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Adenosine mediates relaxation of human small resistance-like coronary arteries *via* A_{2B} receptors

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1 The receptor subtype and mechanisms underlying relaxation to adenosine were examined in human isolated small coronary arteries contracted with the thromboxane A_2 mimetic, 1,5,5-hydroxy-11α, 9α-(epoxymethano)prosta-5Z, 13E-dienoic acid (U46619) to approximately 50% of their maximum contraction to K^+ (125 mM) depolarization (F_{max}). Relaxations were normalized as percentages of the 50% F_{max} contraction.

2 Adenosine caused concentration-dependent relaxations (pEC_{50}, 5.95±0.20; maximum relaxation (R_{max}), 96.7±1.4%) that were unaffected by either combined treatment with the nitric oxide inhibitors, N^G-nitro-L-arginine (L-NOARG; 100 μM) and oxyhaemoglobin (HbO; 20 μM) or the ATP-dependent K^+ channel (K_{ATP}) inhibitor, glibenclamide (10 μM). The pEC_{50} but not R_{max} to adenosine was significantly reduced by high extracellular K^+ (30 mM). Relaxations to the adenylate cyclase activator, forskolin, however, were unaffected by high K^+ (30 mM).

3 Adenosine and a range of adenosine analogues, adenosine, 2-chloroadenosine (2-CADO), 5′-N-ethyl-carboxamidoadenosine (NECA), R(−)-N^6-(2-phenylisopropyl)-adenosine (R-PIA), S(+)-N^6-(2-phenylisopropyl)-adenosine (S-PIA), N^6-cyclopentyladenosine (CPA), 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA), 2-*p*-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamido adenosine hydrochloride (CGS 21680), relaxed arteries with a rank order of potency of NECA = 2-CADO > adenosine = IB-MECA = R-PIA = CPA > S-PIA) > CGS 21680.

4 Sensitivity but not R_{max} to adenosine was significantly reduced approximately 80 and 20 fold by the non-selective adenosine receptor antagonist, 8-(p-sulphophenyl)theophylline (8-SPT) and the A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX). By contrast, the A_1-selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) had no effect on pEC_{50} or R_{max} to adenosine.

5 These results suggest that A_{2B} receptors mediate relaxation to adenosine in human small coronary arteries which is independent of NO but dependent in part on a K^+-sensitive mechanism.

Keywords: Adenosine; A_{2B}-purinoceptors; human coronary artery

Abbreviations: ACh, A_1, A_{2A}, A_{2B}, A_3, ANOVA, 2-CADO, CGS 21680, CPA, D_{100} DMPX, DMSO, DPCPX, F_{max}, Hb0, IB-MECA, K_{ATP}, K_{Ca}, K_{IV}, K_V, KPSS, L-NOARG, NECA, pEC_{50}, pK_B, R_{max}, R-PIA, SNP, S-PIA, 8-SPT, TEA, U46619

Introduction

Adenosine is an endogenous vasodilator and is believed to play important roles in the regulation of coronary blood flow under normal physiological conditions and also during hypoxia and reperfusion after ischaemia (Berne, 1980; Collis & Hourani, 1993; Bouchard & Lamontagne, 1996; Feoktistov & Biaggioni, 1997; Minamino *et al*., 1998). Whilst it is established that adenosine A_{2A} receptors mediate vasodilatation to adenosine throughout the vasculature (Hussain *et al*., 1996; Belardinelli *et al*., 1996; Ongini & Fredholm, 1996; Conti *et al*., 1997; Feoktistov & Biaggioni, 1997), less is known of the roles of A_{2B} receptors. Thus, from limited functional studies, it remains controversial as to whether any vasodilator effects of A_{2B} receptors are endothelium-dependent or -independent and mediated by nitric oxide (NO) or hyperpolarization, particularly *via* the opening of ATP-dependent K^+ channels (K_{ATP}) (Feoktistov & Biaggioni, 1997). Furthermore, there is no information as to the identity of receptor subtypes underlying vasodilatation to adenosine in the human coronary vasculature, although both A_{2A} and A_{2B} receptors have been found on isolated human endothelial cells (Iwamoto *et al*., 1994; Feoktistov & Biaggioni, 1997). Therefore, we investigated both the receptor type and the mechanisms involved in relaxation of human, resistance-like coronary arteries to adenosine *in vitro*. Our data demonstrate that A_{2B} receptors mediate relaxation to adenosine in these clinically relevant arteries independent of nitric oxide (NO) and the endothelium but partly dependent upon the activation of non-K_{ATP} K^+ channels.

