cAMP-Dependent Vasodilators Cross-Activate the cGMP-Dependent Protein Kinase to Stimulate $\text{BK}_{\text{Ca}}$ Channel Activity in Coronary Artery Smooth Muscle Cells

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Abstract—cAMP-dependent vasodilators are used to treat a variety of cardiovascular disorders; however, the signal transduction pathways and effector mechanisms stimulated by these agents are not fully understood. In the present study we demonstrate that cAMP-stimulating agents enhance the activity of the large-conductance, calcium-activated potassium ($\text{BK}_{\text{Ca}}$) channel in single myocytes from coronary arteries by “cross-activation” of the cGMP-dependent protein kinase (protein kinase G, PKG). Single-channel patch-clamp data revealed that 10 $\mu$mol/L isoproterenol, forskolin, or dopamine opens $\text{BK}_{\text{Ca}}$ channels in coronary myocytes and that this effect is attenuated by inhibitors of PKG (KT5823; Rp-8-pCPT-cGMPS), but not by inhibiting the cAMP-dependent protein kinase (protein kinase A, PKA). In addition, a membrane-permeable analog, CPT-cAMP, also opened $\text{BK}_{\text{Ca}}$ channels in these myocytes, and this effect was reversed by KT5823. Direct biochemical measurement confirmed that dopamine or forskolin stimulates PKG activity in coronary arteries but does not elevate cGMP. Finally, the stimulatory effect of cAMP on $\text{BK}_{\text{Ca}}$ channels was reconstituted in a cell-free, inside-out patch by addition of purified PKG activated by either cGMP or cAMP. In contrast, channel gating was unaffected by exposure to the purified catalytic subunit of PKA. In summary, findings from on-cell and cell-free patch-clamp experiments provide direct evidence that cAMP-dependent vasodilators open $\text{BK}_{\text{Ca}}$ channels in coronary myocytes by cross-activation of PKG (but not via PKA). Biochemical assay confirmed this cross-activation mechanism of cAMP action in these arteries. This signaling pathway is a novel mechanism for regulation of potassium channel activity in vascular smooth muscle and other cells. (Circ Res. 2000;86:897-905.)

Key Words: cAMP ▪ protein kinase G ▪ $\text{BK}_{\text{Ca}}$ channel ▪ coronary ▪ cross-activation

Early studies investigating the cellular effects of cyclic nucleotides demonstrated antagonism between cAMP and cGMP in most tissues. An exception to this general rule was vascular smooth muscle (VSM), in which both nucleotides produced the same physiological response, ie, vasodilation; however, the signaling mechanisms by which cAMP or cGMP induced this response were unknown. Most investigations assumed that the vasodilatory response to either nucleotide was mediated via a distinct transduction cascade involving its corresponding nucleotide-activated protein kinase; however, research over the last 10 years has revealed a more complicated signaling process. Findings from the laboratories of Corbin and Lincoln demonstrated that “cross-activation” of the cGMP-dependent protein kinase (protein kinase G, PKG) by cAMP could be a key element in the signal transduction cascade of cAMP-induced vasodilation.

Subsequent studies have established that cGMP can also exert physiological effects by stimulating cAMP-dependent protein kinase (protein kinase A, PKA) activity. For example, PKA appears to mediate nitric oxide–dependent inhibition of aortic smooth muscle cell proliferation and cGMP-stimulated intestinal chloride transport. In addition to identifying which protein kinase(s) mediates cyclic nucleotide–induced vasodilation, characterization of signaling events downstream from kinase activation remains incomplete.

