

Adenosine-A2a Receptor Down-Regulates Cerebral Smooth Muscle L-Type Ca^{2+} Channel Activity via Protein Tyrosine Phosphatase, Not cAMP-Dependent Protein Kinase

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ABSTRACT

Adenosine acting via A2a receptors (A2aR) is a potent cerebral vasodilator that relaxes vascular smooth muscle cells (VSMCs) by a mechanism attributed to activation of cAMP-dependent protein kinase (cAK). We examined effects of adenosine and its mechanism of action on L-type Ca^{2+} channels in native VSMCs from rat basilar artery. Reverse transcription-polymerase chain reaction and immunofluorescence imaging confirmed transcription and expression of A2aR, and in situ hybridization confirmed presence of mRNA for L-type $\text{Ca}_{v1.2b}$ channels. In patch-clamp experiments, adenosine down-regulated Ca^{2+} channel currents in a concentration-dependent manner, with receptor-subtype-specific antagonists [4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM-241385) versus 1,3-dipropyl-8-cyclopentyl-1,3-dipropylxanthine] showing that this was caused by action of A2aR. Down-regulation of channel currents was mimicked by stimulation of cGMP-dependent protein kinase (cGK; 8-Br-cGMP) and by inhibition of tyrosine kinase (AG-18) but not by stimu-

lation of cAK [forskolin and 8-bromo-cAMP (8-Br-AMP)]. Down-regulation of currents by the A2aR agonist 2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethylolcarboxamidoadenosine (CGS-21680) was blocked by inhibiting protein tyrosine phosphatase (PTP; orthovanadate and dephostatin), but not by inhibiting cGK (KT-5823 and H-7). Western blots of lysate or of immunisolated Ca^{2+} channels from arterial segments incubated with CGS-21680 showed 1) increased phosphorylation of vasodilator-stimulated phosphoprotein that was blocked by inhibiting cAK (KT-5720), consistent with activation of cAK by A2aR; and 2) decreased tyrosine phosphorylation of immunisolated $\alpha 1c$ subunit of the Ca^{2+} channel. Our data show that cAK, although activated, was not germane to down-regulation of Ca^{2+} channel activity by A2aR, and they delineate a novel signaling mechanism involving reduced tyrosine phosphorylation of Ca^{2+} channels by A2aR probably caused by PTP activation.

Adenosine is an important vasodilator that regulates cerebrovascular tone in both physiological and pathological conditions such as hypoxia and ischemia (Winn et al., 1981; Rudolph et al., 1992). Two vasodilatory mechanisms of aden-

osine have been broadly defined in various circulatory beds. One involves endothelial receptors, whose occupation results in activation of nitric-oxide synthase (NOS), release of NO from endothelium, and activation of cGMP-dependent protein kinase (cGK) in vascular smooth muscle cells (VSMCs), a mechanism that has been well delineated in coronary vessels (Hein and Kuo, 1999) but that may not be active in the cerebral circulation (West et al., 2003). The second mecha-

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ABBREVIATIONS: NOS, nitric-oxide synthase; cGK, cGMP-dependent protein kinase; VSMC, vascular smooth muscle cell; A2aR, adenosine A2a receptor; cAK, cAMP-dependent protein kinase; PTP, protein tyrosine phosphatase; TEA, tetraethylammonium; A1R, adenosine A1 receptor; DPCPX, 1,3-dipropyl-8-cyclopentyl-1,3-dipropylxanthine; (R)-PIA, (R)-(-)-N⁶-(2-phenylisopropyl) adenosine; 8-Br-cAMP, 8-bromo-cAMP; TK, tyrosine kinase; Na₃VO₄, sodium orthovanadate; RT-PCR, reverse-transcription-polymerase chain reaction; MOPS, 3-(N-morpholino)propanesulfonic acid; VASP, vasodilator-stimulated phosphoprotein; PKC, protein kinase C; Bay k8644, S-(-)-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)-3-pyridine carboxylic acid methyl ester; ZM-241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol; CGS-21680, 2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethylolcarboxamidoadenosine; KT-5720, (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester; KT-5823, (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid methyl ester; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; AG18 (tyrphostin 23), 3,4-dihydroxybenzylidene malononitrile.

nism involves direct activation of receptors on VSMCs resulting in endothelium-independent vasorelaxation. Signaling via this second mechanism remains poorly understood, particularly in the cerebral circulation.

L-type Ca²⁺ channels comprise the most important and highly regulated routes of entry of Ca²⁺ into VSMCs and are clearly implicated in mechanisms of vasoconstriction, with down-regulation of channel activity typically being associated with vasorelaxation. For NO, another small molecule critical for cerebral vasorelaxation, signaling that results in vasorelaxation and that is associated with down-regulation of L-type Ca²⁺ channel activity has been well described (Simard and Li, 2000; Gerzanich et al., 2001). In contrast, a comparable role for adenosine in regulating Ca²⁺ channels has not been reported.

Four distinct adenosine receptors have been identified by molecular cloning (Fredholm et al., 2000; Klinger et al., 2002). Vasodilation in cerebral as well as in other circulations is mediated principally by A2a receptors (A2aR), with A2b but not A1 or A3 receptors also possibly involved (Coney and Marshall, 1998; Shin et al., 2000; Ngai et al., 2001). The importance of A2aR in regulating vascular tone has been confirmed in A2aR-knockout mice (Ledent et al., 1997; Chen et al., 1999). A2aR belong to the family of G-protein-coupled receptors that transfer signals by activating heterotrimeric G proteins (Fredholm et al., 2000; Klinger et al., 2002). A2aR are typically associated with G_s and activation of adenylate cyclase, resulting in accumulation of cAMP and activation of cAMP-dependent protein kinase (cAK). However, A2aR can also activate other G proteins (Fredholm et al., 2000; Klinger et al., 2002), although any role for such noncanonical pathways in vasorelaxation is undetermined.

Activation of the cAK pathway may be associated with relaxation of vascular smooth muscle, but several difficulties exist with attributing A2aR-mediated vasorelaxation exclusively to activation of cAK in VSMCs. First, A2aR-mediated vasorelaxation may not be fully reproduced by activation of cAK with 8-bromo-cAMP (Hong et al., 1999). Second, inhibitors of cAK may not abolish A2aR-mediated vasorelaxation, even though they completely block relaxation caused by forskolin or 8-bromo-cAMP (Hein et al., 2001; West et al., 2003). Third, activation of cAK in VSMCs is not typically associated with down-regulation of L-type Ca²⁺ channel activity, and indeed, in some vascular beds, cAK activation may actually cause up-regulation of VSMC Ca²⁺ channel activity (Tewari and Simard, 1994), an effect that would be expected to oppose A2aR-mediated vasorelaxation.

In the present study, we examined A2aR signaling and regulation of L-type Ca²⁺ channels in native VSMCs from rat basilar artery. Here, we report that adenosine down-regulated activity of VSMC L-type Ca²⁺ channels, that down-regulation occurred via activation of A2aR, that cAK was activated, but that cAK activation was not responsible for down-regulation of channel activity. Rather, our data indicate that down-regulation of channel activity was caused by reduced tyrosine phosphorylation of the channel, probably caused by activation of protein tyrosine phosphatase (PTP). A2aR-mediated down-regulation of L-type Ca²⁺ channels caused by reduced tyrosine phosphorylation is a novel finding.

