The D₅ Dopamine Receptor Mediates Large-Conductance, Calcium- and Voltage-Activated Potassium Channel Activation in Human Coronary Artery Smooth Muscle Cells

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ABSTRACT

Large-conductance, calcium- and voltage-activated potassium (BK_{Ca}) channels hyperpolarize coronary artery smooth muscle cells, causing vasorelaxation. Dopamine activates BK_{Ca} channels by stimulating D₁-like receptor-mediated increases in cAMP in porcine coronary artery myocytes. There are two D₁-like receptors (R), D₁R and D₅R. We hypothesize that the specific D₁-like receptor involved in BK_{Ca} channel activation in human coronary artery smooth muscle cells (HCASMCs) is the D₅R and that activation occurs via cAMP cross-activation of cGMP-dependent protein kinase (PKG), rather than cAMP-dependent protein kinase (PKA). The effects of D₁-like receptor agonists and antagonists on BK_{Ca} channel opening in HCASMCs were examined in the presence and absence of PKG/PKA inhibition by cell-attached patch clamp. In the absence of commercially available ligands specific for D₁R or D₅R, D₁R or D₅R protein was down-regulated by transfecting HCASMCs with human D₁R or D₅R antisense oligonucleotides, respectively: cells transfected with scrambled oligonucleotides and nontransfected HCASMCs

served as controls. The predominant ion channel conducting outward currents in nontransfected HCASMCs was identified as the large-conductance, calcium- and voltage-activated potassium (BK_{Ca}) channel, which was activated by D₁-like receptor agonists despite PKA inhibition with (9R,10S,12S)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*ij*][1,6]benzodiazocine-10-carboxylic acid (KT 5720) (300 nM), but was abolished by inhibiting PKG with 9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*-11*a*-triazadibenzo(*a,g*) cycloocta(*cde*)-trinden-1-one (KT 5823) (300 nM). D₁-like receptor agonists activated BK_{Ca} channels in all transfected cells except those transfected with D₅R antisense oligonucleotides. Thus, the dopamine (D₁-like) receptor mediates activation of BK_{Ca} channels in HCASMCs by D₅R, not D₁R, and via PKG, not PKA. This is the first report of differential D₁-like receptor regulation of vascular smooth muscle function in human cells.

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In addition to being an important neurotransmitter, dopamine exerts paracrine effects on the renal, adrenal, and gastrointestinal regulation of sodium balance and blood pressure. Dopamine acts on two distinct families of G protein-coupled receptors: the D₁-like receptors, which include D₁ and D₅ receptors (D₁R and D₅R), and the D₂-like receptors, which include D₂R, D₃R, and D₄R (Sibley and Monsma, 1992; Jose et al., 2003; Hussain and Lokhandwala, 2003). D₁-like receptors are linked to the stimulatory G protein G_{αs} and stimulate adenylyl cyclase, increasing cAMP production. In contrast, D₂-like receptors are linked to inhibitory G proteins G_{αi} and G_{αo} and inhibit adenylyl cyclase (Missale et al., 1998) and calcium channels, and modulate potassium channels.

Dopamine receptor subtype expression in the vasculature is vessel specific. In pial, renal, mesenteric, and coronary arteries, D₁-like receptors are postjunctional, located in the tunica media (Ozono et al., 1997; Amenta et al., 2002). D₂-

TABLE 1
Primer sequence for RT-PCR

Receptor	Primer (5'–3')	Nucleotide Position	Transcript Size
D ₁ R (GenBank Accession number NM_000794)	F: CCTGTTTCCTATCGCTGCTC R: TGAGGCTGGAGTCACAGTTG	80–99 552–571	bp 491
D ₅ R (GenBank Accession number NM_000798)	F: GACGGTGAACATCAGCAATG R: CCCAGACAGACTCAGCAACA	1551–1570 1743–1762	212

F, forward; R, reverse.

like receptors are pre- and postjunctional, located in the adventitia and the junction between the adventitia and media in pial, renal, and mesenteric vessels (Amenta et al., 2000), and they are associated with dopaminergic nerves in coronary arteries (Cavallotti et al., 2002). Dopamine and its analogs, acting via D₁-like receptors, are coronary vasodilators in animals and humans.

D₁-like receptor-mediated vasodilation is attributed, in part, to activation of hyperpolarizing vasorelaxant potassium channels via cAMP (White et al., 2000). Arterial vascular smooth muscle cells express up to four types of potassium channels (Nelson et al., 1995; Jackson, 2000) at varying densities in different vascular beds: ATP-sensitive (K_{ATP}); inward rectifying; voltage-activated; and calcium-activated, predominantly large-conductance (BK_{Ca}) channels.

