Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 12441–12445, December 1995
Physiology

Adenosine activates ATP-sensitive potassium channels in arterial myocytes via A_2 receptors and cAMP-dependent protein kinase

(vasodilation/glibenclamide/hypoxia/P1 receptors)

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Communicated by C. Ladd Prosser, Marine Biological Laboratory, Woods Hole, MA, August 30, 1995

ABSTRACT **The mechanism by which the endogenous vasodilator adenosine causes ATP-sensitive potassium (K_{ATP}) channels in arterial smooth muscle to open was investigated by the whole-cell patch-clamp technique. Adenosine induced voltage-independent, potassium-selective currents, which were inhibited by glibenclamide, a blocker of K_{ATP} currents. Glibenclamide-sensitive currents were also activated by the selective adenosine A_2-receptor agonist 2-*p*-(2-carboxethyl)-phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS-21680), whereas 2-chloro-N^6-cyclopentyladenosine (CCPA), a selective adenosine A_1-receptor agonist, failed to induce potassium currents. Glibenclamide-sensitive currents induced by adenosine and CGS-21680 were largely reduced by blockers of the cAMP-dependent protein kinase (Rp-cAMP[S], H-89, protein kinase A inhibitor peptide). Therefore, we conclude that adenosine can activate K_{ATP} currents in arterial smooth muscle through the following pathway: (*i*) Adenosine stimulates A_2 receptors, which activates adenylyl cyclase; (*ii*) the resulting increase in intracellular cAMP stimulates protein kinase A, which, probably through a phosphorylation step, opens K_{ATP} channels.**

Adenosine is a potent endogenous vasodilator and is thought to play a major role in adjusting blood flow to metabolic demands (1). Increased adenosine release during hypoxia/reperfusion appears to account for dilations in cerebral, coronary, and retinal arteries (2–6), although this issue is somewhat controversial (cf. ref. 7). Adenosine has also been shown to hyperpolarize arterial smooth muscle (8). Vasodilations as well as hyperpolarizations induced by adenosine are partially reversed by glibenclamide (GLIB), a blocker of ATP-sensitive potassium (K_{ATP}) channels (refs. 8 and 9; for review, see refs. 10 and 11). Therefore, it was suggested that the vasodilatory response to adenosine is at least partly due to activation of smooth muscle K_{ATP} channels. More recently, Dart and Standen (12) provided direct evidence for activation of K_{ATP} channels by adenosine in porcine coronary artery myocytes.

The signal transduction pathway between adenosine receptor(s) and K_{ATP} channels in vascular smooth muscle is not known. K_{ATP} channels in smooth muscle from mesenteric arteries and gallbladder can be activated through stimulation of cAMP-dependent protein kinase A (PKA) (13, 14). However, in coronary artery myocytes, adenosine activates K_{ATP} channels via A_1 receptors (12), which can inhibit adenylyl cyclase (15). A_1 receptor-mediated activation of K_{ATP} currents has been described in cardiac myocytes and has been related to a direct effect of pertussis toxin-sensitive guanine nucleotide binding proteins (G proteins), specifically of the G_i family, on the channel (16). Vasodilatory effects of adenosine, however, have been reported to be pertussis toxin insensitive (17, 18), arguing against a role of G_i protein. Adenosine-induced vasodilations often appear to involve A_2 receptors and accumulation of cAMP (19–23).

Here we provide information on the signal transduction pathway linking adenosine receptors to K_{ATP} channels in arterial smooth muscle. Our results indicate that adenosine acts via A_2 receptors and PKA to stimulate K_{ATP} currents in smooth muscle cells from rabbit mesenteric artery.