Materials and Methods

Patch Clamp. Experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. VSMCs from basilar arteries of adult female Wistar rats were isolated by enzymatic dispersion as described previously (Simard and Li, 2000). Patch-clamp experiments were carried out within 2 to 10 h of cell harvest. The methods used for nystatin-perforated patch recording in this laboratory have been described previously (Simard and Li, 2000). For recordings of macroscopic Ca²⁺ channel currents, the bath solution contained 130 mM TEA-Cl, 1 mM MgCl₂, 10 mM BaCl₂, 10 mM HEPES, 12.5 mM glucose, and 2 mM 4-aminopyridine, pH adjusted to 7.2 with TEA-OH, and the pipette solution contained 130 mM CsCl, 8 mM MgCl₂, and 10 mM HEPES, pH adjusted to 7.35 with CsOH plus nystatin. For cell-attached patch recordings, the pipette contained 100 mM TEA-Cl, 1 mM MgCl₂, 40 mM BaCl₂, 10 mM HEPES, 12.5 mM glucose, and 2 mM 4-aminopyridine, pH 7.2 with TEA-OH, and the bath contained 145 mM KCl, 2 mM MgCl₂, 10 mM HEPES, and 12.5 mM glucose, pH adjusted to 7.4 with NaOH. Pipette resistance was 1.5 to 2.5 MΩ. With nystatin, access resistance was usually ≈20 MΩ, and cells were discarded if it exceeded 60 MΩ. Cell membrane resistance in physiological saline and in the recording solution (Ba²⁺/TEA) was 1 to 3 GΩ and ≈3 to 8 GΩ, respectively. Cell capacitance was 16 ± 2 pF. All patch-clamp experiments were performed at room temperature, 22 to 25°C.

Enzymes used for cell isolation, and other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) or from Fisher Scientific Co. (Pittsburgh, PA). The following pharmacological agents were used: the adenosine type 1 receptor (A1R) antagonist 1,3-dipropyl-8-cyclopentyl-1,3-dipropylxanthine (DPCPX; Tocris Cookson, Bristol, UK); the A1R agonist (*R*)-PIA (Sigma/RBI, Natick, MA); the A2aR antagonist ZM-241385 (Tocris Cookson); the A2aR agonist CGS-21680 (Sigma/RBI). Also used were the adenylate cyclase activator forskolin, the cAK activator 8-Br-cAMP, the cAK inhibitor KT-5720, the cGK activator 8-Br-cGMP, the cGK inhibitor KT-5823, the nonspecific cAK/cGK inhibitor H-7, the tyrosine kinase (TK) inhibitor AG-18, and the PTP inhibitors sodium orthovanadate (Na₃VO₄) and dephostatin, all of which were from Calbiochem (San Diego, CA). For the various enzyme activators and inhibitors, the concentrations used were ≈50 to 100-fold higher than the published EC₅₀ values for the targeted enzyme when drug is applied extracellularly.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). For RT-PCR experiments, vessels were harvested after transcardiac perfusion at room temperature with 100 ml of Krebs' solution plus heparin (1 U/ml) and papaverine (10 μg/ml), followed by Krebs' solution with Triton X-100 (0.1%) plus RNase A (0.1 mg/ml) for 5 min to chemically remove endothelium and degrade endothelial RNA. The RNase activity was terminated by washing, after which the basilar and posterior cerebral arteries were rapidly dissected and placed in RNAlater (Ambion, Austin, TX).

Total RNA was extracted from the arterial homogenate using a DNA-free RNA isolation kit (Ambion) with DNase I application to eliminate DNA contamination, followed by reverse transcription with the oligo-dT primers included in the GeneAmp RNA PCR kit (Roche, Nutley, NJ). For rat A1R, PCR primers were 5'-CGG CAG CAC CCA GAC GAA GA-3' and 5'-CCC ACC ATG CCG CCC TAC AT-3' (Kobayashi et al., 2000). The predicted length of the amplified DNA fragment is 579 base pairs. For rat A2a, PCR primers were 5'-CCA TGC TGG GCT GGA ACA-3' and 5'-GAA GCG GCA GTA ACA CGA ACG-3' (Dixon et al., 1996). The predicted length of the amplified DNA fragment is 150 base pairs. The PCR experiment was conducted with a 480 DNA thermal cycler (Applied Biosystems, Union City, CA). The PCR reactions included 30 cycles with three temperature steps: 95°C, 1 min; 60°C, 1 min, and 72°C, 1.5 min. Products of the amplification reaction were run on a 2.5% agarose gel in parallel with a 0.1-kilobase DNA ladder (Invitrogen, Carlsbad, CA). Amplicons isolated from the gels were sequenced according to

the manufacturer's protocols (ABI 373 stretch sequencer; Applied Biosystems). We used the National Center for Biotechnology Information BLAST program (version 2.1.1; <http://www.ncbi.nlm.nih.gov:80/BLAST/>) to search the National Center for Biotechnology Information GenBank database for nucleotide sequences similar to those of our amplicons. The program determined percentage identity among sequences based on the number of nucleotide substitutions and the number of base pairs being compared.

In Situ Hybridization. A probe specific for nucleotides 3 to 22 of site A, defined as nucleotides -544 to +47 (Biel et al., 1990) of the Cav1.2b sequence (GenBank accession no. X55763), consisted of the synthetic oligonucleotide 5'-CCAGTTACTCTTATGCTCCT-3' (antisense), with the oligonucleotide 5'-GGTCAATGAGAATACGAGGA-3' (sense) used as negative control. Probes were labeled with digoxigenin-11-dUTP using the DIG oligonucleotide tailing kit (Roche Diagnostics, Indianapolis, IN). Labeling efficiency was checked by dot blot analysis. Paraformaldehyde (4%)-fixed frozen sections of the basilar artery, aorta, and left ventricle were pretreated with 0.3% Triton X-100 and 1 μ g/ml proteinase K, acetylated with 0.25% acetic anhydride, prehybridized for 2 h at 37°C with hybridization buffer, and hybridized with digoxigenin-11-dUTP-labeled oligonucleotide probes in a humid chamber for 16 h at 42°C. Slides were treated with 2 \times SSC, 1 \times SSC at room temperature, and 0.1 \times SSC at 50°C after hybridization. Signals were detected by histochemistry procedures with alkaline phosphatase using the 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium reaction.

Immunofluorescence Labeling. For immunolabeling of vessel segments, animals were perfusion fixed with 4% paraformaldehyde in phosphate-buffered saline and brains were removed, sunk in sucrose, and cryosectioned (Frigocut 2800N; Leica, Wetzlar, Germany). Sections (4 μ m) were permeabilized using 0.5% Triton X-100 for 15 min at room temperature. Nonspecific binding was blocked using 1% bovine serum albumin or 1% donkey serum in 0.5% Triton X-100 for 60 min at room temperature, and sections were then incubated with primary antibodies at 4°C for 48 h. Primary antibodies used were directed against: smooth muscle α -actin (1:2,000; Sigma-Aldrich), A1R (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), A2aR (1:1,000; Santa Cruz Biotechnology). Isolated VSMCs obtained as described above for patch clamp were fixed in acetone plus methanol (1:1) for 2 min, nonspecific binding was blocked using 5% goat serum in 0.5% Triton X-100 for 30 min at room temperature, and primary antibodies used were directed against: A1R (1:200; Chemicon International, Temecula, CA) or A2aR (1:100; Chemicon International). After three consecutive 15 min washes with phosphate-buffered saline, sections or cells were incubated with CY3-conjugated species-appropriate secondary antibody (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature in the dark. Corresponding blocking peptides (1:10) were used as negative controls. Immunolabeled sections and cells were examined using a Nikon Eclipse E1000 microscope. Images were captured and processed using a SenSys digital camera (Photometrics, Tucson, AZ) and a personal computer (Dell, Round Rock, TX) with IP Lab software (version 3.01).