Different mechanisms of D₁-like receptor-mediated stimulation of potassium channels in porcine coronary artery cells have been proposed. The vasorelaxant effect of D₁-like receptors was attributed to K_{ATP} channel opening in response to increased cAMP-dependent protein kinase (PKA) activity in one study (Kawano et al., 2008). In contrast, another study on these same arteries (Han et al., 1999) reported that BK_{Ca} channels, activated by D₁-like receptor stimulation, cause vasodilation via cAMP-dependent activation of cGMP-dependent protein kinase (PKG). D₁R and D₅R share similar expression patterns in the vasculature (Amenta et al., 2000, 2002), and both stimulate adenylyl cyclase activity to increase cAMP. D₁-like receptor stimulation also increases phospholipase C (PLC)/protein kinase C (PKC) activity (Felder et al., 1989; Banday and Lokhandwala, 2007), which enhances the ability of the D₁R to increase cAMP production (Yasunari et al., 1993) and vice versa (Yu et al., 1996). The D₅R may also stimulate phospholipase C PLC/PKC activity (Sahu et al., 2009). In contrast, increasing PKC inhibits constitutive and dopamine-mediated stimulation of cAMP production via D₅R (Jackson et al., 2005). Thus, D₁R and D₅R may increase cAMP by unique intracellular signaling pathways.

The ubiquitous BK_{Ca} channels are abundantly expressed in the endothelium and vascular smooth muscle in most arteries (Nelson and Quayle, 1995) and constitute the predominant potassium channel in human and porcine coronary myocytes

(White et al., 1995; Gollasch et al., 1996). The expression of the specific D₁-like receptor subtypes and their role in BK_{Ca} channel activation in human coronary artery smooth muscle cells (HCASMCs) are unknown. The present study aimed to test the hypothesis that stimulation of D₅R, not D₁R, activates BK_{Ca} channels in HCASMCs expressing both D₁R and D₅R and that this activation occurs via PKG not PKA.

Materials and Methods

Cell Culture. HCASMCs from human main coronary arteries (Lonza Walkersville, Inc., Walkersville, MD) were cultured to 80% confluence as described previously (White et al., 2002). Cells (passages 3–4) were plated on to 12-mm coverslips for patch-clamp experiments.

Dopamine Receptor Expression Studies. RT-PCR was performed on total RNA with SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with 15 µl of 2× reaction mix, 1 µl of RNA, 1 µl of SuperScript III RT/Taq, forward and reverse primers for D₁R and D₅R (1 µl each), and 11 µl of deionized H₂O, for a final reaction mixture of 30 µl. PCR conditions were as follows: 55°C for 30 min, 94°C for 2 min, 30 cycles of 94°C for 15 s, annealing temperature of 58°C for 30 s, and amplification at 68°C for 1 min and 68°C for 5 min. Primers (Table 1) were from Integrated DNA Technologies (Coralville, IA).

Quantitative real-time PCR (qRT-PCR) on ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using D₁R and D₅R primers from Integrated DNA Technologies (Coralville, IA) (Table 2) the following primers was performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) and normalized by GAPDH for quantification of D₁R and D₅R mRNA from threshold cycle numbers (C_T, i.e., 2^{−ΔΔCT}). GAPDH primers were obtained from Clontech (Mountain View, CA). Immunoblotting for D₁R and D₅R protein in whole cell lysates from 80% confluent HCASMCs (passages 4–5) was performed using polyclonal anti-rabbit D₁R (1:300, primary, 1 h; and 1:5000, secondary, 3 h; Sanada et al., 1999) and D₅R (1:300, primary, 1 h) and goat anti-rabbit (1:5000, secondary, 3 h). Immunoreactive bands were semiquantified as described previously (Zheng et al., 2003). The polyclonal D₅R antibody was raised against the peptide CRSSAACAPDTSLR and affinity-purified in our laboratory (Gildea et al., 2008).

Gene Silencing Experiments. We incubated 60% confluent HCASMCs in reduced serum media (Opti-MEM; Invitrogen) with D₁R or D₅R scrambled (Scr) or antisense (AS) oligonucleotides (50

ABBREVIATIONS: R, receptor; K_{ATP}, ATP-sensitive; BK_{Ca}, large conductance, calcium- and voltage-activated potassium; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PLC, phospholipase C; PKC, protein kinase C; HCASMC, human coronary artery smooth muscle cell; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Scr, scrambled; AS, antisense; I/O, inside-out; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; NP_o, number of channels × single-channel open probability; SCH 23390, (*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; KT 5720, (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*ij*][1,6]benzodiazocine-10-carboxylic acid; KT 5823, 9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*-11*a*-triazadibenzo(*a,g*) cycloocta(*cde*)-trinden-1-one; SKF 81297, (*R*)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol 1,4,5-trisphosphate; bp, base pair(s); ANOVA, analysis of variance.

