A Catalytically Inactive Mutant of Type I cGMP-dependent Protein Kinase Prevents Enhancement of Large Conductance, Calcium-sensitive K^+ Channels by Sodium Nitroprusside and cGMP*

Richard D. Swayze‡ and Andrew P. Braun§¶

From the Smooth Muscle Research Group, Department of Pharmacology and Therapeutics, Faculty of Medicine, The University of Calgary, Calgary, Alberta, Canada T2N 4N1

The activation of large conductance, calcium-sensitive K^+ (BK_{Ca}) channels by the nitric oxide (NO)/cyclic GMP (cGMP) signaling pathway appears to be an important cellular mechanism contributing to the relaxation of smooth muscle. In HEK 293 cells transiently transfected with BK_{Ca} channels, we observed that the NO donor sodium nitroprusside and the membrane-permeable analog of cGMP, dibutyryl cGMP, were both able to enhance BK_{Ca} channel activity 4–5-fold in cell-attached membrane patches. This enhancement correlated with an endogenous cGMP-dependent protein kinase activity and the presence of the α isoform of type I cGMP-dependent protein kinase (cGKI). We observed that co-transfection of cells with BK_{Ca} channels and a catalytically inactive (“dead”) mutant of human cGKIα prevented enhancement of BK_{Ca} channel response to either sodium nitroprusside or dibutyryl cGMP in a dominant negative fashion. In contrast, expression of wild-type cGKIs supported enhancement of channel activity by these two agents. Importantly, both endogenous and expressed forms of cGKIs were found to associate with BK_{Ca} channel protein, as demonstrated by a reciprocal co-immunoprecipitation strategy. In vitro, cGKIs was able to directly phosphorylate immunoprecipitated BK_{Ca} channels, suggesting that cGKI-dependent phosphorylation of BK_{Ca} channels in situ may be responsible for the observed enhancement of channel activity. In summary, our data demonstrate that cGKIα alone is sufficient to promote the enhancement of BK_{Ca} channels in situ after activation of the NO/cGMP signaling pathway.

The elevation of intracellular cGMP1 in response to endothelium-derived nitric oxide (NO) or clinically prescribed nitrovasodilators, such as nitroglycerin and sodium nitroprusside, is known to play an important role in the hypotensive actions of these agents (1, 2). Similarly, elevation of cGMP by the phosphodiesterase inhibitor sildenafil (Viagra) (3) appears to underlie the smooth muscle-relaxing and anti-impotence effects of this drug. Although the exact mechanism(s) by which elevated cGMP causes smooth muscle relaxation has not been clearly defined, cGMP is known to influence a number of cellular processes (4), such as the levels of cytosolic free calcium, myosin light chain dephosphorylation (5), and the activity of voltage-dependent, L-type calcium channels (6).

In both vascular and nonvascular smooth muscle, activation of large conductance, calcium-sensitive K^+ channels ( maxi-K or BK_{Ca} channels) is reported to occur in response to endogenous NO or exogenous NO donors (7–12). In many cases, addition of exogenous cGMP appears to mimic the effects of NO and NO donors on BK_{Ca} channel activation (8, 10, 12–15), suggesting that cGMP acts downstream of NO. Physiologically, BK_{Ca} channels appear to be important cellular effectors for the vasodilatory actions of the NO/cGMP signaling pathway because blockade of BK_{Ca} channels can interfere with the relaxation-promoting effects of NO (16–18).

A major intracellular target for cGMP in smooth muscle is the type I cGMP-dependent protein kinase (cGKI), a serine/threonine protein kinase that is widely expressed in mammalian tissues (19, 20). This kinase is encoded by a single gene, which gives rise to two alternatively spliced isoforms, α and β, differing only in their N-terminal domains (36% of the first 103 amino acids of the β isoform are identical to those of the α isoform) (19, 21). Both the α and β isoforms of the type I cGMP-dependent protein kinase functionally exist as homodimers (i.e. α/α and β/β), in which each subunit contains a catalytic domain, 2 cGMP-binding sites, and an N-terminal dimerization region (4, 19). Smooth muscle expresses both isoforms (22), although the biological roles of each are not well understood.