In Vitro Assays for Phosphotyrosines. Basilar arteries and aortas were harvested as described above, except that *N*^ω-nitro-L-arginine methyl ester (1 mM) was included in the isolation solution to block endothelial NOS. Vessels were incubated without or with the A2aR agonist CGS-21680 for 30 min at 37°C, and then washed a single time with buffer. Tissues were processed to obtain either total protein from lysates, membrane protein from lysates, or to immunoprecipitate Ca²⁺ channels.

Tissues were lysed in lysis buffer (50 mM Tris-Cl pH 7.5, 250 mM sucrose, 1 mM EDTA, 1% Nonidet-40, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail) for 1 h on ice. To obtain total protein, lysates were centrifuged at 10,000 rpm for 10 min at 4°C. To obtain membrane protein, lysates were centrifuged at

100,000 rpm for 1 h at 4°C. To immunoprecipitate Ca²⁺ channel protein, total protein isolates obtained as described above from six basilar arteries or three aortas were pretreated with 20 μ l/ml rabbit serum and 50 μ l/ml protein A-Sepharose beads for 2 h at 4°C. Supernatant obtained after centrifugation was incubated with 50 μ l/ml protein A-Sepharose beads and antibody directed against the α subunit of the Ca²⁺ channel (pan- α ; Alomone, Jerusalem, Israel) overnight at 4°C. Immunocomplexes were washed three times in buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet-40, 0.1% SDS, and 0.5% sodium deoxycholate) at 4°C and resuspended in 50 μ l of SDS-polyacrylamide gel electrophoresis loading buffer. Antigen was eluted by heating the tubes to 70°C for 10 min.

For Western blots, equivalent amounts of protein were loaded into the wells of 10% NuPAGE Bis-Tris Gel with MOPS running buffer (Novex high-performance precast gel; Invitrogen) for electrophoresis (200 V, 50 min in XcellIII Mini-Cell), and transferred onto polyvinylidene difluoride membranes. Vasodilator-stimulated phosphoprotein (VASP), which was used to gauge activation of cAK, was detected using mouse phospho-specific anti-VASP antibody (serine 239, 1:200; Calbiochem); phosphotyrosines were probed using PY20 antibody (1:1,000, Chemicon International); β -actin was detected using mouse anti- β -actin monoclonal antibody (1:5,000, Sigma-Aldrich). Primary antibodies were visualized using horseradish peroxidase-labeled species-appropriate IgG (1:1,000 or 1:5,000; Amersham Biosciences Inc., Piscataway, NJ) and enhanced chemiluminescence (Amersham Biosciences Inc.). Autoradiographs were scanned and quantified by densitometry (Scion Image software; Scion Corporation, Frederick, MD).

Data Analysis. To quantify the concentration-response relationship (Fig. 2C), data on fractional block were fit to the logistic function: $f_b = (1 - f_{b_{\max}})/[1 + (c/c_o)^{n_H}] + f_{b_{\max}}$, where f_b is fractional block, $f_{b_{\max}}$ is the maximum fractional block, c is concentration, c_o is the concentration at which half of the maximum block is observed, and n_H is the Hill coefficient. Data were fit to the equation using the nonlinear, least-squares method of Marquardt-Levenberg (Origin 7; OriginLab Corp, Northampton MA). For group comparisons, we used a one-way analysis of variance with the Student-Newman-Keuls method for pair-wise multiple comparison. Otherwise, statistical comparisons were evaluated using Student's *t* test. Data are given as mean \pm S.E.

Results

A2aR in Basilar Artery. RT-PCR was used to demonstrate transcription of A2aR and A1R in basilar arteries. After chemical removal of endothelium, total RNA was reverse transcribed with oligo-dT primers to obtain cDNA. Two fragments of cDNA from reverse transcription were amplified by PCR using one primer specific for A2aR and another specific for A1R. Electrophoresis showed that both amplicons were of the predicted sizes, 150 base pairs for the A2aR fragment and 579 base pairs for the A1R fragment (Fig. 1A). After sequencing the amplicons, search of the National Center for Biotechnology Information GenBank database revealed that the two nucleotide sequences exhibited 99% identity with respective published sequences.

Immunofluorescence imaging was used to confirm expression and localization of A2aR on VSMCs. We studied intact vessel segments (Fig. 1B) as well as smooth muscle cells freshly isolated from basilar arteries (Fig. 1C), obtained using the same dissociation methods used for the patch-clamp experiments. Immunolabeling vessel segments demonstrated A2aR on both VSMCs and endothelial cells (Fig. 1B), with labeling on VSMCs being confirmed using isolated cells (Fig. 1C). For comparison, labeling for A1R on an isolated VSMCs is also shown (Fig. 1D). Together, these data con-

firmed transcription and protein expression of A2aR as well as A1R in basilar artery VSMCs.

L-Type Ca_{v1.2b} Channel. Patch-clamp recordings were performed using a nystatin perforated patch whole cell technique to prevent loss of endogenous intracellular signaling pathways that occurs with conventional whole cell recording. With 10 mM Ba²⁺ as the charge carrier, macroscopic currents exhibited kinetics of activation, inactivation, and deactivation, as well as voltage dependence (Fig. 2A) that were typical for L-type Ca²⁺ channels (Simard and Li, 2000; Gerzanich et al., 2001). Macroscopic currents were sensitive to the blocking dihydropyridine nifedipine. Single channel currents obtained with the activating dihydropyridine Bay k8644 showed an underlying single channel conductance of 23 pS (Fig. 2, B and C), as reported previously (Simard and Li, 2000; Gerzanich et al., 2001). In situ hybridization confirmed that the channel expressed in basilar artery was Ca_{v1.2b} (Fig. 2Da), the same as in aorta (Fig. 2Db). Cardiac tissue, in which only Ca_{v1.2a} is expressed, was used as negative control (Fig. 2Dc). No current attributable to any other channel was observed under the recording conditions used.

Adenosine Down-Regulates Ca²⁺ Channel Activity. Under patch clamp, cells were tested using a ramp protocol (Fig. 3A) to elicit pseudo steady-state current voltage curves. Adenosine (10–100 μM) consistently decreased the Ca²⁺

channel current without changing the kinetics or voltage dependence (Fig. 3B). The effect occurred shortly after addition of adenosine to the bath, with current reaching steady state after 7 to 10 min (Fig. 3C). Computation of the concentration-response relationship revealed an EC₅₀ value of 17 μM, with a Hill coefficient of 1.8 (Fig. 3D), consistent with findings on vasorelaxation in rat cerebral vessels (Ngai et al., 2001). The maximum effect observed with 100 μM adenosine was a 27% decrease in current compared with control, which was statistically significant (by analysis of variance, $p < 0.05$) (Fig. 3D).