TABLE 2
Primer sequences for real-time PCR

Receptor	Primer (5'-3')	Nucleotide Position
D ₁ R	F:TCTCCAAGGAGTGCAATCTG	1141-1161
	R:ATGGGTTGGATCTTCTCCAG	1111-1131
D ₅ R	F:AGCTGCCTACATCCACATGA	1220-1240
	R:TCCAGACAGACTCAGCAAC	1347-1367

F, forward; R, reverse.

nM, diluted 1:1000 in serum-free medium: Opti-MEM), tagged with Cy3 red dye (Integrated DNA Technologies, Inc.; Yasunari et al., 1993; Gildea et al., 2008), which had been incubated for 20 to 30 min with Lipofectamine 2000 (1:10 in serum-free medium). AS oligonucleotides were designed from published data (Gildea et al., 2008). Scr oligonucleotide sequences were obtained by using the Genscript website at <http://www.genscript.com>. The investigator was blinded to the identity of the oligonucleotides until final analysis of data.

Patch-Clamp Studies. In cell-attached patches, the recording chamber contained the following solution: 140 mM KCl, 10 mM MgCl₂, 0.1 mM CaCl₂, 10 mM HEPES, and 30 mM glucose, pH 7.4, at 22–25°C. Activity of the predominant ion channel in single HCASMCs was recorded (pCLAMP 9; Molecular Devices, Sunnyvale, CA) in cell-attached patches by filling the patch pipette with Ringer's solution: 110 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, and 10 mM HEPES (White et al., 1995, 2002). Voltage across the patch was controlled by clamping the cell at 0 mV with the high-concentration extracellular (bath) potassium solution. Excised inside-out (I/O) patches were studied with the same pipette solution described above, but a different bath solution containing 60 mM K₂SO₄, 2 mM MgCl₂, 0.16 mM CaCl₂, 1 mM BAPTA, (pCa = 7), 10 mM HEPES, 5 mM ATP, and 10 mM glucose, pH 7.4, at 22–25°C was used to verify calcium sensitivity. Currents were filtered at 1 kHz and digitized at 10 kHz. Average channel activity [number of channels × single-channel open probability (NP_o)] in patches with multiple BK_{Ca} channels was determined as described previously (White et al., 1995). Channel opening was quantified by its NP_o . Most single-channel data were analyzed at a potential of 40 mV, at which BK_{Ca} channels are easily distinguishable from other channel species, thus facilitating accurate statistical analysis. Voltage-clamp and voltage-pulse generation was controlled with an Axopatch 200B patch-clamp amplifier (Molecular Devices). Data were acquired and analyzed with pCLAMP 6.0 and 10.2 software (Molecular Devices). Nontransfected HCASMCs were studied in the cell-attached configuration before and 15 min after incubation with the D₁-like receptor partial agonist fenoldopam (1-aryl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol) (1 μM), and then they were treated with the D₁-like receptor antagonist SCH 23390 (10 μM). Next, nontransfected HCASMCs were pretreated with SCH 23390 (10 μM), the PKA antagonist KT 5720 (300 nM), or the PKG antagonist KT 5823 (300 nM), followed by treatment with D₁-like receptor agonists as described below. Successfully transfected red fluorescent cells were selected for patching. Transfected HCASMCs were incubated serially for 5 to 10 min each with increasing concentrations of the partial D₁-like receptor agonist fenoldopam (1 and 10 μM), the D₁-like receptor full agonist SKF 81297 (1 and 10 μM), and the endogenous dopamine receptor ligand dopamine (1 and 10 μM), and then channel activity was studied in the cell-attached configuration. Subsequently, the cells were treated with SCH 23390 (10 μM).

Drugs. Drugs, purchased from Sigma-Aldrich (St. Louis, MO), were freshly prepared from stock solutions and gently applied to the bath solution (1 ml) in the microscope chamber, to achieve the desired nominal concentration in approximately 5 min.

Statistical Analysis. All data are expressed as the mean ± S.E.M. Statistical significance between two groups was evaluated by Student's *t* test for paired data. Comparison among multiple groups was made by one-way analysis of variance test followed by Tukey's post hoc test. A probability (*P*) of less than 0.05 was considered to indicate a significant difference.

Results

Receptor Expression. D₁R and D₅R mRNA were expressed in HCASMCs, as demonstrated by RT-PCR. D₅R mRNA expression was 7-fold higher than D₁R mRNA expression, as determined by qRT-PCR (Fig. 1A).