To examine the role played by cGKIα in the activation of cellular BK_{Ca} channels by the NO/cGMP signaling cascade, we created a catalytically inactive or “dead” mutant of cGKIα that could be co-expressed with murine BK_{Ca} channels. Utilizing a dominant negative suppression strategy (23, 24), we observed that dead cGKIα prevented activation of BK_{Ca} channels in cell-attached patches of HEK 293 cells in response to the nitrovasodilator sodium nitroprusside (SNP) or dibutyryl cGMP, a membrane-permeable analog of cGMP. Using a reciprocal co-immunoprecipitation strategy, we found that the endogenous and expressed forms of cGKIα were able to associate with BK_{Ca} channel protein. We also observed that cGKIα is able to directly phosphorylate BK_{Ca} channels in vitro, supporting the hypothesis that a similar event may be responsible for the enhancement of channel activity by cGMP in situ. Taken to-
Active PKG Interferes with Enhancement of BKCa Channels

Rabbit polyclonal antibodies against the mouse BKCa channel α subunit and the human type I cGMP-dependent protein kinase were obtained from Chemicon International and Calbiochem, respectively. A horseradish peroxidase-linked, mouse anti-rabbit IgG monoclonal antibody (clone RG-96) was purchased from Sigma Chemical Co. The purified, recombinant cGMP-dependent protein kinase I enzyme was purchased from Calbiochem. The cGMP-dependent protein kinase-selective substrate RRKRRAE was obtained from Peninsula Laboratories. The Lowry protein assay kit (detergent compatible) was purchased from Bio-Rad Laboratories.

Construction and Transfection of eDNA Plasmids—The eDNA coding the mouse BKCa channel (mSto) α subunit (25), the wild-type green fluorescent protein (26), and the human cGKI expression plasmid SRKSSAE was obtained from Peninsula Laboratories. The Lowry protein assay kit (detergent compatible) was purchased from Bio-Rad Laboratories.

Transient transfection of HEK 293 cells (50–80% confluence) was carried out in 35-mm tissue culture dishes using the lipofection technique. Briefly, 6–8 μL of LipofectAMINE (Life Technologies, Inc.) was mixed together with ~1.5 μg of plasmid eDNA in 1 ml of serum-free culture medium (Dulbecco’s modified Eagle’s medium supplemented with L-glutamine and 4.5 g/liter D-glucose) and placed on cells for 4–6 h in a humidified incubator containing 5% CO2 at 37 °C. DNA-containing medium was then aspirated and replaced with serum-containing medium. The following day, cells were detached from the dish by treatment with 0.05% (w/v) trypsin/0.5 mM EDTA and replated onto sterile glass coverslips. Electrophysiological recordings were performed on days 3–5 after transfection (day 1). For biochemical studies, cells detached from 35-mm dishes were replated onto 100-mm dishes to prevent overgrowth. These cells were then harvested on days 3–4 after transfection.

Electrophysiology—Macroscopic currents were recorded at 35 ± 0.5 °C from cell-attached membrane patches of HEK 293 cells using an Axopatch 200B patch clamp amplifier and a Digidata 1200 analog/digital interface. Recording micropipettes were pulled from thin-walled borosilicate glass capillaries (1.2 mm inner diameter; 1.5 mm outer diameter; WPI, Sarasota, FL) using a Sutter P-890 horizontal electrode puller. Micropipettes were filled with a solution containing 5 mM KCl, 140 mM KOH, 1 mM MgCl2, 1 mM CaCl2, and 10 mM HEPES (pH adjusted to 7.3 with methanesulfonic acid) and had tip resistances of 2–3.5 megohms. The bath solution contained 5 mM KCl, 140 mM KOH, 1 mM MgCl2, 1 mM CaCl2, and 10 mM HEPES (pH adjusted to 7.3 with methanesulfonic acid). The recording chamber (~0.3 ml volume) was perfused by gravity flow at a constant rate of 1–1.5 ml/min, using a set of manually controlled solenoid valves to switch between solutions. Reagents were added directly to the solution reservoir tubes at the concentrations indicated.

Transfected HEK 293 cells seeded on coverslips were placed in a temperature-controlled recording chamber on the stage of a Nikon Eclipse TE300 inverted microscope. Individual cells expressing BKCa channels were then identified visually by co-expression of the marker protein green fluorescent protein under epifluorescence using 480 nm excitation and 510 nm emission filters.