MATERIALS AND METHODS

Cell Isolation. The tissue dissection and cell isolation procedures were slightly modified from the procedures described by Quayle *et al.* (13). Briefly, male New Zealand White rabbits (2–3 kg; 3–6 wk old) were anesthetized with sodium pentobarbitol (30 mg·kg^{-1}, i.v.) and exsanguinated. Mesenteric arteries were dissected and put in ice-cold solution containing 137 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 4.17 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 0.44 mM NaH_2PO_4, 5 mM glucose, and 10 mM Hepes (pH 7.4). The arteries were cleaned of connective tissue, cut in four to six pieces, and transferred into a Ca-free isolation solution of the following composition: 60 mM NaCl, 85 mM sodium glutamate, 5.6 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes (pH 7.4). After 10 min, the tissue was placed into the first of two enzyme solutions with the same ionic composition as the Ca-free isolation solution described above, containing 1 mg·ml^{-1} of albumin (Sigma), 0.7 mg·ml^{-1} of papain (Worthington), 1 mg·ml^{-1} of dithioerythritol (Sigma), and digested for 40–50 min at 37°C. The tissue was then transferred into the second enzyme solution containing 0.1 mM $CaCl_2$, 1 mg·ml^{-1} of albumin, 1 mg·ml^{-1} of collagenase type H (Sigma), 1 mg·ml^{-1} of hyaluronidase (Sigma), and digested for another 10–15 min at 37°C. The tissue was then washed in Ca-free fresh isolation solution without enzymes for 10 min before single smooth muscle cells were obtained by gentle trituration with a polished wide-bore pipette. After trituration, cells were stored in the same solution at 4°C to be used the same day. Cells were left to stick to the glass coverslip in the experimental chamber for 15–20 min before starting an experiment.

Electrophysiological Recordings. Potassium currents were measured in the conventional whole-cell configuration of the patch-clamp technique (24) using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Cell capacitance and series access resistance (R_S) were compensated manually. Cells with R_S exceeding 15 MΩ were discarded. The cell capacitance was 14.0 ± 0.4 pF (n = 68). The bathing (external) solution contained 80 mM NaCl, 60 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes (pH 7.4) unless stated otherwise. The pipette (internal) solution contained 102

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Abbreviations: GLIB, glibenclamide; K_{ATP} channel, ATP-sensitive potassium channel; PKA, cAMP-dependent protein kinase A; G protein, guanine nucleotide binding protein; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS-21680, 2-*p*-(2-carboxethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride.

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mM KCl, 38 mM KOH, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 0.1 mM ATP, 0.1 mM ADP, 0.2 mM GTP, 10 mM glucose, and 10 mM Hepes (estimated free concentrations: ATP, 10 μM; Ca^{2+}, 18.7 nM; Mg^{2+}, 0.64 mM; pH 7.2 at 23°C). Under these conditions, the potassium equilibrium potential (E_K), sodium equilibrium potential, and chloride equilibrium potential were −21.6, +55.0, and −6.5 mV, respectively. For some experiments the extracellular potassium was reduced to 20 mM by an equimolar substitution of KCl with NaCl shifting E_K to −49.9 mV. All experiments were conducted at room temperature (23°C). Whole-cell currents were low-pass filtered with a cut-off frequency of 2 Hz and recorded with a sampling frequency of 10 Hz. For analysis, the current amplitude was estimated as the average between two cursors selecting an area of interest. Ion selectivity and voltage dependency of GLIB-sensitive currents in the presence of adenosine were examined using 200-ms voltage-clamp ramps from −100 to +50 mV. Currents evoked during ramp pulses were low-pass filtered with a cut-off frequency of 2 kHz and recorded with a sampling frequency of 10 kHz. Axotape and pCLAMP software (Axon Instruments) were used for digitizing and analyzing all currents.

To minimize any activity of voltage-dependent and large conductance calcium-activated potassium channels, experiments were performed at negative membrane potentials (−70 mV) with intracellular Ca^{2+} buffered to ≈20 nM, while extracellular Ca^{2+} (0.1 mM) was also low. To enhance the amplitude of potassium currents at hyperpolarized potentials, external potassium was raised to 60 mM. Potassium currents are, therefore, inward and seen as downward deflections. Inward rectifier potassium currents were not detected in smooth muscle cells from mesenteric arteries and appear to be present only in smaller arteries (25).