A2aR Function. We used receptor-subtype specific agents to determine which adenosine receptor might be responsible for down-regulating the Ca²⁺ channel current. Using selective blockers for A1R and A2aR, DPCPX and ZM-241385, respectively, we found that the A1R blocker did not prevent adenosine-mediated down-regulation (Fig. 4, A and C, ○), whereas the A2aR blocker blocked it effectively (Fig. 4, B and C, ●). We also studied effects of receptor subtype-specific agonists (*R*)-PIA and CGS-21680, which act selectively at A1R and A2aR, respectively. The A1R agonist produced no

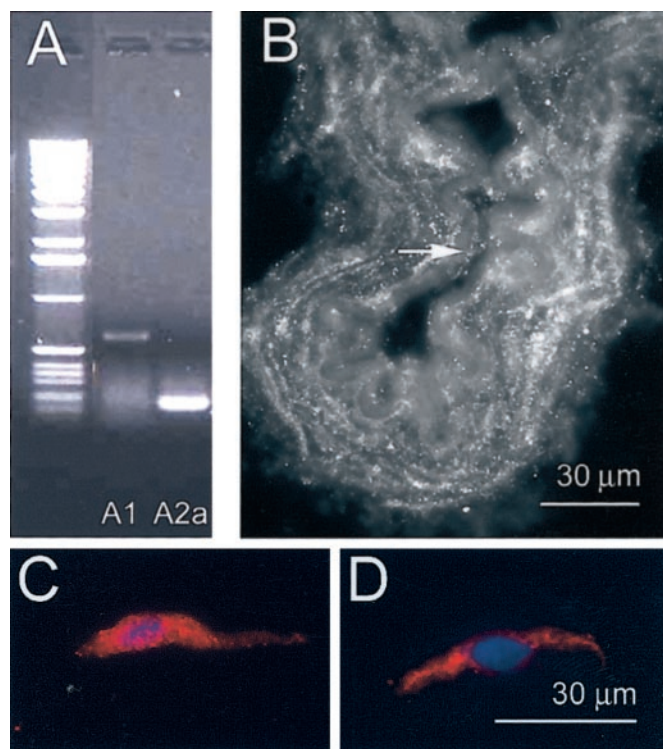


Fig. 1. Expression of A2aRs and A1Rs in smooth muscle cells from rat basilar artery. A, transcripts of adenosine receptors in basilar artery (endothelium removed) assessed by RT-PCR using specific primers for A1R and A2aR (see *Materials and Methods* for details); base pair calibration ladder shown at left. B, localization of A2aR in smooth muscle layers of the basilar artery by immunofluorescence labeling using anti-A2aR-specific antibody; arrow points to endothelium. C and D, single smooth muscle cells enzymatically isolated from basilar artery retain specific immunofluorescence labeling with anti-A2aR (C) and anti-A1R (D) antibodies; the nuclei of the smooth muscle cells are labeled by 4,6-diamidino-2-phenylindole. The data shown in A to D are representative of three independent experiments each for RT-PCR, whole basilar artery immunostaining, and single cell immunostaining.

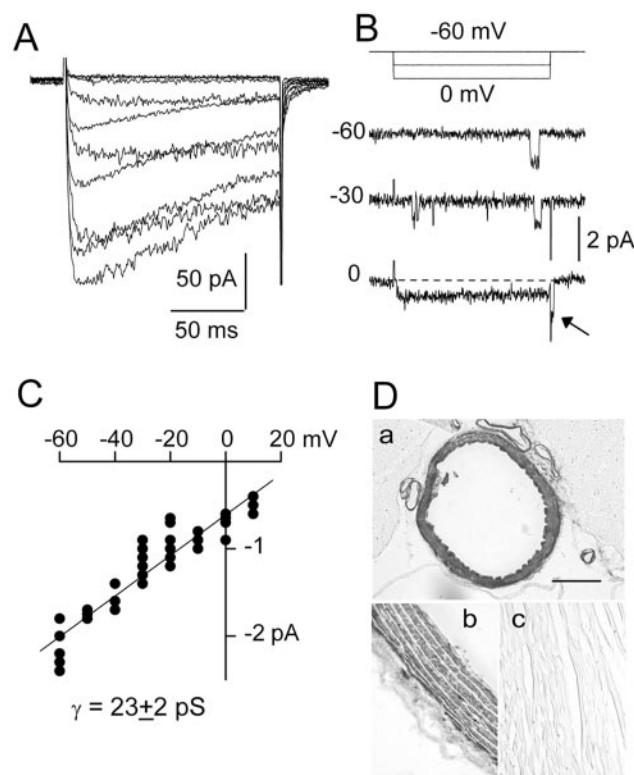


Fig. 2. Smooth muscle cells isolated from rat basilar artery express functional L-type Ca_{v1.2b} channels. A, typical family of whole cell currents recorded during 150-ms step pulses from -60 to $+30$ mV, in 10-mV increments (peak current at $+10$ mV); cells were voltage clamped at a holding potential of -60 mV using a nystatin perforated patch technique with 10 mM Ba²⁺ in the bath and Cs⁺ in the pipette. B, single channel recordings in the cell-attached mode during 150-ms steps to -60 , -30 , and 0 mV from a holding potential of -60 mV; note that the open channel probability increased with depolarization, with the channel being open all the time at 0 mV; arrow indicates “tail current” caused by persistent opening of channel immediately after repolarization to -60 mV. C, plot of the single-channel current amplitude versus test potential yielding a single channel conductance of 23 pS. D, in situ hybridization with probe specific for Ca_{v1.2b} subtype of the L-type Ca²⁺ channel in basilar artery (a) and aorta (b), with cardiac tissue (c), which is known to express the Ca_{v1.2a} subtype of L-type Ca²⁺ channel, being used as a negative control.

diminution of current, but instead, seemed to cause a small transient increase in current (Fig. 4, D and F, ○), whereas the A2aR agonist CGS-21680 mimicked the effect of adenosine, yielding a reduction in current down to about 75% of the original current over the course of 10 min (Fig. 4, E and F, ●). Thus, our data with both antagonists and agonists were consistent with the idea that adenosine-mediated down-regulation is a result of activation of A2aR.

A2aR and cAK. We sought to determine the signaling pathway used by A2aR to down-regulate Ca^{2+} channel currents in basilar artery VSMCs. We screened for involvement of four candidate kinases: cAK, cGK, protein kinase C (PKC), and TK. In our first experiments, we investigated cAK, given that A2aR activation is usually associated with activation of adenylate cyclase. However, addition of forskolin (Fig. 5, A and E, ○) or of 8-Br-cAMP (not shown) resulted in no appreciable down-regulation of Ca^{2+} channel current, indicating that A2aR activation was not mimicked by cAK activation.

To further exclude involvement of cAK, we tested the effect of A2aR activation in the presence of the cAK inhibitor KT-5720. These experiments were complicated somewhat by a reduction in Ca^{2+} channel currents caused by KT-5720 itself (Fig. 6A). This effect was attributed to partial nonspecific block of the channel, rather than an effect mediated by inhibition of cAK, because the chemically similar compound that does not inhibit cAK, KT-5823, exerted the same blocking effect (Fig. 6A). Despite the reduction in current with KT-5720, however, the A2aR agonist CGS-21680 still down-regulated the Ca^{2+} current to the same (fractional) extent as in the absence of cAK inhibition (Fig. 6B). Because of these technical difficulties with KT-5720, we also assessed the effect of CGS-21680 in the presence of another inhibitor of cAK, H-7. H-7 caused less inhibition of the baseline Ca^{2+}

channel current but, like KT-5720, did not prevent the inhibitory effect of A2aR activation, with CGS-21680 inhibiting current to $74 \pm 7\%$ of baseline levels (five cells). Together, these data with cAK activators and inhibitors indicated that cAK was unlikely to be involved in A2aR-mediated down-regulation of Ca^{2+} channels in basilar artery VSMCs.

