or 100 μM MRS1191 (■), or paired concentration–response curves to adenosine (E) in the absence (●) and presence (○) of 100 μM 8-sulphophenyltheophylline and (F) in the absence (●) and presence (○) of 1 μM nitrobenzylthioinosine. All curves were constructed in the presence of 30 nM DPCPX. Data points are average responses (% relaxation of phenylephrine-induced contraction), $n = 4–6$. Where possible the lines through the data were generated by the use of the average logistic fitting parameters. Either average $p[A]_{50}$ and α values are marked together with their associated S.E.M., or the S.E.M. for the maximum relaxation obtained is marked. For abbreviations, see text.

responses occurring at concentrations as low as 10 nM in some preparations, but more usually at concentrations above 10 μM, and none of the curves reached a plateau. Because of the variability in curve shape and location, a paired curve design was adopted to investigate the effects of blockers on these responses. In these experiments, a control concentration–effect curve and a curve after treatment with antagonist were constructed sequentially in a single aortic ring preparation. The IC_{30} ratio for the paired control curves was not significantly different from unity (IC_{30} ratio $= 1.6 + 0.7$, $P > 0.05$), establishing the reproducibility of sequential curves constructed in a single aortic ring. The average pIC_{30} values for the control (first curve) and 8-sulphophenyltheophylline (100 μM) treated (second curve) were 4.3 ± 0.3 and 4.2 ± 0.3, respectively, suggesting that 8-sulphophenyltheophylline did not affect curves to adenosine and this was confirmed by the fact that the IC_{30} ratio was not significantly different from unity (1.0 ± 0.3, $P > 0.05$, Fig. 1E). Likewise, the pIC_{30} values for the control (first curve) and nitrobenzylthioinosine (1 μM) treated (second curve) were 4.5 ± 0.1 and 4.4 ± 0.1, respectively, suggesting that the adenosine uptake inhibitor, nitrobenzylthioinosine, did not affect curves to adenosine and this was again confirmed by the IC_{30} ratio (1.0 ± 0.4, $P > 0.05$, Fig. 1F). Sigmoidal relaxant curves to *R*-PIA and IB-MECA were obtained over a concentration range 1 μM–0.3 mM and achieved maxima

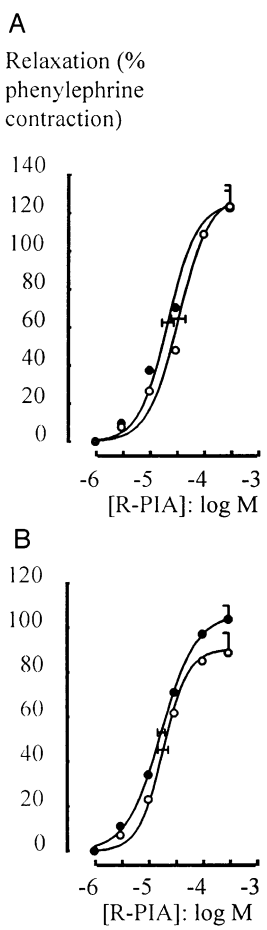

Fig. 2. Concentration–response curves to *R*-PIA (A) in the absence (●) or presence (○) of 100 μM L-NAME and (B) in the presence (●) or absence (○) of endothelium. All curves were constructed in the presence of 30 nM DPCPX. Data points are average responses (% relaxation of phenylephrine-induced contraction), $n = 3–4$. The lines through the data were generated by the use of the average logistic fitting parameters and average $p[A]_{50}$ and α values are marked together with their associated S.E.M. For abbreviations, see text.

in the region of 100% relaxation. These relaxations were also unaffected by 8-sulphophenyltheophylline (100 μM, *R*-PIA $p[A]_{50}$ control = 4.60 ± 0.06; + 8-sulphophenyltheophylline = 4.60 ± 0.04, $P > 0.05$; IB-MECA $p[A]_{50}$ control = 4.80 ± 0.42; + 8-sulphophenyltheophylline = 5.30 ± 0.15, $P > 0.05$, Fig. 1C and D). Responses to IB-MECA were unaffected by the antagonist MRS1191 (30 μM, $p[A]_{50}$ control = 4.80 ± 0.42; + MRS1191 = 4.80 ± 0.36, $P > 0.05$, Fig. 1D).