Decreasing the level of cytosolic calcium in VSM promotes vasodilation. Therefore, it is not surprising that vasodilatory cyclic nucleotides influence the activity of effector mechanisms controlling [$\text{Ca}^{2+}$], and that cross-activation of cyclic nucleotide–dependent protein kinases may contribute to these vascular effects. For example, forskolin-induced phosphorylation of the inositol 1,4,5-triphosphate (IP$_3$) receptor from rat aorta was inhibited more effectively by antagonists of PKG than by PKA blockers. In addition, forskolin-stimulated reduction of agonist-induced calcium release in aortic myocytes required PKG, but not PKA (although both kinases appear to inhibit IP$_3$-dependent calcium release in visceral smooth muscle). Other studies have suggested that kinase cross-activation mediates upregulation of VSM endothelin receptors and phosphorylation of myosin light chain kinase. Thus, there is increasing evidence that a variety of vascular effector mechanisms involve crossover reactivity of cAMP and/or cGMP.
Modulation of ion channel activity has profound effects on vascular tone, and there is evidence for regulation of calcium channels by cyclic nucleotide cross-activation.\(^{13}\) A recent study using selective kinase inhibitors also indicated crossover effects of both cAMP and cGMP in smooth muscle from portal vein, with PKA stimulating and PKG inhibiting calcium channel activity.\(^{14}\) In addition to influencing calcium channel activity, both cGMP and cAMP are important modulators of potassium channel activity in VSM and other cell types,\(^{15}\) but the potential importance of nucleotide cross-activation in regulation of these proteins has received little attention. We recently presented evidence suggesting that dopamine opened potassium channels by cAMP cross-reactivity with PKG.\(^{16}\) The purpose of the present study was to undertake a thorough investigation of this phenomenon by combining single-channel patch-clamp techniques and direct biochemical measurements to investigate the possibility that potassium channel activity in VSM is modulated by cyclic nucleotide cross-activation of protein kinases. Our findings indicate that elevation of either cAMP or cGMP stimulates the activity of the large-conductance, calcium- and voltage-activated potassium (BK\(_{Ca}\)) channel in myocytes isolated from porcine coronary arteries via PKG-dependent phosphorylation.

**Materials and Methods**

**Patch-Clamp Studies**

Fresh porcine hearts were obtained from local abattoirs. The left anterior descending coronary artery was excised and placed into ice-cold Krebs-Henseleit buffer solution of the following composition (in mmol/L): NaCl 122, KCl 4.7, CaCl\(_2\) 1.7, KH\(_2\)PO\(_4\) 1.2, MgCl\(_2\) 1.2, CaCl\(_2\) 1.8, and glucose 11.5. pH 7.2. Myocytes were isolated by a modification of a procedure described previously.\(^{17}\) For Ringer’s solution (in mmol/L), NaCl 110, KCl 5, MgCl\(_2\) 1, CaCl\(_2\) 0.16, BAPTA 1, (pCa 7), HEPES 10. Voltage across the patch was controlled by clamping the cell at 0 mV with the high-concentration extracellular K\(_1^+\) and HEPES 10. ATP currents in cell-attached patches, several drops of cell suspension were placed in a recording chamber containing (in mmol/L) KCl 140, MgCl\(_2\) 10, CaCl\(_2\) 0.1, HEPES 10, and glucose 30 (pH 7.4; 22°C to 25°C). Activity of single potassium channels was recorded (pCLAMP 6.0.4) isolated by a modification of a procedure described previously.\(^{17}\) For Ringer’s solution (in mmol/L), Tris-HCl 50 (pH 7.5), MgCl\(_2\) 20, and MnCl\(_2\) 10; 20 molar ice-cold Krebs-Henseleit buffer solution of the following composition: NaCl 110, KCl 5, MgCl\(_2\) 1, CaCl\(_2\) 0.1, glucose 30 (pH 7.4; 22°C to 25°C). The pipette solution was the same Ringer’s solution described above. Currents were filtered at 2 kHz and digitized at 10 kHz. Average channel activity (number of channels×open probability, NPo) in patches with multiple BK\(_{Ca}\) channels was determined as described previously.\(^{16–19}\) NPo calculations were based on 10 to 15 seconds of continuous recording during periods of stable channel activity.

**Cyclic Nucleotide Measurements**
cAMP and cGMP were measured by using an enzyme immunoassay kit (Biomol), which included all reagents, antibodies, and microtiter plates. Briefly, arteries were exposed to a single concentration of (in mmol/L) dopamine 10 to 100, forskolin 10, or sodium nitroprusside 30 for 30 minutes with 0.1 mmol/L isobutylmethylxanthine (IBMX). Reactions were stopped by adding 0.1N HCl and boiling for 5 minutes. The precipitated protein was removed by centrifugation. After colorimetric analysis, nucleotide levels were expressed as fmol of nucleotide per mg tissue weight.