Chemicals. 2-Chloro-N^6-cyclopentyladenosine (CCPA), 2-*p*-(2-carboxethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS-21680), and pinacidil were purchased from Research Biochemicals. Rp-cAMP[S] was obtained from Biolog (La Jolla, CA). All other chemicals and reagents were obtained from Sigma. For experiments with pinacidil and GLIB, solutions were made from a 10 mM stock with 50% ethanol and 50% dimethyl sulfoxide. Adenosine, CCPA, CGS-21680, and tetraethylammonium chloride were made from concentrated (1000-fold) aqueous stock solutions.

Statistics. Results are expressed as means ± SEM of *n* cells. The unpaired *t* test and Wilcoxon test were used for statistical analysis. The results were obtained from 95 cells of 42 animals.

RESULTS

Adenosine Activates GLIB-Sensitive Potassium Currents. Membrane currents were recorded in myocytes from rabbit mesenteric artery dialyzed with a solution containing 0.1 mM ATP, 0.1 mM ADP, and 140 mM potassium. The membrane potential was −70 mV and external potassium was 60 mM. Under these conditions, adenosine (5 μM) evoked inward currents that were blocked by GLIB (10 μM) (Fig. 1*A*), a selective inhibitor of K_{ATP} channels in pancreatic β-cells and in cardiac, skeletal, and smooth muscle (11, 26). Adenosine (5 μM) increased GLIB-sensitive currents from −10.4 ± 1.6 pA (current density, −0.82 ± 0.06 pA/pF) (n = 37) to −43.2 ± 3.4 pA (current density, −3.23 ± 0.29 pA/pF) (n = 64) or 4-fold.

K_{ATP} channels from mesenteric artery smooth muscle are potassium selective, voltage independent, and blocked by millimolar concentrations of tetraethylammonium ions (TEA^+) (13, 27). The ionic selectivity and voltage dependency of adenosine-induced currents were examined using 200-ms voltage-clamp ramps from −100 to +50 mV. Currents evoked during ramp pulses were recorded with 20 and 60 mM external potassium. The estimated potassium equilibrium potentials