Western Blotting—Transfected cells were detached on day 3 by a brief incubation with sterile phosphate-buffered saline containing 0.05% trypsin/0.5 mM EDTA, centrifuged at 100 × g for 5 min, and stored at −80 °C as intact cell pellets. These pellets were resuspended in 0.5–1 ml of ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, 1% (v/v) Triton X-100, 1 mM EGTA, 2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 μg/ml each of leupeptin, pepstatin A, and aprotinin and then sonicated for 5–10 s to shear the genomic DNA. After measurements of protein concentration were carried out using a modified Lowry procedure (29), lysates were mixed with Laemmli sample buffer containing 1% (v/v) β-mercaptoethanol and incubated for 20–30 min at 70 °C, and the proteins were then separated by SDS-polyacrylamide gel electrophoresis (30). The resolved proteins were electrophoretically transferred to nitrocellulose and probed with rabbit anti-BK Ca channel antibody, followed by further incubation for 2 h with 30 μl of 50% slurry (v/v) of rehydrated protein A-Sepharose beads (Amersham Pharmacia Biotech), followed by rotation at 4 °C for 1 h. Samples were centrifuged for 5 min at 10,000 rpm to pellet the beads, and the soluble material was transferred to a clean microcentrifuge tube. Pre-cleared supernatants were then incubated for 4–16 h at 4 °C with ~1.5 μg of anti-BKCa channel antibody, followed by further incubation for 2 h with 30 μl of protein A-Sepharose beads (50% slurry). The beads were pelleted by centrifugation at 4 °C for 5 min at 3,000 rpm and then washed twice by resuspension in 1 ml of wash buffer containing 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM EDTA, 0.2 mM EGTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.1% (v/v) Triton X-100, and 5 μg/ml each of leupeptin, pepstatin A, and aprotinin and then sonicated for 4–16 h. The pellets were then resuspended in 20–30 μl of a buffer containing 20 mM Tris-HCl, pH 7.5, and 2 mM DTT, 15-μl aliquots were used directly in the assay.

The phosphorylation reaction was carried out for 20 min at 30 °C in a reaction buffer of 40 μl containing (final concentrations) 20 mM Tris-HCl, pH 7.5, 20 mM mG-MgATP, 2 μM cADP, 0.1 mM isobutylmethylxanthine, 20 μM cGMP, 5,000 units of recombinant bovine type I cGMP-dependent protein kinase, and 15 μl of resuspended protein A-Sepharose beads containing immunoprecipitated BKCa subunit. The reaction was started by the addition of [γ-32P]ATP (20 μM; final concentration, 20,000–30,000 cpm/pmol) and stopped by addition of concentrated (4×) Laemmli sample buffer directly to the assay tubes.
Inactive PKG Interferes with Enhancement of BKC\textsubscript{a} Channels

Characterization of Transfected Type I cGMP-dependent Protein Kinase—To examine the functional importance of cGKI\textsubscript{a} in the regulation of BKC\textsubscript{a} channels by cGMP and the nitrovasodilator compound SNP, we hypothesized that a catalytically inactive mutant of cGKI\textsubscript{a} could be used in a dominant negative strategy (23, 24) to suppress or interfere with the actions of endogenous cGKI\textsubscript{a} in an intact cell. Earlier reports have shown that native HEK 293 cells contain an endogenous cGMP-dependent protein kinase activity (15, 34, 35); our new data, described below, strongly suggest that this activity can be accounted for by the presence of the \( \alpha \) isoform of type I cGMP-dependent protein kinase in these cells.

To prepare a catalytically inactive mutant of human cGKI\textsubscript{a} (27), we replaced a critical lysine residue (Lys\textsuperscript{393}) in the ATP-binding motif of the enzyme's catalytic domain with methionine (36, 37). The effectiveness of this substitution was verified using two complementary biochemical approaches. First, an \textit{in vitro} kinase assay was performed using soluble cell lysates from HEK 293 cells transiently transfected with cDNA constructs encoding a BKC\textsubscript{a} channel \( \alpha \) subunit together with either the wild-type or mutant form of cGKI\textsubscript{a}. Fig. 1 shows that the detergent-solubilized lysate from cells transfected with the BKC\textsubscript{a} channel cDNA alone displayed modest cGMP-dependent protein kinase activity, as quantified by measuring phosphorylation of a cGKI-selective peptide substrate (33).