Channel Identification. As expected, both cell-attached and excised inside-out patch-clamp recordings demonstrated that membrane electrical activity recorded from single HCASMCs was dominated by the BK_{Ca} channel. This channel was identified by its high-conductance (approximately 186.5 pS), characteristic large-amplitude (10 pA) and calcium- and voltage-activated outward current (Fig. 1, B and C). Channel activity from a cell-attached patch revealed minimal gating events ($NP_o = 0.001 \pm 0.001$; *n* = 3). In contrast, channel activity increased 1000-fold upon excision of the patch into the I/O configuration, where the cytoplasmic surface of the patch was exposed to an elevated “intracellular” calcium concentration ($NP_o = 1.18876 \pm 0.16$; *n* = 3). The addition of 1 mM tetraethylammonium (a selective inhibitor of BK_{Ca} channels at this concentration) to the bath (i.e., “cytoplasmic”) solution (Fig. 1B) in one set of cells or lowering the calcium concentration of the solution to resting levels (~100 nM) in another set of cells (Fig. 1C) both decreased channel opening probability back to baseline levels within 2 min. These properties, taken together, identify the prominent channel carrying outward currents in HCASMCs as the BK_{Ca} channel (White et al., 1995, 2002; Gollasch et al., 1996).

Transfection Studies. D₁R and D₅R protein expressions were down-regulated by transfection with specific D₁R and D₅R AS oligonucleotides. Oligonucleotide transfection was confirmed by immunoblotting. Nontransfected HCASMC lysates (control) blotted with D₁R antibody revealed a band between 50 and 75 kDa (~60 kDa) (Yu et al., 2004) that was decreased by approximately 50% in D₁R AS-transfected cells compared with nontransfected controls and D₁R Scr-transfected cells in 4/4 immunoblots (Fig. 2A). D₅R expression (~50-kDa bands) was unchanged in D₁R AS-transfected cells (Fig. 2B). Immunoblotting of nontransfected HCASMC lysates (control) with D₅R antibody revealed distinct bands of ~55 kDa (Gildea et al., 2008) that were decreased by approximately 50% in D₅R AS-transfected cells compared with D₅R Scr-transfected cells and nontransfected controls (Fig. 2C). D₁R expression was unchanged in D₅R AS-transfected cells (Fig. 2D). These studies confirm the specificity of D₁R or D₅R down-regulation caused by their specific AS oligonucleotides.

Patch-Clamp Studies. D₁-like receptor agonists activated BK_{Ca} channels in nontransfected HCASMCs. This response was prevented and reversed by the D₁-like receptor antagonist SCH 23390. Only limited channel activity was observed in nontransfected HCASMCs ($NP_o = 0.0225 \pm 0.005$; *n* = 9 cells). In contrast, channel activity increased dramatically (~22-fold) after treatment with fenoldopam ($NP_o = 0.4965 \pm 0.119392$; *n* = 9 cells, each cell was treated serially with both fenoldopam and SCH 23390). D₁-like receptor-stimulated channel activity was reversed by subsequent addition of the D₁-like receptor antagonist SCH 23390 ($NP_o = 0.0157 \pm 0.005$; Fig. 3A). Pretreatment of nontransfected HCASMCs ($NP_o = 0.0038 \pm 0.0001$) with SCH 23390 ($NP_o = 0.0015 \pm 0.0001$) prevented fenoldopam (1 and 10 μM) from increasing BK_{Ca} channel activity ($NP_o = 0.0025 \pm 0.0004$ and 0.00373 ± 0.0003 , respectively; Fig. 3B) in three of three cells, each being treated serially with

Fig. 1. D₁R and D₅R mRNA are expressed in HCASMCs in culture, and the prominent outward current is the BK_{Ca} channel. A, 2% agarose gel electrophoresis of RT-PCR products of D₁R (418 bp) and D₅R (212 bp) mRNA from HCASMC. M, marker (100 bp) (top). D₅R mRNA expression was 7-fold greater than D₁R mRNA expression in HCASMCs by qRT-PCR, normalized to GAPDH, and calculated from threshold cycle numbers [$2^{-\Delta\Delta CT} = 7.07 \pm 0.08$ (mean $\pm$ S.E.M.; $n = 3$; bottom)]. B, representative recordings from the same membrane patch in the cell-attached configuration (100 nM Ca²⁺, +40 mV; control), after excision as an I/O patch (100 μ M Ca²⁺, +40 mV; I/O patch), and 2 min after treatment with 1 mM tetraethylammonium (TEA), which abolished channel activity. C, representative recordings from the same membrane patch in the cell-attached configuration (100 nM Ca²⁺, +40 mV; control) and after excision as an I/O patch (100 μ M Ca²⁺, +40 mV; I/O patch) show a dramatic increase in channel activity in the I/O patch, with outward currents of 5 and 10 pA as per the amplitude histogram. The 5-pA currents may represent nonspecific channels. Two minutes after treatment with inside-out solution (100 nM Ca²⁺, +40 mV; I/O solution), channel activity decreased to baseline levels. Upward deflections are channel openings from the channel closed state (dashed line).