Methods

Tissue source

Small coronary arteries were obtained from the discarded tip of the right atrial appendage from patients (64.4±1.7 years; 31 male, 12 female) undergoing mitral valve (one patient), aortic valve (four patients) or coronary bypass graft surgery (38 patients). Immediately following surgical removal, the atrial appendages were placed in cold, carboxygenated Krebs' solution and transported back to the laboratory. The segments

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of atrial appendage were viewed with a dissecting-light microscope under which the small coronary arteries were carefully freed of surrounding tissue.

Mounting of vessels in myograph

Coronary arteries were cut into 2 mm lengths and mounted horizontally on two parallel stainless steel wires (40 μm) in small vessel Mulvany-Halpern myographs (JP Trading, Denmark) to record changes in isometric force as previously described (Kemp & Cocks, 1997). Vessels were maintained in physiological Krebs' solution at 37°C and continuously gassed with carbogen (95% 0_2, 5% CO_2). The Krebs' solution was composed of (in mM): Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11, pH 7.4.

Normalization

After a 30 min equilibration period, vessel rings were maximally relaxed with sodium nitroprusside (SNP; 10 μM), and then set to passive tensions equivalent to that required to produce 90% of their internal circumference when exposed to a transmural pressure of 100 mmHg (Kemp & Cocks, 1997). In brief, a passive length-tension curve was constructed for each vessel. From this curve, the effective transmural pressure was calculated and the vessel set at a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg (D_{100}).

Experimental protocol

Following normalization, arteries were contracted with isotonic, high K^+ Krebs' solution (124 mM KCl; KPSS) to determine their maximum levels of active force (F_{max}; Kemp & Cocks, 1997). When the tissues reached F_{max}, the bathing solution was replaced with normal Krebs' and the tissues allowed to return to their resting levels of passive force. Regardless of treatment, all arteries were then contracted to $\sim$50% F_{max} with titrated concentrations of the thromboxane A_2 mimetic, 1,5,5-hydroxy-11α, 9α-(epoxymethano)prosta-5Z, 13E-dienoic acid (U46619; 1–30 nM) and in some cases acetylcholine (ACh; 1–3 μM), which in this tissue only causes endothelium-independent contractions (Angus *et al*., 1991). Once these contractions reached steady plateaus, cumulative concentration-relaxation curves to adenosine, adenosine analogues or forskolin were constructed. All concentration-response curves were obtained in the presence of indomethacin (3 μM) to inhibit prostanoid synthesis and SNP (10 μM) to inhibit characteristic spontaneous contraction of these arteries since in preliminary studies we found relaxation to adenosine to be independent of NO. Furthermore, any masking of the vasodilator effects of endothelial adenosine receptors by SNP was unlikely since both endothelial A_{2A} and A_{2B} receptors are coupled *via* Gs to increase levels of cyclic AMP (Iwamoto *et al*., 1994). In a previous study examining endothelium- and NO-dependent relaxations to bradykinin in similar tissue, however, we used isoprenaline to control spontaneous activity and thus avoid such functional block of responses to endothelial NO (Kemp & Cocks, 1997). Thus, isoprenaline was substituted for SNP in the three experiments in which we examined the response to bradykinin in adjacent rings of artery used for the adenosine studies.

Responses to adenosine were obtained in the absence and presence of the NO inhibitors, N^G-nitro-L-arginine (L-NOARG, 100 μM) and oxyhaemoglobin (HbO, 20 μM), high K^+ (30 mM) isotonic Krebs' solution, the ATP-sensitive potassium channel (K_{ATP}) antagonist, glibenclamide (10 μM), and the adenosine receptor antagonist, 8-(p-sulphophenyl)theophylline (8-SPT, 100 μM), 3,7-dimethyl-1-propargylxanthine (DMPX, 100 μM) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 10 nM). These antagonists were added 30 min prior to precontraction with U46619 and ACh. Forskolin relaxations were obtained in the absence and presence of high extracellular K^+ (30 mM).