**Protein Kinase Assays**
Arteries were incubated in Krebs-Henseleit solution with IBMX (0.1 mmol/L) and exposed to a single concentration of 10 µmol/L dopamine, forskolin, or sodium nitroprusside for 15 minutes, after which the smooth muscle tissue was finely chopped and suspended in a 4-volume ice-cold homogenization buffer (in mmol/L, Tris-HCl [pH 7.4] 20, DTT 1, EGTA 1, EDTA 1, and PMSF 1; 10 µg/mL leupeptin; 2 µg/mL aprotinin; and 0.1% Triton X-100), homogenized, and centrifuged at 13,000 g (4°C) for 15 minutes. The supernatant was used as a tissue extract for determination of kinase activity. Protein concentrations were determined by the standard method of Lowry et al.\(^{20}\) Kinase activity was determined by measuring \(^{32}\)P incorporation from [γ-\(^{32}\)P]ATP into the serine residue of the synthetic peptide “Kemptide”, containing a specifically designed sequence that governs high affinity to PKG and PKA.\(^{21}\) Reactions occurred in a total volume of 50 µL that contained (in mmol/L) Tris-HCl 50 (pH 7.5), MgCl\(_2\) 20, and MnCl\(_2\) 10; 20 µL of tissue extract; 100 µmol/L Kemptide; 100 µmol/L ATP, 0.5 µCi [γ-\(^{32}\)P]ATP (4 mCi/µmol); 0.1 µg/mL BSA; and the phosphatase inhibitors (in mol/L) β-glyceroephosphosphate 50, sodium pyrophosphate 1, and sodium vanadate 0.1. Background for PKA and PKG activity was determined from parallel incubations containing the highly selective competitive inhibitor PKI(6–22)-amide (2 µmol/L; for PKA) or KT5823 (300 µmol/L; for PKG) and was always <20% of total Kemptide phosphorylation.\(^{22}\) \(^{32}\)P incorporation was determined by liquid scintillation counting.

**Drugs**
BAPTA, KT5720, and KT5823 were purchased from Calbiochem. The purified catalytic subunit of PKA and purified PKG were purchased from Promega. Guanosine 3’5’-cyclic monophosphorothioate, 8-(4-chlorophenylthio), Rp isomer (Rp-8-pCPT-cGMPs) was purchased from Biolog. [γ-\(^{32}\)P]ATP was purchased from Amersham. All other agents were purchased from Sigma.

**Statistical Analysis**
All data are expressed as mean±SE. Statistical significance between 2 groups was evaluated by Student t test for paired data. Comparison among multiple groups was made by one-way ANOVA test. A probability of <0.05 was considered to indicate a significant difference.

**Results**

**Identification of BK\(_{Ca}\) Channels**
Single-channel patch-clamp studies of coronary myocytes revealed membrane electrical activity to be dominated by a prominent, large-conductance (119±4 pS (n=5) physiological gradients of potassium; 221±11 pS (n=4) symmetrical potassium gradients) channel carrying outward potassium currents. Activity of this channel was enhanced dramatically by increasing [Ca\(^{2+}\)] at the cytoplasmic surface of inside-out membrane patches (Figure 1). Channel activity recorded from a cell-attached patch revealed minimal gating events (NPo~0). In contrast, excising this same patch into an inside-out configuration in which the “cytoplasmic” [Ca\(^{2+}\)] was now 100 µmol/L stimulated channel activity dramatically (Figure 1, middle panel). On average, channel NPo was increased to 0.39±0.05 by calcium (n=4, P<0.001). When the [Ca\(^{2+}\)] of the bath solution was then buffered to more physiological levels (0.1 µmol/L), channel activity was reduced back toward basal levels (NPo 0.007±0.003; n=4). In other experiments on inside-out patches, activity of this channel was nearly abolished by exposure to 1 mmol/L tetraethylammonium (TEA; eg, Figure 7B), which at this concentration is a selective inhibitor for BK\(_{Ca}\) channels.
Therefore, we have identified this protein as the high-conductance, calcium- and TEA-sensitive BK Ca channel, which is the predominant potassium channel species in both porcine17 and human23 coronary smooth muscle.

cAMP-Dependent Vasodilators Stimulate BK Ca Channel Activity

Stimulation of the cAMP signaling pathway with a β-adrenoceptor agonist (isoproterenol) or a direct activator of adenylyl cyclase (forskolin) enhanced BK Ca channel activity in coronary myocytes. In experiments on cell-attached patches, exposure of myocytes to isoproterenol (10 μmol/L) increased channel open probability nearly 20-fold, from an NPo of 0.01 ± 0.008 to 0.174 ± 0.05 (n=4; P<0.03; Figure 2A). Similarly, 10 μmol/L forskolin also increased BK Ca channel activity dramatically in cell-attached patches (NPo 0.01 ± 0.005 to 0.314 ± 0.07; n=8; P<0.003; Figure 2B).