Fig. 2. D₅R protein expression, but not D₁R expression, is down-regulated in HCASMCs transfected with D₅R AS oligonucleotides. A, D₁R protein (~60 kDa) was decreased in D₁R AS-transfected cells compared with nontransfected controls and D₁R Scr-transfected cells (*, $P < 0.05$, one-way ANOVA, Tukey's post hoc test; $n = 4$), normalized by human β -actin (38 kDa). B, D₅R protein expression (~55 kDa) was not decreased in D₁R AS- and D₁R Scr-transfected HCASMCs compared with nontransfected controls, normalized by human β -actin ($n = 4$). C, D₅R protein (~55 kDa) was decreased in D₅R AS-transfected cells compared with nontransfected controls and D₅R Scr-transfected cells (*, $P < 0.05$, one-way ANOVA, Tukey's post hoc test; $n = 4$), normalized by human β -actin ($n = 4$). D, D₁R protein (~60 kDa) was not decreased in D₅R AS- and D₅R Scr-transfected HCASMCs compared with nontransfected controls, normalized by human β -actin ($n = 4$). Data are expressed as mean $\pm$ S.E.M. n refers to the number of immunoblotting experiments in each group.

both drugs. Thus, D₁-like receptors mediate BK_{Ca} channel activation in HCASMCs.

The next series of experiments indicated that D₁-like receptor-mediated activation of BK_{Ca} channels in HCASMCs occurred via PKG and not PKA. Pretreatment of HCASMCs with the PKG inhibitor KT5823 markedly attenuated BK_{Ca} channel response to dopamine and selective D₁-like receptor agonists in the cell-attached configuration. To confirm the presence of BK_{Ca} channels in these patches, calcium-stimulated activity (I/O configuration) was measured as a positive control (Fig. 4A). In contrast, BK_{Ca} channel activation by fenoldopam in nontransfected HCASMCs was neither prevented nor reversed by the PKA inhibitor KT 5720 (300 nM; 20 min). However, subsequent inhibition of PKG by KT 5823 (300 nM) abrogated channel activation within 20 min (Fig. 4B).

D₁-like receptor-mediated activation of BK_{Ca} channels in HCASMCs was not attenuated in cells transfected with D₁R AS

oligonucleotides. HCASMCs transfected with D₁R Scr (Fig. 5A) oligonucleotides served as controls and demonstrated significant concentration-dependent BK_{Ca} channel stimulation with fenoldopam and SKF 81297. Even when the D₁R was down-regulated with D₁R AS oligonucleotide transfection, concentration-dependent BK_{Ca} channel activation was observed after treatment with fenoldopam and SKF 81297, and profound activation was seen with dopamine (10 μ M; Fig. 5B).

Comparable stimulation of BK_{Ca} channel activity was seen in HCASMCs transfected with D₅R Scr oligonucleotides (Fig. 6A), although the magnitude of the increase in channel-gating events varied stochastically between D₁R Scr (Fig. 5A) and D₅R Scr (Fig. 6A) groups. These effects were reversed by SCH 23390 (10 μ M; Figs. 5, A and B, and 6A). In contrast, neither D₁-like receptor agonists nor dopamine could activate BK_{Ca} channels in D₅R AS-transfected HCASMCs (Fig. 6B). Control NP_o (0.00493 $\pm$ 0.005; n = 5) was not altered significantly with increasing concentrations of D₁-like receptor partial or

Fig. 3. The D₁-like receptor partial agonist fenoldopam activates BK_{Ca} channels in HCASMCs. **A**, electrophysiological recordings representing 10 to 15 s of continuous activity from a single HCASMC in the cell-attached configuration before (control) and after treatment with 1 μ M fenoldopam show activation of BK_{Ca} channels ($\sim$ 10 pA). Upward deflections are channel openings from the channel-closed state. Larger deflections ($>$ 10 pA) may represent superimposed BK_{Ca} channel opening on control openings of predominantly BK_{Ca} channels, although amplitudes between 10 and 20 pA may represent other channels. The predominant channel stimulated by fenoldopam conducted 10-pA current as per the amplitude histogram. Subsequent addition of the D₁-like receptor antagonist 10 μ M SCH 23390 (SCH) reversed the stimulatory effect of fenoldopam and decreased channel opening to pretreatment levels ($+40$ mV; *, P < 0.05 versus others, one-way ANOVA, Tukey's post hoc test; n = 5 cells, each cell was treated serially with both fenoldopam and SCH 23390; bottom). **B**, electrophysiological recordings representing 10 to 15 s of continuous activity (top) from a single HCASMC in the cell-attached configuration. BK_{Ca} channel opening in nontransfected HCASMCs (control) was unchanged by treatment with 10 μ M SCH or subsequent treatment with fenoldopam (1 and 10 μ M). Smaller upward deflections may represent nonspecific cationic channels in the patch. BK_{Ca} channel activation was not seen with fenoldopam in five of five SCH-treated cells tested (bottom). Data are expressed as mean $\pm$ S.E.M.