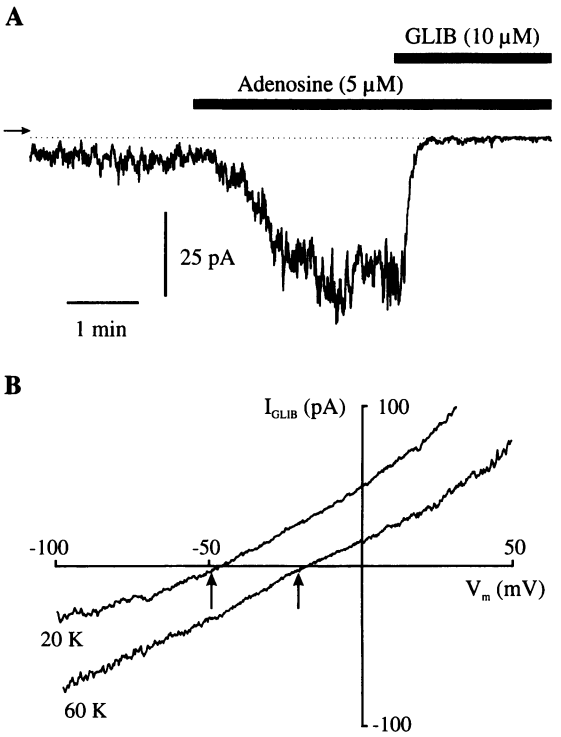

FIG. 1. Adenosine induces GLIB-sensitive, voltage-independent potassium currents in smooth muscle cells from mesenteric artery. (*A*) Original record illustrating the effect of adenosine (5 μM) on whole-cell currents. External potassium was 60 mM, while the cell was dialyzed with 0.1 mM ATP, 0.1 mM ADP, and 140 mM potassium. The holding potential was −60 mV. Zero-current level and the current in the presence of GLIB (10 μM) are indicated by arrow and dotted line, respectively. (*B*) Voltage dependence of adenosine-induced currents. Whole-cell currents were recorded during 200-ms voltage-clamp ramps from −100 to +50 mV. Pipette (internal) solution was the same as in *A*. GLIB-sensitive currents in the presence of adenosine (i.e., difference between currents in the presence of 5 μM adenosine vs. currents in the presence of 5 μM adenosine plus 10 μM GLIB) using 20 and 60 mM extracellular potassium are shown. Arrows indicate theoretical potassium equilibrium potentials with 20 and 60 mM external potassium. Reversal potentials of adenosine-induced currents in 20 and 60 mM potassium were −40.5 ± 1.8 mV (n = 5) and −15.7 ± 0.9 mV (n = 5).

(E_K) with 20 and 60 mM external potassium (internal potassium, 140 mM) were −49.9 and −21.6 mV (arrows in Fig. 1*B*), respectively. The reversal potentials of the GLIB-sensitive currents in the presence of adenosine changed with E_K (Fig. 1*B*), indicating that adenosine-induced currents were through potassium-selective channels. Adenosine-induced GLIB-sensitive currents with 60 mM external potassium were essentially linear over the voltage range from −100 to +50 mV (Fig. 1*B*), which suggests that the open state probability of adenosine-activated potassium channels is voltage independent. Extracellular TEA^+ inhibited adenosine-induced potassium currents in mesenteric artery myocytes: 1, 10, and 20 mM concentrations of this potassium channel blocker reduced GLIB-sensitive currents in the presence of adenosine by 20.7% ± 5.3% (n = 7), 58.5% ± 5.6% (n = 6), and 66.4% ± 6.8% (n = 3), respectively. For comparison, TEA^+ reduced pinacidil-activated K_{ATP} currents in this preparation with a half-block constant of 6.2 mM (27). These results indicate that adenosine activates K_{ATP} channels in smooth muscle cells from mesenteric artery.

Activation of K_{ATP} Currents by the Adenosine A_2 Receptor Agonist CGS-21680. To address the issue of which type of adenosine receptor is involved in activation of K_{ATP} channels, the effects of adenosine receptor agonists on whole-cell po-

tassium currents were examined. The A_1-selective receptor agonist CCPA at 100–250 nM did not increase GLIB-sensitive currents (n = 13) (Fig. 2). CCPA is ≈10,000-fold selective for adenosine A_1 vs. A_2 receptors (K_i, 0.4 and 3900 nM) (for review, see refs. 15 and 28 and references cited therein). In fact, CCPA slightly reduced GLIB-sensitive currents in 3 of 13 cells (see Fig. 2*A*).

The A_2-selective receptor agonist CGS-21680 (200–500 nM) increased GLIB-sensitive currents to −40.5 ± 5.7 pA (n = 12) or 4-fold (Fig. 2). CGS-21680 possesses a 170-fold selectivity for A_2 over A_1 adenosine receptors (K_i, 15 and 2600 nM) (for review, see refs. 15 and 28 and references cited therein). Adenosine, when tested in the same cells, did not significantly alter K_{ATP} currents induced by CGS-21680 (Fig. 2) (mean GLIB-sensitive current in the presence of adenosine, −46.7 ± 7.1 pA; n = 16). These results indicate that adenosine acts through A_2 receptors to stimulate K_{ATP} channels in mesenteric artery.

Inhibition of Adenosine-Induced Currents by the cAMP Analog Rp-cAMP[S] and the Protein Kinase Inhibitors H-89 and PKI-(5–24)amide. The finding that adenosine activates GLIB-sensitive currents *via* A_2 receptors suggests a role for elevation of intracellular cAMP and subsequent activation of the PKA. Another vasodilator, calcitonin gene-related peptide, has been shown to activate K_{ATP} currents in vascular and nonvascular smooth muscle through this pathway (13, 14).