Although it seemed that cAK was not involved in A2aR-mediated down-regulation of Ca^{2+} channels, we considered that A2aR activation might nevertheless still be associated with cAK activation in basilar artery VSMCs. We performed Western immunoblots of total protein from control vessel segments and from segments incubated with CGS-21680 and evaluated the blots for VASP, which can be phosphorylated by either cAK or cGK (Ibarra-Alvarado et al., 2002). Exposure to CGS-21680 was found to result in a clear increase in phospho-VASP (Fig. 6E), an effect that was completely blocked by incubation with the cAK inhibitor KT-5720 (Fig. 6E). These data indicated A2aR activation was in fact activating cAK, as might be expected, even though activation of this pathway could not account for the effect on Ca^{2+} channels.

A2aR and PKC. Activation of PKC by phorbol 12-myristate 13-acetate had little effect on the current, suggesting no

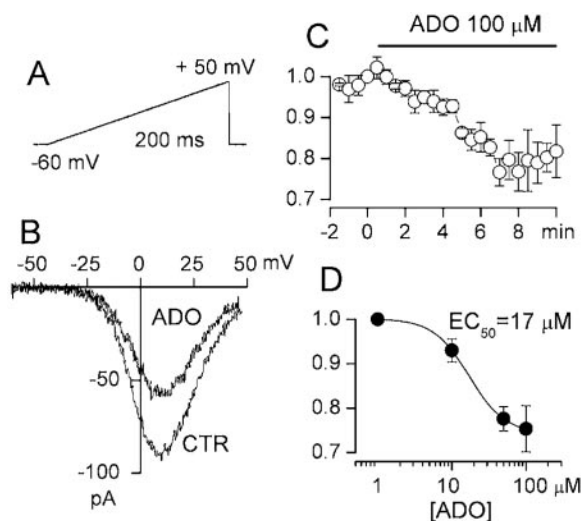


Fig. 3. Adenosine down-regulates L-type Ca^{2+} channel activity in smooth muscle cells from the basilar artery. A, ramp voltage protocol (0.45 mV/ms) used to elicit pseudo steady-state current voltage curves. B, Ca^{2+} channel currents recorded before (CTR) and 6 min after (ADO) addition of $100 \mu\text{M}$ adenosine to the bath solution. C, time course of the adenosine effect; averaged data from seven cells (mean \pm S.E.) are shown, with the maximum peak current as shown in B being normalized to the peak current immediately before addition of adenosine; horizontal bar indicates adenosine ($100 \mu\text{M}$) perfusion. D, concentration-response curve for the effect of adenosine obtained by averaging data from three to five cells at each concentration; data (mean \pm S.E.) were fit to a sigmoidal logistic equation, yielding an EC_{50} value of $17 \mu\text{M}$ and a Hill coefficient of 1.8.

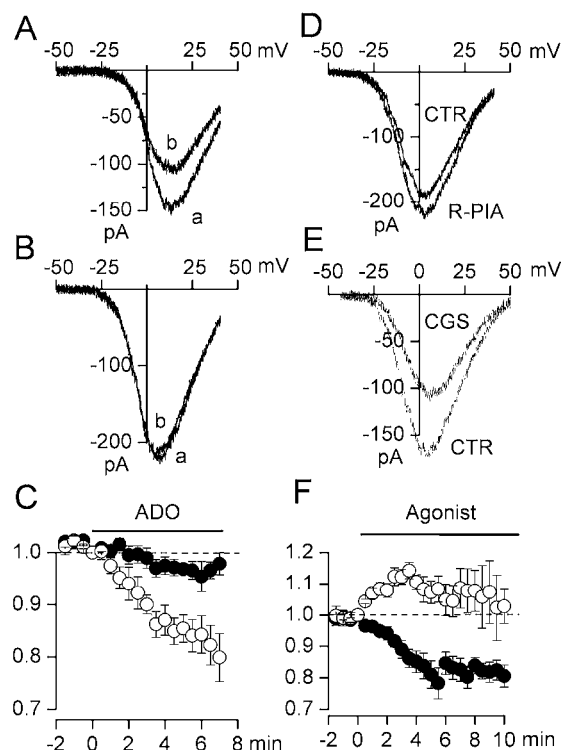


Fig. 4. Pharmacological profile of the adenosine receptor involved in down-regulation of the Ca^{2+} channel current. A and B, Ca^{2+} channel currents before (a) and 6 min after (b) addition of adenosine ($100 \mu\text{M}$) in the presence of the selective A1R antagonist DPCPX ($1 \mu\text{M}$) (A) or the selective A2aR antagonist ZM-241385 ($1 \mu\text{M}$) (B). C, averaged data (mean \pm S.E.) as a function of time for the experiments shown in A and B; horizontal bar indicates application of adenosine (ADO) in the presence of DPCPX (○) or ZM-241385 (●). D, Ca^{2+} channel currents before (CTR) and 6 min after [(R)-PIA] addition of the A1R-selective agonist (R)-PIA ($10 \mu\text{M}$). E, Ca^{2+} channel currents before (CTR) and 6 min after (CGS) addition of the A2aR-selective agonist CGS-21680 (500 nM). F, averaged data (mean \pm S.E.) as a function of time for the experiments shown in D and E; horizontal bar indicates application of the agonists (R)-PIA (○) or GS-21680 (●). For C and F, data were obtained from six to nine cells for each drug; peak Ca^{2+} channel currents were normalized to the peak current obtained immediately before agonist application.

involvement of PKC in A2aR-mediated down-regulation of Ca²⁺ channels (Fig. 5, B and E, ●).

A2aR and cGK. In contrast, activation of cGK with 8-Br-cGMP down-regulated Ca²⁺ channels (Fig. 5, C and E, □), mimicking down-regulation observed with CGS-21680. We thus used the cGK inhibitor KT-5823 to assess potential involvement of cGK in channel down-regulation. As with KT-5720, KT-5823 reduced the Ca²⁺ channel current (Fig. 6A), an effect that could not be ascribed to cGK inhibition, given that cGK activation causes down-regulation. Despite the reduction in current with KT-5823, CGS-21680 still down-regulated the Ca²⁺ current to the same (fractional) extent as in the absence of cGK inhibition (Fig. 6B), suggesting that cGK was not involved in the A2aR-mediated effect.

Block of Ca²⁺ channel activity by KT-5823 was also observed when recording Ca²⁺ channels using a cell-attached patch configuration (Fig. 6, C and D). Again, however, despite partial channel block with KT-5823, CGS-21680 still down-regulated Ca²⁺ currents (Fig. 6, C and D). Notably, the magnitude of the inhibitory effect of CGS-21680 observed with a cell-attached technique (Fig. 6D, KT-5823 versus CGS) was appreciably larger than the magnitude observed

with a whole-cell nystatin patch technique (Fig. 6B). This presumably reflected a healthier physiological state of the cells, as suggested by the observation that run-down of Ca²⁺ channel current was virtually absent with the cell attached patch configuration. As noted above, our experiments with the nonspecific inhibitor H-7, which blocks cGK as well as cAK, showed that effects of CGS-21680 were still observed in the presence of this agent. Thus, although activation of cGK seemed to mimic effects of A2aR-activation, our data with two different cGK inhibitors indicated that cGK was not involved.