Drugs

U46619 ([1,5,5-hydroxy-11α, 9α-(epoxymethano)prosta-5Z, 13E-dienoic acid], Upjohn, Kalamazoo, MI, U.S.A.); ACh, indomethacin, N^G-nitro-L-arginine (L-NOARG), adenosine, bovine haemoglobin (Sigma, U.S.A.); 5′-N-ethyl-carboxamidoadenosine (NECA), 2-*p*-(2-carboxyethyl)phenethylamino-5′ N-ethylcarboxamido adenosine hydrochloride (CGS 21680), 2-chloroadenosine (2-CADO), 1-deoxy-1-[6-[[(3-iodophenyl)-methyl]amino] -9H -purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA), R(−)-N^6-(2-phenylisopropyl)-adenosine (R-PIA), S(+)-N^6-(2-phenylisopropyl)-adenosine (S-PIA), N^6-cyclopentyladenosine (CPA), 8-(p-sulphophenyl)theophylline (8-SPT), 3,7-dimethyl-1-propargylxanthine (DMPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), forskolin (RBI, U.S.A.); bradykinin triacetate (Fluka, U.K.); sodium nitroprusside (DBL., Australia). Stock solutions of U46619 (1 mM) were made up in absolute ethanol, L-NOARG (100 mM) in 1 M $NaHCO_3$, and indomethacin (100 mM) in 1 M Na_2CO_3. Adenosine, S-PIA, R-PIA, NECA, 2-CADO, CGS 21680, CPA and DPCPX were made up as stock solutions (100 mM) in dimethyl sulphoxide (DMSO). Haemoglobin was dissolved in 0.9% NaCl to make up a 1 mM stock solution. The stock solution was subsequently reduced to oxyhaemoglobin (HbO) by the addition of a small amount (<0.1 g) of sodium dithionite. Excess sodium dithionite was extracted by running the solution through a sephadex (PD-10) column equilibrated with 0.9% NaCl. All subsequent dilutions of stock solutions were in distilled water and all other drugs were made up in distilled water.

Statistical analysis

Responses were expressed as percentage reversal of the U46619 and ACh contraction (Kemp & Cocks, 1997). Contractile responses were measured as a percentage of the maximum contraction to KPSS (F_{max}). The individual relaxation curves were fitted (Graphpad Prism, version 1.00) to the sigmoidal logistic equation,

$$Y = \text{BOTTOM} + (\text{TOP} - \text{BOTTOM}) / 1 + 10^{(pEC_{50} - X).\text{HILLSLOPE}}$$

where X = the logarithm of the agonist concentration and Y = the response. BOTTOM = the lower response plateau, TOP = the upper response plateau and pEC_{50} is the X value when the response is halfway between BOTTOM and TOP. The variable HILLSLOPE controls the slope of the curve. From this relationship, computer estimates of pEC_{50} values were determined and expressed as $-\log$ M.

Differences between mean pEC_{50} and maximum relaxation (R_{max}) values were tested either with a two-tailed Student's unpaired *t*-test or one way analysis of variance (ANOVA) with comparisons to control or between all experimental groups made *via* Dunnett's and Tukey's modified *t*-tests, respectively. Results are expressed as mean ± s.e.mean and statistical significance was accepted at the $P<0.05$ level. *n* values refer to number of rings from separate patients.

Results

The effect of L-NOARG, HbO and high K^+ on responses to adenosine

Adenosine caused concentration-dependent relaxations (R_{max}; 96.7±1.4%, pEC_{50}; 5.95±0.20) in small coronary arteries with diameters (D_{100}) of 207.3±6.2 μm ($n=20$). The response to adenosine was unaffected by a combination of HbO (20 μM) and L-NOARG (100 μM) (Figure 1). By contrast, in separate arteries from the same patients, endothelium-dependent relaxation to bradykinin, as we previously reported (Kemp & Cocks, 1997), was markedly attenuated by this treatment ($n=3$, data not shown). Raising the extracellular concentration of K^+ to 30 mM, however, caused a significant 12 fold ($P<0.05$) decrease in the sensitivity (pEC_{50}; 4.88±0.32, $n=4$) to adenosine with no change in R_{max} (92.5±2.1%, $n=4$) (Figure 1). Glibenclamide (10 μM) had no effect on either the sensitivity (pEC_{50}, 6.07±0.21, $n=5$) or R_{max}; (92.5±2.1%, $n=5$) to adenosine (Figure 1) and relaxations to forskolin (pEC_{50}; 7.43±0.22, R_{max}, 79.7±5.1%, $n=4$) were unchanged in the presence of high K^+ (30 mM; Figure 1).