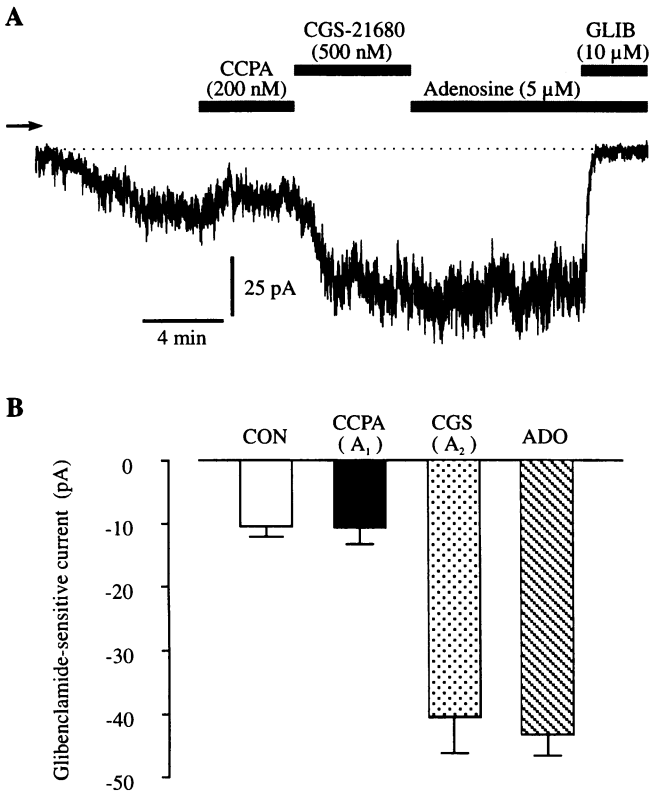

FIG. 2. GLIB-sensitive currents in mesenteric artery smooth muscle are activated by the adenosine A_2 receptor agonist CGS-21680 but not by the adenosine A_1 receptor agonist CCPA. (*A*) Original record illustrating effects of CCPA (200 nM), CGS-21680 (500 nM), and adenosine (5 μM) on membrane currents. Same conditions as in Fig. 1. Zero-current level and the current in the presence of GLIB (10 μM) are indicated by arrow and dotted line, respectively. (*B*) Summarized data of GLIB-sensitive currents recorded in the presence of CCPA (200 nM; n = 13), CGS-21680 (CGS; 500 nM; n = 12), and adenosine (ADO; 5 μM; n = 16). GLIB-sensitive currents in the absence of agonists (control currents) (CON; n = 37) were estimated from the difference between the current in the absence of any adenosine receptor agonist and the current in the presence of GLIB with a receptor agonist present.

Diastereomers of adenosine-3′,5′-cyclic monophosphorothioate (cAMP[S]), which are membrane permeable and highly resistant to cyclic nucleotide phosphodiesterases, can be used to study the role of cAMP and PKA. Sp-cAMP[S] activates K_{ATP} currents in arterial smooth muscle (13). Rp-cAMP[S], which competes for the cAMP binding sites at the regulatory subunits and prevents dissociation of the PKA holoenzyme into catalytic and regulatory subunits, inhibited K_{ATP} currents activated by adenosine (Fig. 3). On average, Rp-cAMP[S] (100–500 μM) reduced GLIB-sensitive currents in the presence of adenosine by 50.3% (from −62.6 ± 18.1 to −31.1 ± 2.7 pA; n = 5). This finding suggests that adenosine activates K_{ATP} currents by elevating intracellular cAMP and thereby stimulating PKA.