Neither removal of endothelium nor blockade of nitric oxide synthase using L-NAME (100 μM) affected curves to *R*-PIA ($p[A]_{50}$ in the absence and presence of L-NAME = 4.48 ± 0.13 and 4.67 ± 0.11, respectively, $P > 0.05$; $p[A]_{50}$ in the presence and absence of endothelium = 4.74 ± 0.10 and 4.77 ± 0.07, respectively, $P > 0.05$, Fig. 2).

4. Discussion

In preliminary experiments, contractile responses to both adenosine and *R*-PIA were observed, which were abolished by both 8-sulphophenyltheophylline and DPCPX, suggesting the presence of contractile adenosine A_1 receptors. However, in this study, we wanted to characterise relaxant effects of adenosine and its analogues, therefore, all further experiments were performed under conditions of selective adenosine A_1 receptor blockade (30 nM DPCPX). This concentration of DPCPX did not affect relaxations to adenosine and *R*-PIA, therefore, they are not adenosine A_1 receptor mediated, even though *R*-PIA is normally considered to be a selective adenosine A_1 receptor agonist. Under these conditions, NECA and CGS 21680 caused only relaxations in the mouse isolated aorta at relatively high concentrations and these were unaffected by 8-sulphophenyltheophylline, suggesting that they were not mediated by adenosine A_2 receptors. This observation contrasts with data previously obtained in aortic preparations from other mammalian species where adenosine A_2 receptors mediate vasodilation (rat aorta, Lewis et al., 1994; Prentice and Hourani, 1996; guinea pig aorta, Collis and Brown, 1983; hamster aorta, Prentice and Hourani, 2000).

Although responses to adenosine varied between aortic preparations, it was possible to obtain, sequentially, two reproducible curves from a single preparation. In order to see if curves to adenosine were affected by 8-sulphophenyltheophylline, sequential curves were therefore constructed before and after incubation with the antagonist in a single aortic preparation. Like relaxations to NECA and CGS 21680, those to adenosine were unaffected by 8-sulphophenyltheophylline, suggesting that they were also not mediated by adenosine A_2 receptors.

Whereas relaxations to NECA and CGS 21680 were small, sigmoidal curves to *R*-PIA and IB-MECA were obtained, which could be fitted to a logistic function, suggesting a receptor-mediated effect. However, these relaxations were also resistant to blockade by 8-sulphophenyltheophylline. As all of the experiments were carried out in the presence of an adenosine A_1 receptor-selective concentration of DPCPX (which did not affect the relaxations), and relaxations to all of the agonists were resistant to blockade by 8-sulphophenyltheophylline, we can conclude that adenosine, NECA, CGS 21680, *R*-PIA and IB-MECA activate a site distinct from adenosine A_1 and A_2 receptors to cause relaxation of the mouse aorta. The adenosine A_3 receptor-selective antagonist MRS1191 was used to investigate the involvement of adenosine A_3 receptors, however, at a concentration approximately 10-fold higher than its K_i at rat-cloned adenosine A_3 receptors ($K_i = 3$ μM, Jiang et al., 1997), it did not alter curves to IB-MECA. Therefore, it is unlikely that the relaxations are mediated by adenosine A_3 receptors either.

There is evidence that adenosine and some of its analogues are able to produce both antagonist-sensitive and antagonist-resistant relaxations in other vascular preparations, for example in the rat aorta (Prentice and Hourani, 1996), rat mesenteric artery (Prentice et al., 1997), guinea-pig aorta (Collis and Brown, 1983), rat renal artery (Martin and Potts, 1994) and hamster aorta (Prentice and Hourani, 2000). The lack of effect on curves to *R*-PIA of endothelium denudation or of nitric oxide synthase inhibition by L-NAME in the mouse aorta suggests that the mechanism by which this agonist mediates its effects is neither endothelium- nor nitric oxide-dependent. This result is consistent with data obtained in other vascular preparations where antagonist-resistant sites have also been found to be non-endothelium dependent, for example, in the rat aorta, mesenteric artery and renal artery (Prentice and Hourani, 1996; Prentice et al., 1997; Martin and Potts, 1994), and hamster aorta (Prentice and Hourani, 2000).