Fig. 4. PKG inhibition, but not PKA inhibition, prevented and reversed D₁-like receptor-mediated activation of BK_{Ca} channels in HCASMCs. **A**, electrophysiological recordings in the cell-attached configuration representing 10 to 15 s of continuous activity from a single HCASMC with the PKG inhibitor KT 5823 (300 nM; 30 min), the D₁-like receptor partial agonist fenoldopam (1 and 10 μ M), the D₁-like receptor full agonist SKF 81297 (1 and 10 μ M), and the endogenous dopamine receptor ligand dopamine (10 and 100 μ M) demonstrated no increase in BK_{Ca} channel activity (*, $P < 0.001$ versus others, one-way ANOVA, Tukey's post hoc test; $n = 6$ cells, each being treated serially with all drugs mentioned above; bottom). **B**, electrophysiological recordings in the cell-attached configuration representing 10 to 15 s of continuous activity (top) from a single HCASMC in untreated cells (control) and after serial incubation with the PKA inhibitor KT 5720 (300 nM; 20 min), fenoldopam (1 μ M; 5 min), and a second treatment with KT 5720 (300 nM; 20 min) show neither prevention nor reversal of fenoldopam-induced increases in BK_{Ca} channel activity with KT 5720. Finally, addition of the PKG inhibitor KT 5823 (300 nM; 20 min) into the recording chamber reversed channel stimulation within 15 min in all cells tested (*, $P < 0.05$ versus others, one-way ANOVA, Tukey's post hoc test; $n = 6$ cells, each being treated serially with all drugs mentioned above; bottom). Data are expressed as mean $\pm$ S.E.M.

full agonists, or even 100 μ M dopamine ($NP_o = 0.00124 \pm 0.0003$; $n = 5$ cells, each being serially treated with all drugs mentioned). Excision of patches from D₅R AS-transfected cells into the I/O configuration, which exposed the cytoplasmic surface of the membrane to 100 μ M calcium demonstrated substantial calcium-stimulated channel activity in D₅R AS-transfected cells (Fig. 6B, inset, inside-out), confirming the presence of the channel in the patch.

Discussion

In summary, D₁-like receptor-mediated activation of BK_{Ca} channels in HCASMCs occurs by the stimulation of the D₅R, not D₁R, signaling via PKG, not PKA. This study is the first report of BK_{Ca} channel activation in HCASMCs by D₁-like receptor stimulation. The results obtained differ from the

previously reported dopamine-mediated activation of K_{ATP} channels in porcine coronary myocytes (Kawano et al., 2008). One possible reason for this apparent difference could be the use of calcium-free (EGTA-containing) intra- and extracellular solutions in the earlier study. Because BK_{Ca} channels require and are very sensitive to intracellular calcium levels, it is quite possible that activation of these channels might have been artificially suppressed in the study. Therefore, dopamine-induced activation of BK_{Ca} channels could not be demonstrated in that study, nor could there be an identification of the specific D₁-like receptor (D₁R versus D₅R) mediating the response.

Cellular functions of the specific D₁-like receptor (D₁R versus D₅R) have eluded explanation as they are currently pharmacologically indistinguishable with commercially available ligands. We used receptor gene silencing to ad-

Fig. 5. Dopamine and D₁-like receptor agonists activate BK_{Ca} channels in HCASMCs transfected with D₁R Scr and AS oligonucleotides. A, electrophysiological recordings in the cell-attached configuration representing 10 to 15 s of continuous activity (top) from a single HCASMC transfected with D₁R Scr oligonucleotides (control) and 5 to 10 min after serial addition of increasing concentrations of D₁-like receptor partial agonist fenoldopam (1 and 10 μ M) and full agonist SKF 81297 (1 and 10 μ M) show progressive increases in BK_{Ca} channel activity, which were reversed 10 min after treatment with the D₁-like receptor antagonist 10 μ M SCH 23390 (SCH) (*, $P < 0.05$, one-way ANOVA versus control and SCH-treated cells, Tukey's post hoc test; $n = 5$ cells, each being treated serially with drugs mentioned above; bottom). B, electrophysiological recordings in the cell-attached configuration representing 10 to 15 s of continuous activity (top) from a single HCASMC transfected with D₁R AS oligonucleotides (control) and 5 to 10 min after serial addition of increasing concentrations of the D₁-like receptor partial agonist fenoldopam (1 and 10 μ M), full agonist SKF 81297 (1 and 10 μ M), and endogenous dopamine receptor ligand dopamine (10 μ M) show progressive increases in BK_{Ca} channel activity, which were reversed 10 min after treatment with 10 μ M SCH (*, $P < 0.05$, one-way ANOVA versus control and SCH-treated cells, Tukey's post hoc test; $n = 5$ each being treated serially with drugs mentioned above; bottom). Data are expressed as mean $\pm$ S.E.M.