To provide additional support for this mechanism, the effects of two other PKA inhibitors, H-89 and the PKI-(5–24)amide, which bind to the active center of the catalytic subunits and suppress kinase activity, were examined. H-89 (1 μM), extracellularly applied, effectively inhibited GLIB-sensitive currents induced by adenosine (from −43.2 ± 5.8 to −14.4 ± 2.9 pA; n = 10) as well as by the A_2 receptor agonist CGS-21680 (from −25.4 ± 5.1 to −7.0 ± 1.1 pA; n = 4) (Fig. 4 *A*, *B*, and *D*). Unlike adenosine-induced currents, GLIB-sensitive currents activated by the synthetic potassium channel opener pinacidil (5 μM), which is thought to directly open K_{ATP} channels, were not significantly reduced by the protein kinase inhibitor (−90.8 ± 16.1 pA in the absence of H-89, −76.6 ± 4.5 pA in the presence of H-89; n = 8) (Fig. 4 *C* and *D*). This observation argues against a nonspecific effect of H-89 (see also ref. 13). Adenosine (5 μM) activation of K_{ATP} currents was completely abolished in seven cells dialyzed for ≈10 min with a pipette (internal) solution containing the specific inhibitor peptide PKI-(5–24)amide (4 μM) (Fig. 5). GLIB-sensitive currents in cells dialyzed with the kinase inhibitor peptide were, however, still activated by pinacidil (Fig. 5). These results suggest that adenosine and the A_2 adenosine receptor agonist CGS-21680 open K_{ATP} channels through stimulation of PKA.

DISCUSSION

Adenosine is thought to play an important role in hypoxic vasodilation and ischemia/reperfusion-induced hyperemia (2, 4, 6, 29). Adenosine appears to act in part through stimulation of K_{ATP} channels in vascular smooth muscle, since vasodilatory effects of adenosine are often attenuated by GLIB (7, 8). Adenosine-induced stimulation of large conductance calcium-activated potassium channels may also contribute to observed smooth muscle relaxations (see, e.g., refs. 30–32). Activation of

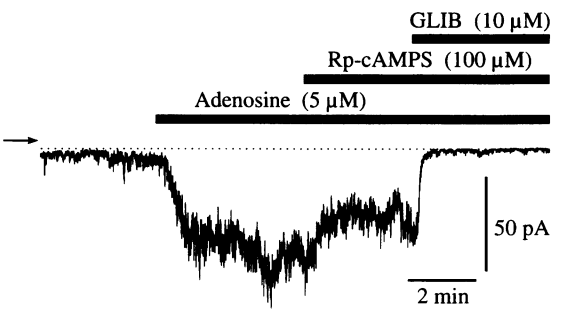

FIG. 3. The cAMP analog Rp-cAMP[S] inhibits adenosine-induced GLIB-sensitive currents. Original records illustrating the effect of Rp-cAMP[S] (100 μM) on the GLIB-sensitive current in the presence of adenosine (5 μM). Rp-cAMP[S] was applied externally. Same conditions as in Fig. 1. Zero-current level and the current in the presence of GLIB (10 μM) are indicated by arrow and dotted line, respectively.