Responses to adenosine receptor agonists and antagonists

All the adenosine analogues used caused concentration-dependent relaxation in human small coronary arteries with a rank order of potency (pEC_{50} values in parentheses; $n=4-5$) of NECA (6.98±0.20) = 2-CADO (6.98±0.20) > adenosine (6.07±0.21) = IB-MECA (5.93±0.09) = R-PIA (5.72±0.10) = CPA (5.63±0.09) > S-PIA (5.27±0.09) > CGS 21680 (<5.00) (Figure 2). All analogues caused near maximal relaxation except CGS 21680 (30 μM) which reversed the level of contraction by 32.3±14.3%.

The response to adenosine was unchanged in the presence of the selective A_1 adenosine receptor antagonist, DPCPX (10 nM) (pEC_{50}, 5.89±0.21; R_{max}; 94.0±1.9%, $n=4$; Figure 2). By contrast, the A_2 adenosine receptor antagonist, DMPX (100 μM), and the non-selective adenosine receptor antagonist, 8-SPT (100 μM), caused significant 20 ($P<0.01$) and 80 fold ($P<0.01$) decreases respectively in the sensitivity to adenosine, with no change in R_{max} (Figure 2).

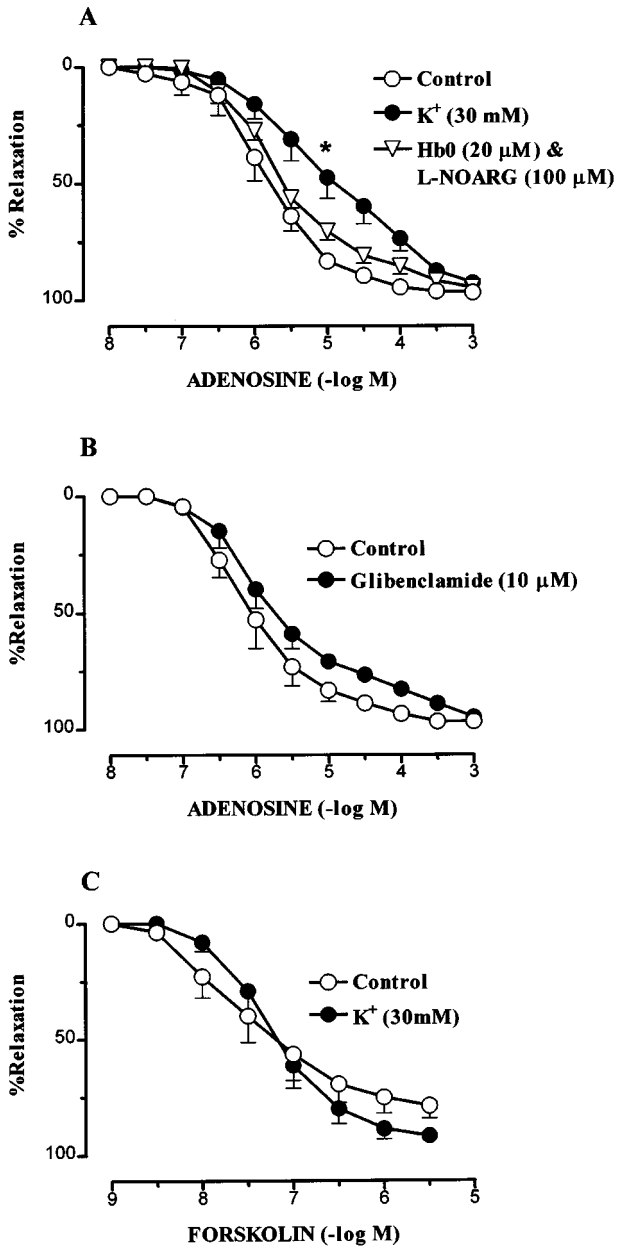

Figure 1 Relaxations in human isolated small coronary arteries to (A) adenosine in normal Krebs' (control), high K^+ (30 mM KCl) and a combination of oxyhaemoglobin (HbO) and L-NOARG ($n=4-5$), (B) adenosine in the presence of normal Krebs' (control, $n=5$) or glibenclamide ($n=5$) and (C) forskolin in normal Krebs' (control, $n=4$) and high K^+ ($n=4$). Values are mean±s.e.mean. (*) indicates pEC_{50} value significantly different from control ($P<0.05$, Dunnett's modified *t*-statistic).