Stimulating the cAMP signaling cascade with 10 μmol/L dopamine also enhanced BK Ca channel activity significantly, from an average NPo of near 0 to 0.332 ± 0.07 in 3 of 3 cell-attached patches (Figure 3).

It might be assumed that the stimulatory effect of these cAMP-dependent vasodilators involved stimulation of PKA; however, treating cells with inhibitors of PKA did not attenuate cAMP-stimulated BK Ca channel activity. For example, 300 nmol/L KT5720 did not affect dopamine-stimulated channel activity significantly (NPo: dopamine, 0.332 ± 0.07; dopamine + KT5720, 0.464 ± 0.16; n=3; Figure 3), and we had shown previously that Rp-8-pCPT-cAMPS did not antagonize the effect on dopamine on BK Ca channels.16 In contrast, subsequent addition of 10 μmol/L Rp-8-pCPT-cGMPS, an inhibitor of PKG, completely reversed the stimulatory effect of dopamine on channel activity (NPo 0.04 ± 0.04; n=3; P=0.001; Figure 3). In similar experiments 300 nmol/L KT5823 reversed forskolin-stimulated BK Ca channel activity in cell-attached patches by an average of 95.7 ± 3% (n=3). At this concentration, KT5823 exhibits high selectivity for PKG over PKA. Control experiments indicated that neither KT5720, KT5823, nor Rp-8-pCPT-cGMPS had any direct effects on BK Ca channel activity in inside-out patches (n=4). These findings suggested that increasing intracellular levels of cAMP stimulated the activity of BK Ca channels via cross-activation of PKG. Subsequent biochemical and electrophysiological experiments were performed to test this hypothesis.

Cyclic Nucleotide Levels

Results from enzyme immunoassay demonstrated ~5 times more cAMP than cGMP in coronary artery smooth muscle.
under nonstimulated conditions, and these results are consistent with those of Francis et al., demonstrating that basal levels of cAMP were 5 times higher than those of cGMP in these same arteries. Dopamine increased cAMP accumulation in coronary smooth muscle in a concentration-dependent fashion (Figure 4A). At the concentration of dopamine used in our patch-clamp studies (10 μmol/L), dopamine elevated [cAMP] by 6-fold (n=4; P<0.05), whereas higher dopamine concentrations produced a greater augmentation (50 μmol/L, 10-fold; 100 μmol/L, 14-fold). In addition to dopamine, 10 μmol/L forskolin increased cAMP accumulation 133-fold (n=4; P<0.05). In contrast to their effects on cAMP accumulation, neither dopamine (10 to 100 μmol/L; Figure 4B) nor forskolin (10 μmol/L; data not shown) increased the concentration of cGMP (n=4). As a positive control, either 0.5 or 10 μmol/L sodium nitroprusside increased [cGMP] 17- or 30-fold, respectively, over control levels (n=4; P<0.05). As a negative control, neither concentration of sodium nitroprusside affected [cAMP] (n=4). Therefore, cAMP-dependent vasodilators increase cAMP levels in coronary arteries but do not cross-stimulate accumulation of cGMP.

Cross-Activation of PKG by cAMP-Dependent Vasodilators: Intact Cells

In addition to the variety of cAMP-dependent vasodilators used, direct application of cAMP to coronary myocytes stimulated BKCa channel activity (Figure 5A). Chlorophenylthio (CPT)-cAMP is a membrane-permeable cAMP derivative that increases intracellular [cAMP] directly, and a 20-minute exposure to 100 μmol/L CPT-cAMP stimulated BKCa channel activity by >20-fold, on average (NPo 0.008±0.008 to 0.192±0.03 (n=3) P<0.05). In the time-course experiment illustrated in Figure 5A, the stimulatory effect of CPT-cAMP was completely reversed by 300 μmol/L KT5823. In addition to cAMP, treating myocytes with a membrane-permeable derivative of cGMP (8-bromo-cGMP, 1 mmol/L) stimulated BKCa channel activity by nearly 60-fold (n=3; Figure 5B). These electrophysiological and pharmacological studies suggested that PKG was the enzyme mediating the effects of cAMP-dependent vasodilators on BKCa channels. Subsequent biochemical studies measured the effects of these agents on kinase activity directly.