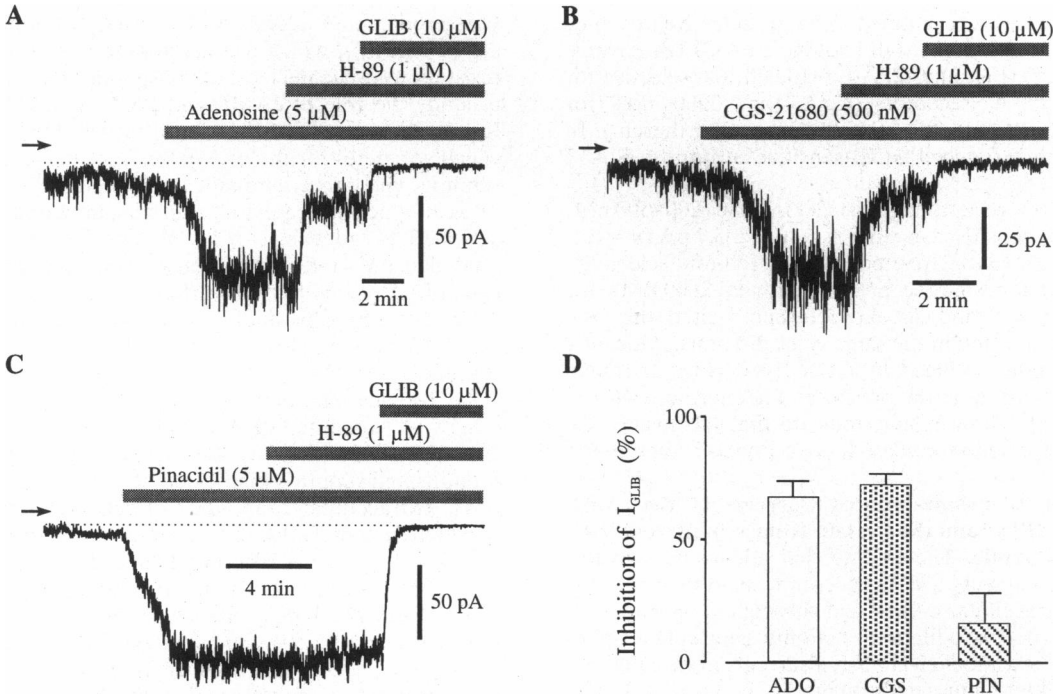

FIG. 4. Blocker of cAMP-dependent protein kinase H-89 inhibits currents induced by adenosine and A_2 receptor agonists. Original records illustrating the effect of externally applied H-89 (1 μM) on the GLIB-sensitive current in the presence of adenosine (5 μM) (*A*), CGS-21680 (500 nM) (*B*), and the K_{ATP} channel opener pinacidil (5 μM) (*C*). Same conditions as in Fig. 1. Zero-current level and the current in the presence of GLIB (10 μM) are indicated by arrow and dotted line, respectively. (*D*) Summary of the inhibitory effect of H-89 on GLIB-sensitive currents recorded in the presence of adenosine (ADO; 5 μM; $n = 10$), CGS-21680 (CGS; 0.5 μM; $n = 4$), and pinacidil (PIN; 5 μM; $n = 8$). Whereas reduction in currents activated by adenosine and CGS-21680 was statistically significant ($P < 0.01$), pinacidil-induced currents were not significantly decreased by H-89.

potassium channels causes membrane potential hyperpolarization, which leads to vasodilation by closing voltage-dependent calcium channels (11, 33). The goal of this study was to elucidate the mechanism by which adenosine stimulates smooth muscle K_{ATP} channels. Here we provide evidence that adenosine activates K_{ATP} channels in arterial smooth muscle through the following signal transduction pathway: (*i*) Stimulation of A_2 receptors leads to activation of adenylyl cyclase and elevation of intracellular cyclic AMP ($[cAMP]_i$). (*ii*) Increase in $[cAMP]_i$ results in activation of PKA. (*iii*) PKA activates K_{ATP} currents, presumably by phosphorylating the channel or an associated regulatory protein.

The current nomenclature of adenosine (P1) purinergic receptors includes four classes (A_1, A_2, A_3, A_4) (15, 28). Vasodilation to adenosine has been mostly attributed to A_2 receptors (for review, see refs. 15 and 28). A_1 receptors are linked to the pertussis toxin-sensitive G protein G_i and inhibit adenylyl cyclase. A_2 receptors, in contrast, couple to the pertussis toxin-insensitive, cholera toxin-sensitive G protein G_S, which activates adenylyl cyclase. Stimulation of K_{ATP} current by adenosine was described first in cardiac myocytes, where it is mediated through A_1 adenosine receptors and a membrane delimited effect of G proteins, specifically of the G_i family (16). A similar mechanism has been proposed for smooth muscle cells from porcine coronary artery (12). Our data, however, show that A_1 adenosine receptors do not mediate activation of K_{ATP} channels in mesenteric artery smooth muscle. A_3 and A_4 receptors are also unlikely to be involved, since these receptors possess very low affinities for the agonist CGS-21680 (15, 28), which was effective in mesenteric artery myocytes. Our findings are in accord with reports that adenosine relaxes mesenteric arteries through A_2 receptors (23) and stimulates adenylyl cyclase in cultured smooth muscle cells from this preparation (21). The PKA-inhibitor peptide completely blocked adenosine activation of K_{ATP} currents in mesenteric artery myocytes (Fig. 5), arguing against a role of direct G-protein activation of K_{ATP} channels in this preparation. The PKA inhibitor H-89 applied externally reduced the GLIB-sensitive potassium currents in the presence of adenosine to a level (≈14 pA) similar to the level observed in the absence of adenosine (≈12 pA). This suggests that H-89 effectively blocked the adenosine-induced current. However, Rp-cAMP[S] was less effective in inhibiting the adenosine-induced current, perhaps reflecting competition between Rp-cAMP[S] and endogenous cAMP for the binding site on PKA.