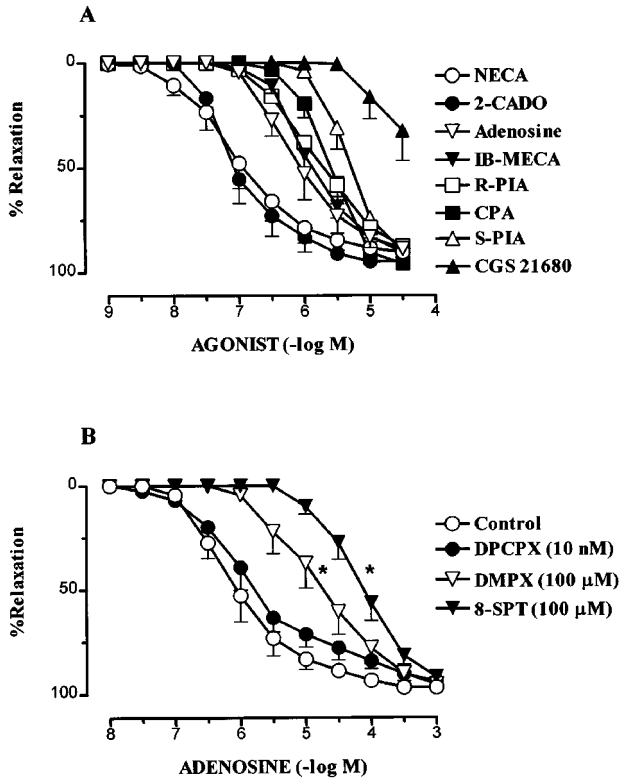

Figure 2 Relaxations to (A) the adenosine agonists; NECA, 2-CADO, adenosine, IB-MECA, R-PIA, CPA, S-PIA and CGS 21680 ($n=4-5$) and (B) adenosine in the absence (control) and presence of the adenosine antagonists, DPCPX, DMPX and 8-SPT ($n=4-5$) in human isolated small coronary arteries. Values are mean±s.e.mean. (*) indicates pEC_{50} value significantly different from control ($P<0.01$, Dunnett's modified *t*-statistic).

Discussion

This study is the first to demonstrate that adenosine mediates relaxation of human small isolated coronary arteries *via* A_{2B} receptors. Furthermore, the response to adenosine in these clinically important arteries appeared to be independent of NO and thus the endothelium, but partly mediated by a K^+-sensitive mechanism.

Four subtypes of adenosine receptors, A_1, A_{2A}, A_{2B} and A_3 have been cloned (Collis & Hourani, 1993; Tucker & Linden, 1993; Burnstock, 1995; Feoktistov & Biaggioni, 1997) and adenosine and its analogues display characteristic orders of potencies at each of these receptors. In the present study, the adenosine analogue, NECA, was significantly more potent than both CPA and R-PIA suggesting that A_2 rather than A_1 receptors were involved (Collis & Hourani, 1993). Also, the >100 fold higher potency of NECA compared with CGS 21680 indicated the presence of A_{2B} rather than A_{2A} receptors since these agonists are equipotent at A_{2A} receptors (Feoktistov & Biaggioni, 1997). Thus, the rank order of potency of adenosine and its analogues in the present study was NECA = 2-CADO > adenosine = IB-MECA = R-PIA = CPA > S-PIA > CGS 21680, which is characteristic of A_{2B} receptors (Collis & Hourani, 1993; Feoktistov & Biaggioni, 1997). Furthermore, the potency ratios relative to NECA for NECA, adenosine, CPA, R-PIA, S-PIA and CGS21680 observed here were 1, 0.15, 0.06, 0.07, 0.02 and <0.01 respectively. These compare favourably with values from two separate studies with similar adenosine analogues at A_{2B} receptors in the guinea-pig aorta. Thus, Martin (1992) found values of 1, 0.16, 0.03, and <0.001 for NECA, adenosine, R-PIA and CGS21860 respectively, whilst in the same tissue, Gurden *et al*. (1993) reported values of 1, 0.03, 0.03, 0.003 and 0.002 for NECA, CPA, R-PIA, S-PIA and CGS21860 respectively.