In the previous experiment in which we assessed phosphorylation of VASP by CGS-21680, we also performed an experiment in which vessel segments were incubated with

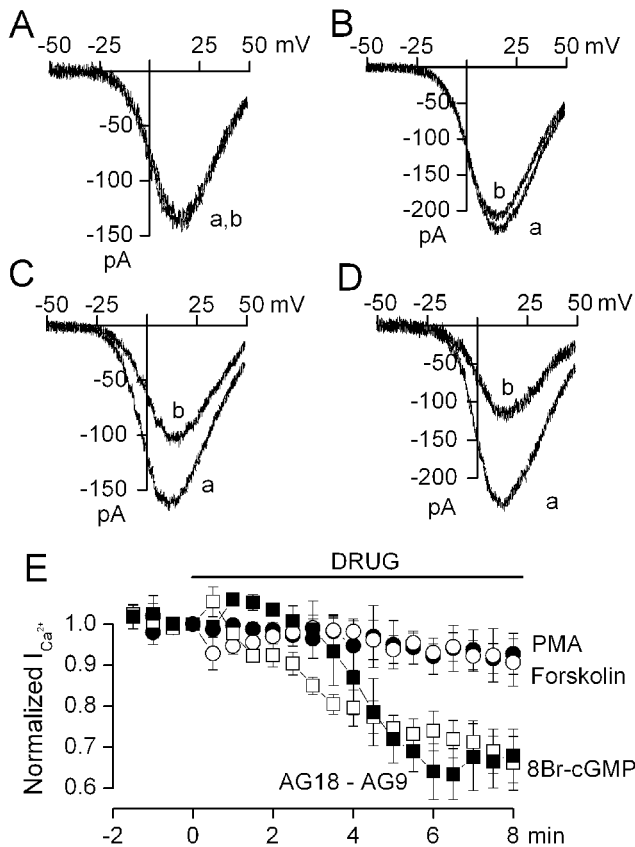


Fig. 5. Assessment of possible second messengers involved in down-regulation of the Ca²⁺ channel currents. A to D, Ca²⁺ channel currents before (a) and 6 min after (b) addition of the cAK activator forskolin (100 μM) (A), the PKC activator phorbol 12-myristate 13-acetate (100 nM) (B), the cGK activator 8-Br-cGMP (100 μM) (C), or the TK inhibitor tyrphostin AG-18 (100 μM) (D). E, averaged data (mean ± S.E.) for the experiments shown in A to D (5–9 cells for each drug); the data labeled AG18-AG9 represent the difference observed between inhibition with AG-18 (100 μM; 6 cells) and that observed with the kinase-inactive analog, AG-9 (100 μM; 4 cells); horizontal bar indicates application of the drug studied; peak Ca²⁺ channel currents were normalized to the peak current observed immediately before drug application.

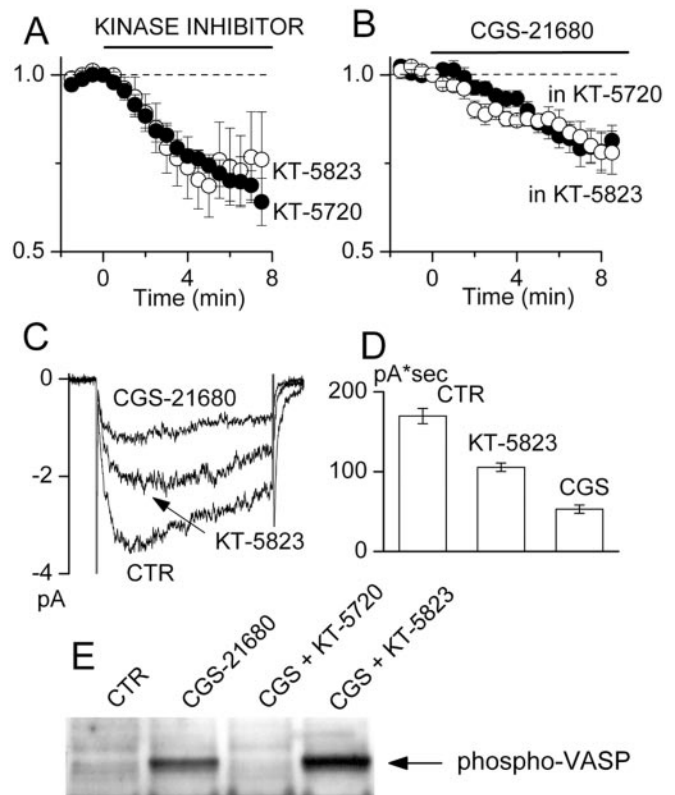


Fig. 6. Selective cAK and cGK inhibitors do not prevent down-regulation of the Ca²⁺ channel currents after A2aR activation. A, effect of the cAK (KT-5720, 1 μM, ●) and cGK (KT-5823, 2 μM, ○) inhibitors on Ca²⁺ channel currents; peak currents were normalized to the current recorded before addition of the inhibitor. B, effect of the selective A2aR agonist CGS-21680 on the Ca²⁺ channel currents in cells exposed to cAK (KT-5720, ●) and cGK (KT-5823, ○) inhibitors; peak currents were normalized to the currents recorded 6 to 8 min after addition of the kinase inhibitor (shown in A) and immediately before the addition of CGS-21680; same cells as in A; in A and B, data are averages (mean ± S.E.) obtained from seven and five cells for KT-5720 and KT-5823, respectively. C, multichannel traces recorded in cell-attached mode in control (CTR), after addition of the cGK inhibitor KT-5823 (2 μM) for 10 min and after addition of CGS-21680 (500 nM) in the continued presence of KT-5823; traces shown were obtained by averaging 20 consecutive Ca²⁺ channel currents activated every 20 s by voltage steps to 0 mV in a cell-attached patch held at –60 mV. D, summary of data obtained from five patches (mean ± S.E.) in the experiment shown in C; values of open probability of the Ca²⁺ channel currents were quantified by calculating the area under current traces obtained as described above (C). E, effect of the cAK and cGK inhibitors on phosphorylation of VASP induced by the activation of the A2aR; Western blot of phospho-VASP in isolated control basilar arteries (first lane), in arteries exposed to CGS-21680 alone (second lane), in arteries exposed to CGS-21680 plus KT-5720 (third lane), and in arteries exposed to CGS-21680 plus KT-5823 (fourth lane); time of incubation, 30 min.

CGS-21680 plus the cGK inhibitor KT-5823. Unlike the complete block observed with KT-5720, KT-5823 had no effect on CGS-21680-mediated phosphorylation of VASP (Fig. 6E), further indicating that cGK was not involved in A2aR signaling.

A2aR and PTP. Our screening experiments for potential involvement of different kinases indicated that the TK inhibitor AG-18 (100 μ M) reduced Ca^{2+} channel activity to $\approx 50\%$ of baseline values (Fig. 5D). It was previously shown that TK inhibitors can block Ca^{2+} channels by a TK-independent mechanism (Belevych et al., 2002). To confirm that the inhibitory effect of AG-18 that we observed was caused by inhibition of TK, we evaluated the chemically similar compound AG-9 (100 μ M), which does not inhibit TK. AG-9 reduced Ca^{2+} channel activity to $\approx 80\%$ of baseline values. The effect of AG-9 was rapidly and completely reversible, whereas the effect of AG-18 was only slowly and partially reversed by washing. Together, these data suggested that the effect of AG-18 was caused by two mechanisms and that the best estimate for the effect of AG-18 attributable to TK inhibition would be obtained by subtracting the effect of AG-9 from that of AG-18 (Fig. 5E, ■). Based on these experiments, we concluded that Ca^{2+} channel activity in cerebral VSMCs is regulated by TK, and thus that CGS-21680 could be causing a reduction in tyrosine phosphorylation caused either by inhibition of TK or by activation of PTP.

We used the PTP inhibitors sodium orthovanadate and dephostatin to assess for potential involvement of PTP. We used multichannel recordings in cell-attached patches to assess effects of the PTP inhibitors (Fig. 7). In these experiments, no channel block by the inhibitors was evident, and both agents completely prevented any down-regulation of Ca^{2+} channels by CGS-21680 (Fig. 7, A–D). These data suggested that A2aR-mediated down-regulation of channel activity was caused by activation of PTP.