Smooth muscle cells from coronary artery have been reported to express both A_1 and A_2 adenosine receptors (34). Pharmacological studies in arterial smooth muscle, including coronary artery, have suggested that adenosine-induced relaxations are largely mediated by A_2 receptors (20, 23, 35–37; for review, see refs. 15 and 28; however, see also ref. 38). Adenosine-induced relaxations of coronary arteries are asso-

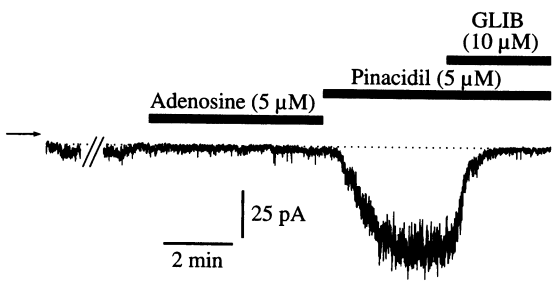

FIG. 5. Specific PKA inhibitor peptide PKI-(5–24)amide abolishes adenosine-induced currents in mesenteric arterial smooth muscle cells. Original record illustrating effects of adenosine (5 μM) and pinacidil (5 μM) in cells dialyzed with a pipette (intracellular) solution containing the PKA inhibitor PKI-(5–24)amide (4 μM). Same conditions as in Fig. 1. Zero-current level and current in the presence of GLIB (10 μM) are indicated by arrow and dotted line, respectively.

ciated with an elevation in intracellular cAMP and activation of PKA (19, 22) as expected for activation of A_2 receptors. Furthermore, if stimulation of A_1 receptors was significant in coronary arteries this could lower cAMP and tend to oppose vasorelaxation. Our study supports the idea that adenosine-induced GLIB-sensitive vasodilations in various vascular beds, including coronary arteries, are due to stimulation of K_{ATP} channels mediated by A_2 receptors, elevation of cAMP, and activation of PKA.

A number of other endogenous vasodilators, including calcitonin gene-related peptide (39), vasoactive intestinal peptide (40), prostaglandins (9, 41), and β_1-adrenoceptor agonists (42, 43), have been shown to act in part through a GLIB-sensitive mechanism. These smooth muscle relaxants are also known to stimulate adenylyl cyclase and elevate intracellular cAMP (44–46). Calcitonin gene-related peptide has been previously shown to activate K_{ATP} channels in arterial as well as gallbladder smooth muscle via cAMP-dependent protein kinase (13, 14). We therefore propose that adenosine and various other vasodilators converge on a common intracellular pathway leading to membrane hyperpolarization and ultimately vasodilation.

We would like to thank Drs. A. Bonev for valuable discussions on the project and Harm Knot for helpful comments on the manuscript and Mr. A. Stevenson for technical assistance. T.K. is a Feodor-Lynen Fellow of the Alexander von Humboldt-Stiftung. This work was supported by grants from the National Institutes of Health (HL-44455 and HL-51728) and the National Science Foundation (DCB-9019563).

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