Direct evidence for cross-activation of PKG by cAMP-dependent vasodilators was obtained from studies measuring 32P incorporation. Enzyme activity of both PKG and PKA was measured in coronary arteries under basal and stimulated conditions (n=3, each value being an average of 4 to 6 individual measurements). Basal activity of PKA (2.0±0.08 pmol P/min×mg protein−1) was ~4-fold higher than that of PKG (0.55±0.04 pmol P/min×mg protein−1). Interestingly, as illustrated in Figure 6A, dopamine (10 μmol/L) produced a 3-fold stimulation of PKG activity. In a similar fashion, forskolin (10 μmol/L) increased PKG activity 6-fold compared with control levels (P<0.05). As a positive control, treatment with 10 μmol/L sodium nitroprusside increased PKG activity by 7-fold. As anticipated, the same concentrations of both dopamine and forskolin stimulated activity of PKA, producing a 2.3-fold and 6-fold, respectively, increase above control levels (Figure 6B). An unexpected finding, however, was that 10 μmol/L sodium nitroprusside “cross-activated” PKA activity (1.8-fold; P<0.05). Therefore, kinase cross-activation can occur for both PKG and PKA in coronary myocytes.
Figure 5. cAMP or cGMP stimulate BK<sub>Ca</sub> channel activity in coronary myocytes. Activity plots of BK<sub>Ca</sub> channel open probability (NPo) recorded from cell-attached patches, as calculated for a series of 100-ms test pulses to +40 mV, and plotted as a vertical bar on the activity histogram. Total recording time under each condition was 10 to 11 seconds, as indicated on the time axes. Breaks in the time axes represent drug incubation periods. Periods of drug exposure are indicated by horizontal lines above histograms. A, Activity plot of channel NPo before and 15 minutes after application of 100 μmol/L CPT-cAMP and 30 minutes after subsequent addition of 300 nmol/L KT5823. B, Activity plot of channel NPo before and 20 minutes after application of 1 mmol/L 8Br-cGMP.

Cross-Activation of PKG by cAMP-Dependent Vasodilators: Reconstitution Studies

To complement and extend our biochemical measurements, the functional importance of cyclic nucleotide/kinase effects was studied in a reconstituted cell-free (inside-out patch) system using the BK<sub>Ca</sub> channel as a sensitive molecular assay. As illustrated in Figure 7A, addition of the catalytic subunit of PKA (400 U/mL) to the cytoplasmic surface of an inside-out membrane patch had no significant effect on BK<sub>Ca</sub> channel activity (NPo: control, 0.014±0.014; PKA, 0.001±0.001; n=5). Subsequent addition of 50 μmol/L cGMP also had no effect (n=5). In contrast, addition of purified PKG (400 U/ml in the presence of cGMP to “activate” the holoenzyme) produced a dramatic stimulatory effect on BK<sub>Ca</sub> channel activity (average NPo 0.410±0.19; n=5; P<0.03; Figure 7A). In the absence of cGMP, “unactivated” PKG did not affect channel activity (3 of 3 inside-out patches). In an additional experiment on a single inside-out patch (Figure 7B), purified PKG produced the expected stimulation of BK<sub>Ca</sub> channel activity (NPo from 0.01 to 0.65), but channel gating was completely abolished by subsequent addition of 1 mmol/L TEA to again verify that the activity of the BK<sub>Ca</sub> channel is modulated by PKG-dependent phosphorylation mechanisms.

A final series of experiments provided direct molecular confirmation of cAMP cross-activation of PKG. In these experiments purified PKG was activated by cAMP, but not cGMP as before, in Figure 7. In inside-out patches, increasing [cAMP] at the cytoplasmic surface of the membrane to 10 μmol/L had no effect on BK<sub>Ca</sub> channel activity (Figure 8A). However, subsequent addition of purified “unactivated” PKG, in the presence of cAMP, now stimulated BK<sub>Ca</sub> channel activity dramatically: NPo control, 0.001±0.001; cAMP, 0.001±0.001; cAMP+PKG, 0.336±0.16 (n=3, P<0.03). A summary histogram of the results obtained from reconstitution experiments performed on cell-free patches is illustrated in Figure 8B. BK<sub>Ca</sub> channel activity under control conditions was generally minimal (NPo 0.002±0.002; n=5), and activity was not affected significantly by application of either 10 μmol/L cAMP (NPo 0.001±0.001; n=3), 50 μmol/L cGMP (0.001±0.001; n=5), the purified catalytic subunit of PKA (NPo 0.014±0.014; n=5), or “unactivated” purified PKG (NPo 0.009±0.009; n=3). In contrast, BK<sub>Ca</sub> channel activity was enhanced significantly (P<0.03) by purified PKG activated by either 50 μmol/L cGMP (NPo 0.265±0.062; n=5) or 10 μmol/L cAMP (NPo 0.336±0.161; n=3). PKG produced a similar magnitude of stimulation when activated by either nucleotide.