In the presence of cGMP, substrate phosphorylation was increased \( \sim 2 \times \) fold above background levels observed in the absence of cGMP, and this activity was comparable to that present in mock-transfected cells. However, cGMP-dependent substrate phosphorylation was strongly enhanced (\( \sim 8 \times \) fold) in the lysate from cells co-transfected with BKC\textsubscript{a} channels and wild-type cGKI\textsubscript{a}, demonstrating expression of intact, biochemically active protein kinase. In contrast to this situation, cells transiently transfected with BKC\textsubscript{a} channels and the catalytically inactive or dead form of cGKI\textsubscript{a} displayed a level of cGMP-dependent kinase activity that was very similar to that of cells transfected with BKC\textsubscript{a} channels alone. This finding is thus consistent with results using other protein kinases (36, 38), in which mutation of this invariant lysine residue within the catalytic domain is sufficient to block expression of enzymatic activity. It is interesting that co-expression of dead cGKI\textsubscript{a} did not lower the total observable cGMP-dependent protein kinase activity measured \textit{in vitro}. This observation may be due to: \( a \) the transfection efficiency of HEK 293 cells with the LipofectAMINE reagent (Life Technologies, Inc.) ranges from 20–30% in our hands; therefore, endogenous cGMP-dependent activity in the majority of cells remains unaffected, and \( b \) the assay mixture for cGMP-dependent protein kinase activity contains saturating concentrations of Mg-ATP, cGMP, and peptide substrate, therefore the presence of inactive kinase molecules would not be expected to interfere with the activity of native kinase by depletion of essential reagents \textit{in vitro}.

FIG. 1. Cyclic GMP-dependent protein kinase activities in HEK 293 cells expressing wild-type or catalytically dead cGKI\textsubscript{a}.

HEK 293 cells were transfected with BKC\textsubscript{a} channel \( \alpha \) subunit alone (BK alone) or together with either wild-type cGKI\textsubscript{a} (BK + cGKI\textsubscript{a}) or catalytically inactive cGKI\textsubscript{a} (BK + dead cGKI\textsubscript{a}); mock-transfected cells (Mock) were transfected with an equal amount of empty plasmid (\textit{i.e.} no insert cDNA present). Detergent-soluble cellular lysates were prepared from each group of cells, and cGMP-dependent protein kinase activity was quantified using an \textit{in vitro} kinase assay (see “Materials and Methods”). Results are expressed as the means \( \pm \) S.E. of three to four separate experiments, each carried out in duplicate.

Using an anti-cGKI antibody, we observed modest expression of an endogenous immunoreactive protein with a molecular mass of \( \sim 75 \) kDa, corresponding to the \( \alpha \) isoform of cGKI (22). No expression of a 78–80-kDa \( \beta \) isoform was detected in our HEK 293 cells, although both \( \alpha \) and \( \beta \) isoforms were readily observed under the same Western blottings conditions in isolated smooth muscle myocytes from rabbit aorta (data not shown). In contrast, HEK 293 cells co-transfected with cDNAs encoding either the wild-type or mutant form of cGKI\textsubscript{a} showed very strong expression of a similar 75-kDa band, consistent with the presence of exogenous cGKI\textsubscript{a} protein. The lower immunoreactive bands with a molecular mass of \( \sim 60 \) kDa most likely represent proteolytic fragments of the full-length wild-type and mutant cGKI\textsubscript{a} (19). Taken together, the results shown in Figs. 1 and 2A demonstrate that both the wild-type and mutant forms of recombinant human cGKI\textsubscript{a} can be strongly expressed in HEK 293 cells, with only the wild-type enzyme displaying significant cGMP-dependent kinase activity. We then examined whether co-transfection of cGKI\textsubscript{a} influenced the expression pattern of BKC\textsubscript{a} channels themselves. Fig. 2B shows a Western blot of the same cellular extracts used in Fig. 2A, which was probed with an antibody against the BKC\textsubscript{a} channel \( \alpha \) subunit. A single immunoreactive band of \( \sim 125 \) kDa was observed, the level of which was comparable under all three transfection conditions. Similar results were obtained in two additional experiments. Taken together, these observations demonstrate that co-transfection of HEK 293 cells with either wild-type or mutant cGKI\textsubscript{a} cDNA leads to the expected expression of both protein and kinase activity, without altering the expression pattern of BKC\textsubscript{a} channel protein.