The previous experiments provided pharmacological evidence suggesting that A2aR signaling in VSMCs involved reduced tyrosine phosphorylation. To further examine this, we performed immunoblots of lysate from basilar artery, both total protein and the membrane fraction, with the blots being probed using an antibody directed against phosphotyrosine. Basilar artery segments were incubated in the presence of CGS-21680, either without or with sodium orthovanadate, to reproduce the salient features of the experiment of Fig. 7D. In two separate experiments, we found that total phosphotyrosines were reduced with CGS-21680 compared with control, and PTP inhibition by sodium orthovanadate prevented reduction of tyrosine phosphorylation by CGS-21680 (Fig. 7E). In the membrane fraction of protein, CGS-21680 also reduced tyrosine phosphorylation, including a band near 200 kDa corresponding to the molecular mass of the α_{1c} L-type Ca^{2+} channel subunit (Fig. 7F). Quantifying the optical density of the 200-kDa band of the membrane protein indicated a significant decrease (by *t* test, $p < 0.05$; $n = 4$) associated with CGS-21680 (Fig. 7G).

Finally, we performed immunoisolation experiments to examine more specifically the status of tyrosine phosphorylation of the α_{1c} subunit. For these experiments, we studied channels from basilar artery and aorta, both of which were shown to express the $\text{Ca}_{v1.2b}$ channel (Fig. 2D). Basilar artery and aorta segments were incubated either alone, with adenosine or with CGS-21680. Basilar arteries from six rats yielding 500 μ g of total protein did not yield sufficient immunoiso-

lated Ca^{2+} channel protein for evaluation, but aorta from three rats yielding 2.5 mg of total protein did. In two separate experiments, we found that Western blots of Ca^{2+} channel α subunit immunisolated from aorta using pan- α antibody showed two bands at ≈ 200 and ≈ 185 kDa, as described previously (Hell et al., 1993) and that exposure to both adenosine and CGS-21680 resulted in substantial reduction of tyrosine phosphorylation of these bands (Fig. H).

Discussion

A2aR activation is a potent mechanism for relaxation of cerebral and other blood vessels (Coney and Marshall, 1998;

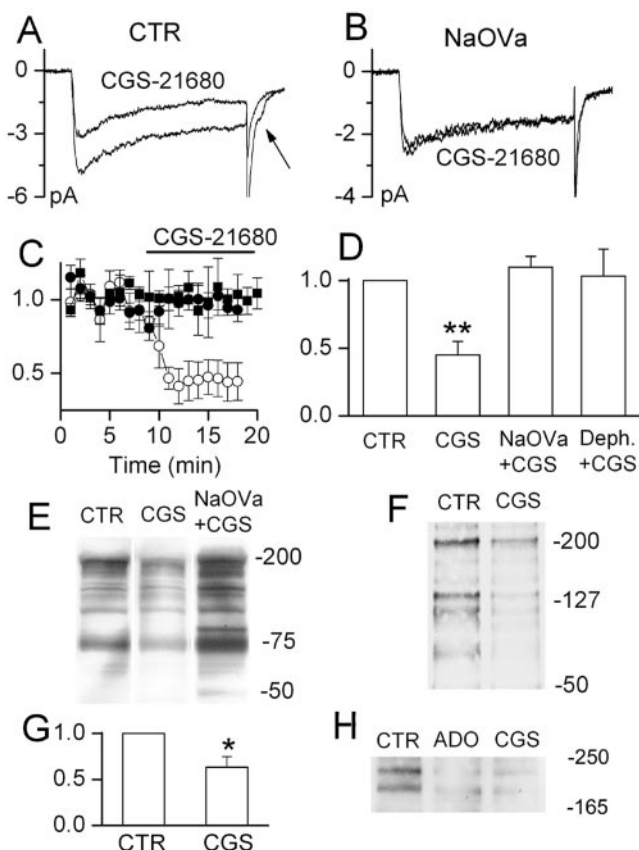


Fig. 7. A2aR down-regulates Ca^{2+} channel current via protein tyrosine phosphatase. A, effect of the A2aR agonist CGS-21680 on multi- Ca^{2+} channel currents recorded in a cell-attached patch; current traces were obtained by voltage steps from -60 to 0 mV as described in Fig. 6C; tail currents after repolarization are indicated by the arrow. B, effect of CGS-21680 after 10-min exposure of the patch to tyrosine phosphatase inhibitor Na_3VO_4 (100 μ M). C, open probability of the Ca^{2+} channel currents after addition of 500 nM CGS-21680; open probability was calculated as described for Fig. 6D and was normalized to the mean open probability before addition of CGS-21680; \circ , CGS-21680 alone; \bullet , CGS-21680 added after 10-min incubation with Na_3VO_4 ; \blacksquare , CGS-21680 added after 10-min incubation with dephostatin (50 μ M); data are mean \pm S.E. for five patches for each experiment. D, bar graph of the Ca^{2+} channel open probability in control (CTR), after addition of CGS-21680 (CGS), after addition of CGS-21680 with prior incubation with Na_3VO_4 or dephostatin; $**$, $p < 0.01$. E and F, Western blots of total protein (E) or the membrane fraction of protein (F) from basilar arteries blotted with anti-phosphotyrosine antibodies in CTR, after incubation with 500 nM CGS-21680 (CGS) or CGS-21680 plus 100 μ M Na_3VO_4 . G, plots of the normalized optical density of the band at 200 kDa in "F" corresponding to molecular mass of the Ca^{2+} channel α_1 subunit in CTR and after CGS; $n = 4$; $*$, $p < 0.05$. H, Western blots of immunisolated Ca^{2+} channel α_1 subunit from rat aorta blotted with anti-phosphotyrosine antibodies in CTR, after incubation with 100 μ M adenosine (ADO) or 500 nM CGS.

Shin et al., 2000; Ngai et al., 2001). An important finding from the present experiments came from combined RT-PCR, Western blot, immunofluorescence, and pharmacological data demonstrating that A2aR was transcribed, was expressed, and was functional in basilar artery VSMCs. Previous experiments with vascular smooth muscle have used pharmacological data to identify adenosine receptor subtypes. In addition, molecular techniques have been used to verify transcription and expression of A2aR in coronary vessels (Hein et al., 2001), but this is the first report to use the combination of these techniques in cerebral vessels. For the receptor subtype-specific functional experiments that we carried out, we used CGS-21680, which is a high-affinity A2aR agonist (Hutchison et al., 1989) that exhibits long receptor occupancy (Gao et al., 2001). Our data, obtained from a combination of methods, reaffirm previous reports of functional involvement of A2aR in cerebral vessels.

Relaxation of VSMCs may occur by one of several mechanisms, but relaxation is generally accompanied by diminished influx of Ca²⁺ via L-type Ca²⁺ channels. Reduced influx of Ca²⁺ can be brought about by a voltage-dependent mechanism, in which K⁺ channel activity is up-regulated, leading to polarization of the cell membrane and deactivation of L-type Ca²⁺ channels, or by a voltage-independent mechanism, in which the Ca²⁺ channel itself as well as intermediate regulatory phosphoproteins are phosphorylated or dephosphorylated (Gerzanich et al., 2001). Studies of ion channels in VSMCs have shown that activity of Ca²⁺-activated K⁺ channels (Li and Cheung, 2000) as well as K_{ATP} channels (Kleppisch and Nelson, 1995; Hein et al., 2001) may be up-regulated by adenosine-receptor activation, with effects on K_{ATP} channels typically being mediated by activation of A2aR. A2R-induced down-regulation of Ca²⁺ channel activity by a mechanism independent of voltage has been found in PC12 cells (Park et al., 1998) and in rod photoreceptors (Stella et al., 2002) but has not previously been reported in VSMCs.