It is unlikely that A_3 receptors mediated the response to adenosine (Hannon *et al*., 1995) in the human small coronary artery given the low potency of the A_3-selective agonist, IB-MECA, compared with its affinity of 1 nM at rat A_3 receptors (Jacobson *et al*., 1995) and A_3 receptors are resistant to inhibition by the A_1, A_2 non-selective antagonist, 8-SPT (10 μM; Hannon *et al*., 1995). 8-SPT, however, antagonized the response to adenosine with an estimated affinity of 5.9 which is similar to that reported for 8-SPT at A_{2B} receptors in the guinea-pig aorta ($pK_B = 5.3$; Hargreaves *et al*., 1991). Furthermore, the A_2 antagonist, DMPX, inhibited the relaxation curve to adenosine in an apparent competitive manner yet in contrast the selective A_1 antagonist, DPCPX, failed to have any effect. Therefore, our findings indicate that A_{2B} receptors mediate relaxation to adenosine in human small coronary arteries. This is in contrast to substantial published literature which demonstrates that adenosine mediates most of its vasodilator responses *via* A_{2A} receptors (Ongini & Fredholm, 1996; Feoktistov & Biaggioni, 1997; Shryock & Belardinelli, 1997). For example, A_{2A} receptors appear to mediate relaxation to adenosine in large coronary arteries of the pig (Conti *et al*., 1997), cow (Conti *et al*., 1993) and vasodilation in the isolated perfused guinea-pig heart (Vials & Burnstock, 1993; Poucher *et al*., 1995; Belardinelli *et al*., 1996, 1998; Shryock *et al*., 1998). Also, although an undefined A_2 receptor has been reported in human large coronary arteries, it was not further subtyped as either A_{2A} or A_{2B} (Ramagopal *et al*., 1988).

In the present study, we confirmed our earlier finding in similar human small coronary arteries that endothelium-dependent relaxation to bradykinin was largely blocked by combined L-NOARG and HbO treatment (Kemp & Cocks, 1997). Therefore, the inability of the same NO inhibitors to block relaxation to adenosine in adjacent, similar-sized arteries suggests that adenosine did not mediate its effect *via* either NO or the endothelium. Also, it was unlikely that any endothelial A_{2A}-mediated responses were masked by functional antagonism caused by the presence of SNP to inhibit spontaneous activity since CGS 21680 was still virtually inactive in tissues treated with isoprenaline instead of SNP to control spontaneous activity (Kemp & Cocks, unpublished data). Similar endothelium-independent vasodilator responses to adenosine in the rat mesenteric bed as those described here have also been reported to be mediated by A_{2B} receptors (Rubino *et al*., 1995). The direct smooth muscle relaxing effect of adenosine, however, appeared to involve K^+ channels and thus a hyperpolarization-dependent mechanism given that it was partially inhibited by high extracellular K^+. In coronary vascular preparations where K_{ATP} have been implicated in relaxation to adenosine *via* hyperpolarization (Randall, 1995; Bouchard & Lamontagne, 1996; Nakae *et al*., 1996; Mutafovayambolieva & Keef, 1997), the opening of these channels is thought to be regulated by changes in intracellular levels of cyclic AMP (Miyoshi & Nakaya, 1993; Kleppisch & Nelson, 1995; Feoktistov & Biaggioni, 1997). Our data, however, indicate that cyclic AMP-regulated K_{ATP} do not underlie relaxation to adenosine in the human small coronary arteries since the response was refractory to glibenclamide and relaxation to forskolin was unaffected by high extracellular K^+. A role for other K^+ channels such as inward rectifying (K_{IV}), voltage-dependent (K_V) and Ca^{2+}-activated K^+ channels (K_{Ca}), however, cannot be excluded since Cabell *et al*. (1994) found that tetraethylammonium (TEA) and the more selective inhibitor of large-conductance K_{Ca}, iberiotoxin, blocked adenosine-induced relaxation in canine coronary arteries. Thus, adenosine may cause smooth muscle relaxation, in part, *via* the opening of K_{Ca} channels as has been reported in large coronary arteries of the pig and human (Olanrewaju *et al*., 1995) and small coronary arteries of the dog (Cabell *et al*., 1994).

In conclusion, the present study provides strong circumstantial evidence that relaxation of human small resistance-like coronary arteries by adenosine is mediated by A_{2B} receptors which are coupled to K^+ channels to mediate part of the response. A more definitive demonstration that A_{2B} receptors mediate coronary vasodilatation to adenosine in humans, however, awaits the development of selective A_{2B} receptor antagonists.

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