Discussion

Cross-activation is a special form of biochemical cross-talk in which a ligand that is highly specific for a particular receptor interacts with another receptor that is highly specific for a different ligand under physiological conditions. We had previously demonstrated that dopamine relaxed porcine coronary arteries by opening BK<sub>Ca</sub> channels via activation of the DA-1 receptor, and subsequent pharmacological studies sug-
activated K channels in VSM,2,3,7,9,13,14,25,26 and, consistent findings that now establish the importance of cyclic nucleotide cross-activation in regulating BKCa channel activity in coronary myocytes. Findings from both on-cell and cell-free patches demonstrated that cAMP, cAMP-dependent vasodilators, and cGMP opened this potassium channel. This stimulation was reversed by selective inhibitors of PKG but was unaffected by PKA inhibition. In addition to these pharmacological studies, we were able to reconstitute cyclic nucleotide stimulation of channel activity in a cell-free system by addition of purified (cAMP-activated) PKG, whereas the purified catalytic subunit of PKA was ineffective at restoring channel activity. Lastly, direct biochemical measurements confirmed that cAMP-dependent vasodilators cross-activated PKG in coronary myocytes. These findings are consistent with previous studies suggesting and/or demonstrating cyclic nucleotide–dependent cross-activation of protein kinases in VSM.1,7,9,13,14,25,26

Figure 7. Purified PKG, but not PKA, stimulates BKCa channel activity in cell-free patches (+40 mV) from coronary myocytes. A, Recordings from the same inside-out patch before and 30 minutes after application of the purified catalytic subunit of PKA (PKAc; 400 U/mL). In contrast to PKAc, subsequent addition of purified PKG (400 U/mL; activated with 50 μmol/L cGMP) stimulated channel activity. Channel openings are upward deflections from baseline (dashed line). B, Activity plot of BKCa channel activity recorded from an inside-out patch before and after application of purified PKG (400 U/mL; activated with 50 μmol/L cGMP), and 10 minutes after cumulative addition of 1 mmol/L TEA. Total recording time under each condition was 5 to 7 seconds, as indicated on the time axes. Period of drug exposure is indicated by the horizontal lines above the histogram.

Stimulation of potassium channel activity is a very powerful means of relaxing smooth muscle, and it is no surprise that both cAMP and cGMP promote vasodilation, at least in part, by modulation of K+ channel activity. Calcium-activated K+ channels are particularly suited for this role because they respond to increases in intracellular [Ca2+] by attenuating calcium influx via repolarization-induced closure of voltage-dependent calcium channels. Activity of these channels is important for reducing active tension and also for maintaining basal levels of tone. Studies have demonstrated that under “resting” conditions, there is sufficient BKCa channel activity to regulate membrane potential.27 Furthermore, we have demonstrated previously that blockade of these channels with iberiotoxin (a highly selective inhibitor) induced spontaneous contraction of otherwise quiescent porcine coronary arteries17 and inhibited dopamine-induced coronary relaxation by >90%.16 These studies suggest that in porcine coronary arteries, the majority of relaxation induced by cAMP-elevating agents can be attributed to opening of BKCa channels; however, these studies do not preclude the importance of other effector mechanisms in these or other vessels. For example, cross-activation of PKG by cAMP inhibits L-type calcium channel activity in myocytes from rabbit portal vein,14 whereas cAMP may also stimulate the activity of other potassium channels (eg, KATP) by PKA-dependent phosphorylation.28

As summarized by Lincoln et al,29 there is simply no “specific” effect of a cyclic nucleotide analog or a cyclic nucleotide–dependent kinase in cells. In addition, there are multiple targets and feedback mechanisms at nearly every step in the transduction cascade. Therefore, elucidating the complicated signaling mechanisms of cyclic nucleotide–dependent vasodilation is an ongoing challenge. For example, there are 3 major intracellular targets of cGMP, as follows: ion channels, phosphodiesterase (PDE), and PKG.15 Although molecular studies have suggested expression of a cyclic nucleotide–gated channel in rabbit aorta,30 the present findings excluded direct effects of either cAMP or cGMP on BKCa channel gating in coronary myocytes: addition of either nucleotide to the cytoplasmic surface of inside-out patches did not stimulate membrane electrical activity. On the other
channel activity was stimulated dramatically by cAMP activation of PKG. Therefore, cAMP cross-activation of PKG activity appears to mediate the effects of cAMP-dependent vasodilators on BK<sub>Ca</sub> channels in coronary smooth muscle.