Effects of the NO/cGMP Signaling Pathway on BKC\textsubscript{a} Channel Activity—The functional importance of cGKI\textsubscript{a} in the regulation of BKC\textsubscript{a} channels was examined by using patch clamp techniques to record BKC\textsubscript{a} channel activity in cell-attached membrane patches of transfected HEK 293 cells in the absence...
and presence of 100 μM SNP. Fig. 3A shows BKCa channel activity before and during bath application of 100 μM SNP.

In the absence of SNP, a modest level of BKCa channel activity was observed, the magnitude of which varied from cell to cell. However, after ~4 min of exposure to SNP, we observed a large increase in the amplitude of BKCa channel macroscopic current; this increase typically peaked during 2–6 min of exposure and was reversible over several minutes upon washout of SNP from the bath (Fig. 3C). SNP produced an average increase of ~4-fold in current magnitude compared with control, as quantified in Fig. 3C. This observation is thus consistent with recent findings reported by Fukao et al. (15) using whole cell voltage clamp methodologies that SNP could augment the activity of canine colonic BKCa channels expressed in HEK 293 cells. Using our dominant negative strategy, we observed a much greater in cells transfected with cGKIα cDNA. The lower band at ~60 kDa most likely represents a proteolytic fragment of full-length cGKIα (19). After detection of cGKIα, the blot was stripped and re-probed with an antibody recognizing the BKCa channel α subunit. A single band of ~125 kDa was detected in each lysate (B). The electrophoretic mobility of molecular mass markers (in kDa) is indicated to the right of each panel.

Although SNP is known to elevate intracellular cGMP in several cell types via the NO-dependent activation of guanylyl cyclase (39), it is also capable of generating chemical products (i.e. peroxynitrates, S-nitrosothiols, and ferrocyanoates) that may directly influence BKCa channel activity (40, 41). To address whether SNP may initiate other cellular mechanisms not dependent upon activation of cGKI, we examined the effect of dibutyryl cGMP (db-cGMP) produced an increase (4–5-fold above control) in BKCa channel activity that was qualitatively similar to that seen with exposure to SNP (compare Fig. 4, A and C with Fig. 3, A and C). In the absence of db-cGMP, no significant change augment this already maximal response.

Fig. 2. Protein expression levels of wild-type and catalytically dead cGKIα in transiently transfected HEK 293 cells. A shows a Western blot of equal amounts of total cellular lysates (~75 μg/lane) from HEK 293 cells transfected with the BKCa channel α subunit alone (BK alone) or together with either wild-type cGKIα (BK + cGKIα) or catalytically inactive cGKIα (BK + dead cGKIα). An antibody specifically recognizing the type I cGMP-dependent protein kinase detected a ~75-kDa band in each lane; however, the intensity of this band was much greater in cells transfected with cGKIα cDNA. The lower band at ~60 kDa most likely represents a proteolytic fragment of full-length cGKIα (19). After detection of cGKIα, the blot was stripped and re-probed with an antibody recognizing the BKCa channel α subunit. A single band of ~125 kDa was detected in each lysate (B). The electrophoretic mobility of molecular mass markers (in kDa) is indicated to the right of each panel.

Fig. 3. Effect of transiently expressed wild-type and catalytically dead cGKIα on the enhancement of BKCa channel activity by sodium nitroprusside. A and B show cell-attached patch clamp recordings of macroscopic BKCa channel currents from HEK 293 cells transiently transfected with BKCa channel α subunit alone (A) or together with catalytically inactive (dead) cGKIα (B). BKCa channel currents in cell-attached membrane patches were evoked by voltage clamp steps from ~90 to +150 mV, in 10-mV increments; the membrane patch was held at 0 mV (refer to the inset). The top set of traces in each panel was recorded shortly after the formation of a gigaohm seal (designated as 0 min). Using continuous bath superfusion, cells were then exposed to 100 μM SNP, and current families were recorded every 2 min for up to 6–7 min after the start of exposure (the bottom set of traces in each panel). The fold change in current amplitude at +100 mV in the absence or presence of SNP exposure versus initial control level is plotted in C for cells transfected with BKCa channels alone or together with either catalytically inactive (dead) cGKIα or wild-type cGKIα. The reversibility of stimulated current amplitude in cells expressing BKCa channels alone after SNP wash-out (SNP W/O) for 6–8 min is also indicated. Current amplitudes were quantified by measuring the average steady-state current over the last 5 ms of the depolarizing pulse. Data are presented as the means ± S.E. (n = 4–6 cells in each group). An asterisk indicates that these values are significantly different from the control value in the absence of SNP, p < 0.05.
Inactive PKG Interferes with Enhancement of BK<sub>Ca</sub> Channels

In BK<sub>Ca</sub> channels expressed alone, the level of endogenous cGKI<sub>α</sub> is sufficient to produce maximal enhancement of BK<sub>Ca</sub> channel activity.