In PC12 cells and in rod photoreceptors, activation of A2aR leads to down-regulation of Ca²⁺ channel activity via cholera toxin-sensitive G-protein and activation of cAK (Park et al., 1998; Stella et al., 2002). In coronary VSMCs, A2aR-induced up-regulation of K_{ATP} channel activity is mediated by adenylyl cyclase and elevated levels of cAMP (Kleppisch and Nelson, 1995). Potential involvement of cAK in adenosine-mediated effects in the cerebral circulation has previously been suggested by demonstrating an increase in cAMP levels in microvessels from rabbit and feline cerebral cortex (Li and Fredholm, 1985). In our experiments with basilar artery VSMCs, we also obtained evidence that cAK was activated, as indicated by VASP phosphorylation that was blocked by the cAK inhibitor KT-5720. However, cAK is not invariably or exclusively involved in A2aR-induced effects. Most importantly, A2aR-induced relaxation of cerebral vessels has recently been found not to be blocked by inhibition of cAK (West et al., 2003). Similarly, we found that the cAK signaling pathway did not contribute to down-regulation of Ca²⁺ channel activity in basilar artery VSMCs, as shown by the lack of effect of forskolin and 8-Br-cAMP on Ca²⁺ channel availability and by the lack of effect of the cAK inhibitors KT-5720 and H-7 in preventing A2aR-mediated down-regulation of Ca²⁺ channels.

A2aR-mediated activation of two distinct signaling path-

ways has been reported in neurons (Gubitz et al., 1996). Similarly, our data suggested that A2aR not only activated cAK but also activated a second pathway, resulting in reduced tyrosine phosphorylation that was associated with down-regulation of Ca²⁺ channel activity. First, we showed that inhibition of TK with AG-18 caused down-regulation of channel activity, establishing that in cerebral VSMCs specifically, TK phosphorylation is critical for Ca²⁺ channel activity. Subsequent pharmacological experiments with both patch-clamp and Western blot measurements were consistent with involvement of PTP in the effect of CGS-21680. We found that inhibitory effects of CGS-21680 on Ca²⁺ channel currents could be prevented by two molecularly distinct PTP blockers, sodium orthovanadate or dephostatin, and similarly, that inhibitory effects of CGS-21680 on tyrosine phosphorylation could be prevented by sodium orthovanadate. Western blots showed that A2aR activation led to reduced levels of phosphotyrosines in total cell lysates and in membrane fractions of lysates from basilar artery, including at the molecular mass of the $\alpha 1c$ subunit of the L-type Ca²⁺ channel, as well as in immunisolated $\alpha 1c$ subunits from aorta. Together, these data provide strong evidence of a critical association between reduced tyrosine phosphorylation of the channel and a decrease in channel activity. Although reduced tyrosine phosphorylation can arise from either TK inhibition or PTP activation, the most parsimonious explanation of our data, specifically the data with sodium orthovanadate and dephostatin, is that CGS-21680 leads to activation of PTP, not inhibition of TK. This interpretation would accord with previous reports of adenosine-induced responses involving PTP activation (Abe and Saito, 1998; Harrington et al., 2000; Harrington et al., 2001; Crist et al., 2001).

We did not identify any specific PTP that might be involved, nor did we characterize the G protein that is involved in A2aR-mediated down-regulation of Ca²⁺ channel activity in basilar artery VSMCs. The PTPs constitute a family of more than 75 enzymes, with as many as 18 identified in rat vascular smooth muscle (Schaapveld et al., 1997; Wright et al., 2000). The conventional PTPs are classified into two broad groups, the receptor-like and the cytoplasmic or non-membrane groups (Wright et al., 2000; Tonks and Neel, 2001). The receptor-like PTPs exhibit transmembrane components that are directly activated by integrins and similar intrinsic molecules, whereas the cytoplasmic PTPs are activated by signaling cascades that can involve classic receptor occupancy and G protein intermediates. Activation of PTP is involved in inhibition of VSMCs contraction (Melis et al., 2000), but overall this signaling cascade remains poorly characterized in VSMCs. Although effects of PTP inhibitors on Ca²⁺ channels in VSMCs have been investigated (Wijetunge et al., 1998; Kimura et al., 2000), the present study is the first to tentatively demonstrate a receptor-mediated mechanism involving activation of a PTP that specifically targets Ca²⁺ channels in VSMCs. Numerous complex interactions between various G proteins and PTPs remain as possible mechanisms for the effects that we observed. An exhaustive examination of these individual possibilities, however, is beyond the scope of the present work and remains for future studies to address.

Regulation of Ca²⁺ channel activity by tyrosine phosphorylation in VSMCs is complex and is not completely under-

stood, in part because many of the pharmacological agents typically used have multiple effects. Our data showed that the TK inhibitor AG-18 reduced channel activity by two mechanisms, not only inhibiting TK activity but also acting as a channel blocker. Fortunately, the PTP inhibitors sodium orthovanadate and dephostatin showed no effect on basal channel activity, making interpretation of data obtained with these agents easier. Overall, our observations suggested that normally, there is ongoing tyrosine phosphorylation and dephosphorylation of the channel, that the dynamic balance between the two processes is dominated by TK, and that ongoing PTP activity is "revealed" by inhibiting TK.

Not all isoforms of the L-type Ca^{2+} channel are phosphorylated by tyrosine kinase. Here, we showed using *in situ* hybridization that rat basilar artery VSMCs expresses the $\text{Ca}_{v1.2b}$ isoform, as identified in other VSMCs (Ertel et al., 2000), and which are subject to tyrosine phosphorylation (Wijetunge et al., 1998; Kimura et al., 2000). Tyrosine kinase-dependent regulation of smooth muscle and neuronal isoforms of the L-type Ca^{2+} channel involves phosphorylation of the tyrosine residue at position 2122 of the $\alpha 1$ subunit (Bence-Hanulec et al., 2000). The cardiac isoform of the L-type channel lacks this residue, a finding that is believed to correlate with absence of tyrosine kinase activation of cardiac Ca^{2+} channels (Belevych et al., 2002) and that might explain why adenosine by itself has no direct effect on Ca^{2+} conductance in cardiac myocytes, although it does antagonize the isoproterenol-induced increase in cAMP, which leads indirectly to a decrease in Ca^{2+} conductance (Isenberg and Belardinelli, 1984; West et al., 1986).

In summary, we have shown that A2aR activation caused reduced activity of Ca^{2+} channels in cerebral VSMCs and that this effect was mediated not by activation of cAK but by reduced tyrosine phosphorylation of the channel, probably caused by activation of PTP. Multiple converging mechanisms seem to contribute to adenosine's strong vasodilatory influence in various blood vessels, with many of these mechanisms acting in concert to down-regulate VSMC Ca^{2+} channels and reduce Ca^{2+} influx, including activation of cGK via endothelial NOS, activation of cAK to open K^{+} channels, hyperpolarize the cell and turn off Ca^{2+} channels by virtue of their voltage dependence, and as shown here, tyrosine dephosphorylation of the Ca^{2+} channel to directly decrease its probability of opening. The vasorelaxation that ensues from one or more of these actions in cerebral vessels is likely to be a powerful contributor to augmented cerebral blood flow in conditions of hypoxia.

Acknowledgments

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