dress this issue using AS oligonucleotides of D₁R and D₅R (Gildea et al., 2008) to distinguish their individual effects on specific receptor-channel pathways. To our knowledge, this is the first report of single-channel recordings from Lipofectamine-transfected HCASMCs. These studies demonstrate a reliable method for evaluating the real-time effects of receptor-specific stimulation on signaling pathways that ultimately influence ion channel function in human cells. Although total cellular D₅R protein expression was decreased by only 50% by AS transfection, a dramatic functional difference in electrophysiological properties with complete abrogation of the BK_{Ca} channel activation in response to D₁-like receptor agonists was observed. The receptor protein expression was measured using protein from whole cell lysates and not exclusively membrane fractions and thus may not directly correlate with ion channel function. Even so, the unequivocal effect of decreasing D₅R expression suggests that a "critical threshold" level of D₅ receptor expression is neces-

sary to trigger the cascade of intracellular events that lead to BK_{Ca} channel activation and supports the notion that there are no dopamine "spare" receptors in HCASMCs, similar to previous reports in other molecular expression systems (Watts et al., 1995). Furthermore, the absence of activation of BK_{Ca} channels in D₅R AS-transfected cells (five of five cells) compared with D₅R Scr-transfected cells, D₁R Scr-, and D₁R AS-transfected cells even with high concentrations of dopamine (100 μ M) indicates that an intact and full complement of D₅R is essential for dopamine to activate BK_{Ca} channels in HCASMCs in vitro. The above-mentioned phenomena thus support a primary role of the D₅R in D₁-like receptor-mediated BK_{Ca} channel activation.

Classically, cAMP/PKA mediates most D₁-like receptor-mediated cellular responses. (Sibley and Monsma, 1992; Yasunari et al., 1993; Sanada et al., 1999; Jose et al., 2003; Hussain and Lokhandwala, 2003; Zheng et al., 2003; Gildea et al., 2008). PKA and/or PKG could activate the BK_{Ca} channel by modulat-

Fig. 6. D₁-like receptor agonists and dopamine activate BK_{Ca} channels in HCASMCs transfected with D₅R AS oligonucleotides. A, electrophysiological recordings in the cell-attached configuration representing 10 to 15 s of continuous activity (top) from a single HCASMC transfected with D₅R AS oligonucleotides (control) and 5 to 10 min after serial addition of increasing concentrations of D₁-like receptor partial agonist fenoldopam (1 and 10 μM) and full agonist SKF 81297 (1 and 10 μM) show progressive increases in BK_{Ca} channel activity, which were reversed 10 min after treatment with the D₁-like receptor antagonist 10 μM SCH 23390 (SCH) (*, $P < 0.05$, one-way ANOVA versus control and SCH-treated cells, Tukey's post hoc test; $n = 5$ cells, each being treated serially with drugs mentioned above; bottom). B, electrophysiological recordings in the cell-attached configuration representing 10 to 15 s of continuous activity (top) from a single HCASMC transfected with D₅R AS oligonucleotides (control) and 5 to 10 min after serial addition of increasing concentrations of the D₁-like receptor partial agonist fenoldopam (1 and 10 μM), full agonist SKF 81297 (1 and 10 μM), and nonspecific dopamine receptor agonist dopamine (10 and 100 μM) show no increases in BK_{Ca} channel opening in D₅R AS-transfected HCASMCs (+40 mV; $n = 5$ cells, each being treated serially with drugs mentioned above), whereas subsequent treatment with the D₁-like receptor antagonist 10 μM SCH tended to decrease channel opening (bottom); the apparent decrease was not statistically significant. Data are expressed as mean $\pm$ S.E.M. Inset shows inside-out configuration demonstrating the presence of a calcium-sensitive channel in the patch at the end of the experiment in HCASMCs transfected with D₅R AS oligonucleotides.

ing its calcium and voltage sensitivities (Schubert and Nelson, 2001). PKA has been reported previously to be ineffective at stimulating BK_{Ca} channel activity in porcine coronary arteries (White et al., 2000). However, PKG is known to mediate BK_{Ca} channel activation (Brenner et al., 2000). Our findings in human cells support the essential role of PKG in BK_{Ca} channel activation: PKG inhibition with KT 5823 prevented channel activation in nontransfected HCASMCs in response to partial and full D₁-like receptor agonists and dopamine (100 μM) and also reversed fenoldopam-stimulated BK_{Ca} channel activity. In contrast, a PKA inhibitor KT 5720 neither prevented nor reversed BK_{Ca} channel stimulation in response to fenoldopam, consistent with previous observations in porcine coronary artery cells. The specificity of KT 5720 (300 nM) and KT 5823 (300 nM) on PKA and PKG activity, respectively, has been confirmed by enzyme assays in previous studies (White et al., 2000; Zhu et al., 2002).