**Direct Phosphorylation of BK<sub>Ca</sub> Channels by Purified cGKI<sub>α</sub>**—Whereas it has been generally hypothesized that the regulation of BK<sub>Ca</sub> channels by cGMP may involve a phosphorylation event, only recently have results appeared in the literature directly supporting such a mechanism (9, 14, 15, 42, 43). To examine whether BK<sub>Ca</sub> channels expressed in HEK 293 cells may undergo direct phosphorylation in the presence of cGKI<sub>α</sub>, we isolated expressed BK<sub>Ca</sub> channels by immunoprecipitation and then incubated these purified channels in either the absence or presence of purified cGKI<sub>α</sub>. Fig. 5A shows an autoradiogram of an in vitro phosphorylation assay using immunoprecipitates from cells transfected with BK<sub>Ca</sub> channel cDNA or cells transfected with empty vector alone (mock-transfected cells).

In the lane containing immunoprecipitated BK<sub>Ca</sub> channels plus purified cGKI<sub>α</sub>, a major phosphorylated band of ~125 kDa was observed, most likely corresponding to the BK<sub>Ca</sub> channel α subunit. The second major phosphoprotein observed in the same lane, migrating at ~75 kDa, corresponds to the purified cGKI<sub>α</sub> after autophosphorylation (4). As shown in Fig. 5A, addition of purified cGKI<sub>α</sub> to the immunoprecipitate from mock-transfected cells produced only a single phosphoprotein of ~75 kDa, again corresponding to the autophosphorylated cGKI<sub>α</sub> enzyme. The absence of this 75-kDa band from both reactions lacking addition of the purified cGKI<sub>α</sub> further supports this conclusion. To demonstrate the presence of BK<sub>Ca</sub> channel protein in our reactions, we performed a Western blot on the total cellular lysates and immunoprecipitates from both sets of transfected cells. Fig. 5B shows that the BK<sub>Ca</sub> channel α subunit is strongly expressed in the total cellular lysate of positively transfected cells, with significant recovery in the immunoprecipitate. However, a similar immunoreactive band was not detected in either the lysate or immunoprecipitate of mock-transfected cells, consistent with the observed lack of a phosphoprotein of ~125 kDa in the autoradiogram of Fig. 5A.

**Interaction of cGKI with BK<sub>Ca</sub> Channels in Situ**—To address the potential mechanism by which expression of the catalytically inactive form of cGKI<sub>α</sub> interfered with stimulation of BK<sub>Ca</sub> channel activity by SNP and db-cGMP, BK<sub>Ca</sub> channels and cGKI<sub>α</sub> (wild-type or catalytically inactive forms) were transiently expressed either alone or together in HEK 293 cells. BK<sub>Ca</sub> channels were then directly immunoprecipitated, and the immunoprecipitates were probed by Western blot for the presence of associated cGKI<sub>α</sub>.