However, the results obtained in this study with nitrobenzylthioinosine contrast with data obtained previously where nitrobenzylthioinosine abolished antagonist-resistant relaxations to adenosine, suggesting that they may be mediated by an intracellular mechanism (guinea-pig aorta, Collis and Brown, 1983; rat mesenteric artery, Prentice et al., 1997; hamster aorta, Prentice and Hourani, 2000). In the mouse aorta, nitrobenzylthioinosine did not affect responses to adenosine. Nitrobenzylthioinosine has been shown to bind with high affinity (low nM range) in a reversible, specific and saturable manner to uptake sites in the mouse, just as in other mammalian species (Verma and Marangos, 1984). However, nitrobenzylthioinosine-insensitive uptake sites exist (for review, see Jennings et al., 1998), so although the data suggest that adenosine responses are not dependent on nitrobenzylthioinosine-sensitive uptake into an intracellular compartment, access of adenosine by simple diffusion or by a nitrobenzylthioinosine-insensitive uptake site cannot be ruled out.

Overall, the data suggest the absence of relaxant adenosine A_1, A_{2A}, A_{2B} or A_3 receptor, but the presence of another as yet undefined mechanism(s), resistant to block-

ade by 8-sulphophenyltheophylline or MRS1191, which is activated by adenosine, NECA and CGS 21680 at high concentrations, and by *R*-PIA and IB-MECA at lower concentrations. The mechanism mediating the relaxations is not endothelium- or nitric oxide-dependent. Thus far, the mouse isolated aorta is the only mammalian vascular preparation shown to lack functional, currently cloned adenosine receptors, which mediate relaxation and, as such, it is an ideal tissue in which to study the undefined mechanism by which adenosine and analogues induce smooth muscle relaxation.

Data obtained in vivo in mice lacking the adenosine A_{2A} receptor suggest an involvement of this receptor in the tonic control of blood pressure since knockout mice were hypertensive compared to wild types (Ledent et al., 1997). Even though there are no adenosine A_{2A} receptors in the largest artery, these receptors may be present in smaller branches of the murine vascular network where they may play a role in regulation of blood pressure. The involvement of central A_{2A} receptors in the tonic control of blood pressure also cannot be ruled out.

Acknowledgements

This work was funded by The Wellcome Trust (reference 057088).

References

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.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., Williams, M., 1994. Nomenclature and classification of purinoceptors. Pharmacol. Rev. 46, 143–156.

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 Future 20, 689–699.

Jennings, L.L., Cass, C.E., Ritzel, M.W.L., Yoa, S.Y.M., Young, J.D., Griffiths, M., Baldwin, S.A., 1998. Adenosine transport: recent advances in the molecular biology of nucleoside transporter proteins. Drug Dev. Res. 45, 277–287.

Jiang, J.-L., Van Rhee, A.M., Chang, L., Patchorik, A., Evans, P., Melman, N., Jacobson, K.A., 1997. Structure activity relationships of 4-phenylethynyl-6-phenyl-1,4-dihydropyridines as highly selective A_3 adenosine receptor antagonists. J. Med. Chem. 40, 2596–2608.

Knight, G.E., Burnstock, G., 1996. The effects of purine compounds on the isolated aorta of the frog *Rana temporaria*. Br. J. Pharmacol. 117, 873–878.

Ledent, C., Vaugeois, J.M., Schiffman, S.N., Pedrazzii, T., El Yacoubi, M., Vanderhaeghen, J.J., Costentin, J., Heath, J.K., Vassart, G., Parmentier, M., 1997. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A_{2A} receptor. Nature 388, 674–678.

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.

Martin, P.L., Potts, A.A., 1994. The endothelium of the rat renal artery plays an obligatory role in A_2 adenosine receptor mediated relaxation induced by 5′-*N*-ethylcarboxamidoadenosine and N^6-cyclopentyladenosine. J. Pharmacol. Exp. Ther. 270, 893–899.

Prentice, D.J., Hourani, S.M.O., 1996. Activation of multiple sites by adenosine analogues in the rat isolated aorta. Br. J. Pharmacol. 118, 1509–1517.

Prentice, D.J., Hourani, S.M.O., 2000. Characterisation of adenosine receptors mediating relaxation in hamster isolated aorta. Naunyn-Schmiedeberg's Arch. Pharmacol. 362, 427–434.

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.

Prentice, D.J., Payne, S.L., Hourani, S.M.O., 1997. Activation of two sites by adenosine receptor agonists to cause relaxation in rat isolated mesenteric artery. Br. J. Pharmacol. 122, 1509–1515.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.

Verma, A., Marangos, P.J., 1984. Nitrobenzylthioinosine binding in brain: an interspecies study. Life Sci. 36, 283–290.