PKG appears to be the primary receptor protein for cGMP in VSM, and the present study has demonstrated that cAMP-stimulating agents increased PKG activity in coronary arteries. Although PKG exhibits ~20-fold higher affinity for cGMP over cAMP in vitro, cAMP levels are typically 5- to 10-fold greater than those of cGMP. Moreover, like many protein kinases, autophosphorylation of PKG occurs, resulting in a 15-fold increased affinity for cAMP. On the basis of these in vitro measurements, one would expect both PKG and PKA to be activated by cAMP under stimulated, and possibly basal, conditions. In intact cells, however, the situation is most likely much more complicated, and we used antagonists of both PKA and PKG to evaluate the relative importance of each kinase in mediating cAMP-stimulated BK<sub>Ca</sub> channel gating in a “physiological” cellular system—the single myocyte. We used 2 different types of kinase inhibitors in these studies. The KT series of compounds competitively inhibits ATP binding to the kinase catalytic site and exhibits clear concentration-dependent selectivity in vitro. KT5823 inhibits PKG activity with a K<sub>i</sub> of 234 nmol/L, whereas the K<sub>i</sub> for PKA inhibition is >10 μmol/L. On the other hand, KT5720 is much more selective for PKA (K<sub>i</sub> of 60 nmol/L) than for PKG (K<sub>i</sub> of >2 μmol/L). Therefore, the lower nanomolar concentrations used in the present study are well within the selectivity range of these inhibitors. In addition, we also attenuated PKG activity by using a different type of inhibitor, Rp-8-pCPT-cGMPS. This analog inhibits cyclic nucleotide binding in a competitive fashion with an in vitro K<sub>i</sub> of 0.5 μmol/L, but has only limited effects on PKA activity. Our pharmacological studies on intact cells were completely consistent with results obtained from direct biochemical measurement of kinase activity and nucleotide levels and strongly suggested a physiological role for cAMP-stimulated PKG activity. Nonetheless, we performed additional reconstitution studies with purified kinases to confirm that cAMP activated PKG-stimulated BK<sub>Ca</sub> channel activity. These studies also cast doubt on the possibility that significant kinase activity could be intrinsic to the BK<sub>Ca</sub> channel protein complex or due to a membrane-associated kinase. If such a colocalized cyclic nucleotide–stimulated kinase or other membrane-delimited mechanism was present, then either cAMP or cGMP should have enhanced channel activity in excised patches. This, however, was not the case. Therefore, these studies strongly suggest that cytosolic PKG is the key enzyme that mediates cyclic nucleotide regulation of BK<sub>Ca</sub> channel activity in coronary arteries.

It is apparent that cAMP induces vasodilation by several distinct mechanisms, and cross-activation of PKG could contribute to this diversity of action. There are multiple potential targets for PKG-induced phosphorylation, including the IP<sub>3</sub> receptor, cytoskeletal proteins, vasodilator-stimulated phosphoprotein, Ca<sup>2+</sup>-ATPase, calcium channels, and BK<sub>Ca</sub> channels. The present findings also indicated that cAMP-dependent vasodilators stimulated PKA activity in coronary myocytes, but the role(s) this enzyme
plays in cAMP-induced vasodilation is not fully defined. For example, introduction of the PKA catalytic subunit into rat aortic smooth muscle cells had no effect on KCl-stimulated increases in cytosolic \([\text{Ca}^{2+}]\). Interestingly, in this same study cAMP-dependent vasodilators decreased calcium levels only in the presence of PKG; in the absence of PKG, either forskolin or isoproterenol increased \([\text{Ca}^{2+}]\). These findings suggested that activated PKA may actually elevate cytosolic \([\text{Ca}^{2+}]\) in VSM, and subsequent patch-clamp studies have demonstrated increases in calcium (ie, \([\text{Ba}^{2+}]\)) currents in the presence of lower concentrations of forskolin, isoproterenol, or cAMP in myocytes from portal vein. In contrast, both electrophysiological studies reported that higher concentrations of these cAMP-dependent vasodilators inhibited \([\text{Ba}^{2+}]\) currents, as did cGMP. Therefore, it appears that cAMP cross-activation of PKG can depress calcium influx by both direct and indirect (BK\(_{\text{Ca}}\) channel) action on calcium channel activity. This hypothesis is entirely consistent with results from the original work of Francis et al demonstrating that analogs of cAMP that were potent, specific activators of PKA were only weak relaxants of porcine coronary arteries. On the other hand, more recent findings from Eckly-Michel et al reported that low concentrations of isoproterenol relaxed rat aorta, and this response was blocked by PKA inhibition. In light of these findings, it is clear that both cGMP- and cAMP-dependent vasodilators can relax VSM by stimulating PKG activity; however, defining the relative importance of PKA in mediating cAMP-induced vascular relaxation will require further study.