Using this co-immunoprecipitation strategy, we found a small amount of expressed cGKI<sub>α</sub> associated with isolated BK<sub>Ca</sub> channels, and we found that brief stimulation by dibutyryl cGMP modestly enhanced this interaction (Fig. 6A). Under these same conditions, we further observed that the catalytically inactive form of cGKI<sub>α</sub> associated with immunoprecipitated BK<sub>Ca</sub> channels to a much greater extent compared with the expressed wild-type kinase. However, in cells transfected with BK<sub>Ca</sub> channels alone, we were unable to detect the presence of endogenous cGKI<sub>α</sub> in BK<sub>Ca</sub> channel immunoprecipitates. This inability to capture such a steady-state interaction may reflect the combination of relatively low amounts of endogenous kinase present in these cells compared with BK<sub>Ca</sub> channels and the transient nature of interaction of cGKI<sub>α</sub> with the BK<sub>Ca</sub> channel substrate. Similarly, in their recent study, Wang et al. (44) could not detect either endogenous cAMP-dependent protein kinase or cSrc tyrosine kinase in immunoprecipitates of recombinant Drosophila Slo channels transiently expressed in HEK 293 cells. However, when either kinase was co-expressed along with the channel, channel-kinase interactions could be observed using such a co-immunoprecipitation strategy.
When BK<sub>Ca</sub> channel immunoprecipitates were probed for the presence of channel protein, we observed that similar amounts were recovered from cells transfected under each condition (Fig. 6B). This finding indicates that unequal immunoprecipitation of BK<sub>Ca</sub> channel protein cannot account for the difference observed in the levels of co-immunoprecipitated cGKI<sub>α</sub> shown in Fig. 6A. To further verify this result, we then probed equal amounts of the starting whole cell lysates for the expression of type I cGMP-dependent protein kinase to ensure that differences in the level of cGKI<sub>α</sub> expression between conditions did not account for the differential co-immunoprecipitation of cGKI<sub>α</sub> shown in Fig. 6A. Our observation that expression of the transiently expressed, wild-type cGKI<sub>α</sub> was greater than that of the catalytically inactive form of the kinase, which was greater than that of endogenous cGKI<sub>α</sub> (see Fig. 6C), indicates that gross differences in cGKI<sub>α</sub> expression can not account for the differential co-immunoprecipitation of wild-type and dead cGKI<sub>α</sub> with BK<sub>Ca</sub> channels presented in Fig. 6A. In Fig. 6C, the amount of endogenous type I cGMP-dependent protein kinase was below detection in cells transfected with BK<sub>Ca</sub> channels alone, likely due to the low amount of whole cell lysate loaded per lane (i.e. ~15 μg). By restricting the amount of protein loaded per lane in this particular experiment, we were able to achieve a better comparison of the expression levels between the wild-type and catalytically inactive forms of cGKI<sub>α</sub> in the whole cell lysates shown in Fig. 6A.

This important association between BK<sub>Ca</sub> channels and cGKI<sub>α</sub> was further examined by performing reciprocal co-immunoprecipitation, in which we probed anti-cGKI immunoprecipitates for the presence of co-associated BK<sub>Ca</sub> channel protein. As expected, we observed that BK<sub>Ca</sub> channels co-immunoprecipitated with co-expressed wild-type or catalytically inactive cGKI<sub>α</sub> (Fig. 6D), although there was not the same marked difference as seen in Fig. 6A. Importantly, we also observed that the endogenous form of cGKI<sub>α</sub> in HEK 293 cells is able to co-associate with expressed BK<sub>Ca</sub> channels, as demonstrated by the presence of these two proteins in the same anti-cGKI<sub>α</sub> immunoprecipitates. When these immunoprecipitates were probed for the presence of cGKI<sub>α</sub> protein, we observed similar levels of either expressed wild-type or inactive cGKI<sub>α</sub>, along with modest amounts of the endogenous form of cGKI<sub>α</sub> (Fig. 6E). A Western blot of the initial whole cell lysates probed for the BK<sub>Ca</sub> channel α subunit demonstrates similar expression of BK<sub>Ca</sub> channel protein in the four groups of transfected cells (Fig. 6F), thus confirming equal starting conditions for the immunoprecipitation results shown in Fig. 6D.

Finally, we believe that the difference in co-immunoprecipitation data shown in Fig. 6, A and D, may reflect the relative expression of cGKI<sub>α</sub> versus BK<sub>Ca</sub> channels. If we consider that cGKI<sub>α</sub> is expressed to a greater level than BK<sub>Ca</sub> channel protein under the conditions of our transient co-transfection, then we would anticipate that there is a greater likelihood to observe cGKI<sub>α</sub> co-associated with BK<sub>Ca</sub> channel immunoprecipitates because the kinase molecules are present in excess quantity. However, for cGKI<sub>α</sub> immunoprecipitation, the majority of either wild-type or inactive kinase molecules would not be associated with BK<sub>Ca</sub> channel protein, which decreases the probability that immunoprecipitated cGKI<sub>α</sub> will have a channel molecule bound to it. This situation leads to a low recovery of kinase co-associated with the channel, which effectively dilutes any observable differences in the detected co-associations. This