The above-mentioned findings suggest that BK_{Ca} channel stimulation by D₁-like receptor agonists occurs via cAMP-

dependent cross-activation of PKG rather than PKA. Although D₁-like receptor stimulation activates cGMP in canine renal glomeruli (Wargo et al., 1982), previous studies of BK_{Ca} channel stimulation by dopamine and other ligands in various vascular beds support the cross-activation hypothesis (Zhu et al., 2002; Barman et al., 2003). Furthermore, dopamine has a greater stimulatory effect on PKG rather than PKA activity in porcine coronary arteries, despite the fact that it increases cAMP, but not cGMP levels, in this tissue (White et al., 2000). Vascular myocytes are a rich source of PKG, which phosphorylates the BK_{Ca} channel to regulate vascular tone (Robertson et al., 1993). In a reconstituted cell-free (I/O) system, treatment with "purified" PKG led to dramatic BK_{Ca} channel activation in porcine coronary artery cells (White et al., 2000). After balloon angioplasty, human coronary arteries exhibit decreased vasodilation and a predisposition for restenosis, together with decreased PKG expression (Anderson et al., 2000). The present study sug-

gests a possible role for PKG in coronary vasodilatation, probably by its effect on BK_{Ca} channels; however, further studies are needed to confirm this mechanism in intact human coronary arteries in vivo.

Shared signaling pathways of D₅R and D₁R have been described in the literature, but there are differences reported too. Activation of D₁-like receptors (D₁R or D₅R) increases cAMP generation, activates PKA (Sibley and Monsma, 1992; Jose et al., 2003; Hussain and Lokhandwala, 2003; Gildea et al., 2008) and also stimulates PLC activity via cAMP-independent mechanisms (Felder et al., 1989; Yasunari et al., 1993; Hussain and Lokhandwala, 1997; Banday and Lokhandwala, 2007). However, D₁-like receptor agonists stimulate PLC activity in discrete brain regions in mice lacking D₁R (Friedman et al., 1997) but not in mice lacking D₅R (Sahu et al., 2009), which suggests that D₅R, not D₁R, is responsible for D₁-like receptor activation of PLC, at least in neural cells. D₅R-mediated increase in PLC activity should decrease levels of phosphatidylinositol 4,5-bisphosphate (PIP₂), an independent activator of BK_{Ca} channels in the cerebral vasculature (Vaithianathan et al., 2008). Thus, D₅R probably does not activate these channels in HCASMCs via PIP₂. Hydrolysis of PIP₂ yields 1,2-diacylglycerol (which activates PKC) and inositol 1,4,5-trisphosphate (IP₃). BK_{Ca} channels are inhibited by PKC-dependent and -independent pathways, so the stimulatory effects of D₅R on BK_{Ca} channels cannot be attributable to an increase in PKC. Although hydrolysis of PIP₂ also yields IP₃, which increases BK_{Ca} channel activity (Cai et al., 2005), D₅R has been shown to decrease IP₃ levels (White et al., 1999), decreasing the likelihood of IP₃ mediating effects of D₅R on BK_{Ca} channels. Our findings support the notion that D₅R-mediated activation of the BK_{Ca} channel occurs via cAMP/PKG pathways (Robertson et al., 1993; White et al., 1995, 2000; Han et al., 1999), which must be confirmed in future studies. For example, given the exquisite calcium sensitivity of the BK_{Ca} channel, the effects of D₅R stimulation on intracellular calcium also beg elucidation in future studies. A limitation of the present study is that it is performed on cells in culture: culture conditions could influence BK_{Ca} channel activity. However, our observations are entirely consistent with previously reported research performed in freshly obtained porcine coronary myocytes (Han et al., 1999). Furthermore, channel opening in response to drug treatments was compared with control measurements in the same cell, and all groups of cells were studied under identical culture conditions. Although relative concentrations/affinity of D₁R and D₅R may also be affected by culture conditions, all experiments were performed on early passaged cells (passages 3–4) in all groups. No significant differences in receptor protein expression or control BK_{Ca} channel opening were observed between cells within the respective groups studied, other than inherent stochastic variations in channel-gating events.

Hypertension, diabetes, aging, and coronary artery disease are all associated with endothelial dysfunction. Therefore, hyperpolarization of vascular smooth muscle cells via BK_{Ca} channels may be an important compensatory mechanism to help maintain flow-mediated vasodilatation in human coronary arterioles (Miura et al., 2001). A better mechanistic understanding of the differential effects of D₁R and D₅R on coronary vascular function could lead to the emergence of novel therapeutic targets for coronary artery disease in humans.

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