It is well known that stimulation of PKG activity increases the open probability of BK\(_{\text{Ca}}\) channels in VSM and other cells, and the present results are entirely consistent with the literature in this regard. In contrast, the present findings with both pharmacological inhibitors and purified kinases demonstrated that PKA did not stimulate BK\(_{\text{Ca}}\) channel activity in either on-cell or cell-free patches from coronary arteries. Previous studies have demonstrated that PKA inhibits BK\(_{\text{Ca}}\) channel activity in neuroendocrine cells or in uterine smooth muscle cells. Furthermore, PKA-mediated relaxation of mesenteric arteries does not involve potassium channel activity. In contrast, there is evidence that PKA may stimulate BK\(_{\text{Ca}}\) channel opening in other types of VSM. For example, the catalytic subunit of PKA opens single BK\(_{\text{Ca}}\) channels in patches from rat tail artery myocytes. In artificial lipid bilayers, BK\(_{\text{Ca}}\) channels derived from coronary arteries were stimulated by PKA, and studies on inside-out patches from short-term cultured aortic myocytes demonstrated increased BK\(_{\text{Ca}}\) channel NP, after PKA. In the present study we used only freshly dissociated myocytes and did not observe PKA stimulation of BK\(_{\text{Ca}}\) channel activity, nor did we observe an effect of selective PKA inhibitors on intact cells. Interestingly, Song and Simard reported that PKA stimulated BK\(_{\text{Ca}}\) channel activity in freshly dissociated cells from guinea pig basilar artery, but this stimulation was consistent only when \([\text{Ca}^{2+}]\) was \(\leq 0.1 \text{ mmol/L}\). Therefore, it is clear that both cAMP and cGMP can stimulate BK\(_{\text{Ca}}\) channel activity in VSM; however, the identification of the specific kinase(s) involved in the response appears to be heterogeneous with respect to vessel and species. Clearly, the molecular basis of cyclic nucleotide–dependent vasodilation requires further study.

In summary, the present study presents consistent evidence from a diversity of experimental procedures that cAMP cross-activates PKG to stimulate BK\(_{\text{Ca}}\) channel activity in myocytes from porcine coronary arteries. In light of these and previous studies on this phenomenon, it would appear that cAMP cross-activation of PKG can provide an answer to the question of why cAMP and cGMP both relax coronary arteries, whereas their effects are often antagonistic in other tissues. Whether this model is applicable to other VSMs remains to be confirmed, although portal vein appears to exhibit a similar cross-activation pathway. Classically, stimulation of cAMP mediates the vasodilatory effects of certain catecholamines (eg, isoproterenol or dopamine), and with the more recent discoveries that important vasoactive peptides (eg, adrenomedullin or calcitonin gene-related peptide) may also stimulate cAMP-dependent vasodilation, it is clear that a more thorough understanding of how cyclic nucleotides relax VSM is needed. Future research into understanding vascular signaling mechanisms will continue to focus on physiological and therapeutic means of stimulating and/or interdicting the signal transduction cascades of cAMP and cGMP in VSM.

Acknowledgments

This work was supported by grants from the American Heart Association (to G.O.C. and to R.E.W.) and awards from the National Heart, Lung, and Blood Institute (HL54844 to R.E.W. and HL64779 to R.E.W. and G.O.C.).

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cAMP-Dependent Vasodilators Cross-Activate the cGMP-Dependent Protein Kinase to Stimulate BK Ca Channel Activity in Coronary Artery Smooth Muscle Cells
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Circ Res. 2000;86:897-905
doi: 10.1161/01.RES.86.8.897

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