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**Fig. 5. Direct phosphorylation of BK<sub>Ca</sub> channels by cGKI<sub>α</sub> in vitro.** A, immunoprecipitates from HEK 293 cells transiently transfected with cDNA encoding either BK<sub>Ca</sub> channel α subunit (IP from BK<sub>Ca</sub> transfected cells) or empty vector (IP from mock transfected cells) were used as substrates in an in vitro phosphorylation reaction. The presence or absence of purified recombinant bovine cGKI<sub>α</sub> in each reaction is indicated by + or −, respectively, above the lanes. After termination, reaction mixtures were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. B shows a Western blot of the total cellular lysates and immunoprecipitates (IP) used in the experiment described in A. The blot was probed with an antibody recognizing the BK<sub>Ca</sub> channel α subunit. The positions of molecular mass standards are indicated to the right of each panel.
idea is supported by our observation that expressed BKCa channels can be detected in immunoprecipitates of endogenous cGKI (Fig. 6D), which is present at much lower levels than the expressed forms of the kinase. This low expression ratio between endogenous kinase and expressed channel thereby increases the likelihood that an immunoprecipitated kinase molecule will be co-associated with a channel protein. Therefore, immunoprecipitation of the lesser of these two proteins in-
increases the likelihood of detecting an interaction with the more abundant partner.

**DISCUSSION**

In this study, we have examined the importance of the cGKI in the regulation of a BK$_{Ca}$ channel by the NO/cGMP signaling pathway in intact cells. To do so, we created a catalytically inactive mutant of the $\alpha$ isoform of cGKI (see "Materials and Methods") that could be transfected and expressed in mammalian cells. We anticipated that this mutant would selectively target and interfere with the function of endogenous cGKI in a dominant negative fashion (23, 24), which is not possible with the nonselective cGMP-dependent protein kinase inhibitor KT5823 (15) or the disruption of the cGKI gene (45), leading to loss of both $\alpha$ and $\beta$ isoforms. The results shown in Figs. 1 and 2 demonstrate that HEK 293 cells transiently transfected with BK$_{Ca}$ channel cDNA expressed a measurable level of endogenous cGMP-dependent protein kinase activity that correlated with the presence of the $\sim$75-kDa $\alpha$ isoform of cGKI, as detected by Western blotting. Transient expression of wild-type cGKI produced a large increase in cGMP-dependent protein kinase activity, as measured in total cell lysates, which correlated with a large increase in immunoreactive cGKI. In contrast, transient expression of the catalytically inactive or dead mutant of cGKI produced no change in measurable cGMP-dependent protein kinase activity compared with control (BK$_{Ca}$ channel alone) but led to a similar large increase in the expression of immunoreactive type I cGMP-dependent protein kinase, indicating the presence of transfected cGKI protein.

Having established both the activity and expression of endogenous cGMP-dependent protein kinase in our HEK 293 cells, we examined whether stimulation of this intrinsic pathway by the NO donor SNP or membrane-permeable db-cGMP could result in altered BK$_{Ca}$ channel activity. Using the cell-attached recording mode of the patch clamp technique (to keep dependent protein kinase activity compared with control (BK$_{Ca}$ channel alone) we observed that exposure of cells to either SNP (Fig. 3A) or db-cGMP (Fig. 4A) significantly increased the magnitude of macroscopic BK$_{Ca}$ channel currents, in agreement with recent observations of other investigators (14, 15). Taken together with our biochemical data above, these electrophysiological data would be consistent with a role for endogenous cGMP-dependent protein kinase in the augmentation of BK$_{Ca}$ channel activity by SNP and db-cGMP in these cells. Given that the primary function of protein kinases is to phosphorylate selected substrates, we anticipated that BK$_{Ca}$ channels would undergo serine/threonine phosphorylation in the presence of cGKI. The results shown in Fig. 5 clearly demonstrate that purified cGKI can readily phosphorylate immunoprecipitated BK$_{Ca}$ channels in vitro, in agreement with the findings of others (14, 42). Such direct phosphorylation of BK$_{Ca}$ channels by cGKI in situ would thus serve as the basis for the enhancement of BK$_{Ca}$ channel activity observed electrophysiologically after the addition of cGMP, Mg-ATP, and cGKI to excised membrane patches (9, 15, 43, 46) and would also explain how enhancement could be maintained after excision of inside-out membrane patches from stimulated cells (14).

2 A. P. Braun, unpublished observations

If cGKI is indeed a critical component in the regulation of BK$_{Ca}$ channel activity by the NO/cGMP signaling pathway, then we would predict that expression of the catalytically inactive cGKI mutant described above would selectively prevent such augmentation in response to SNP or db-cGMP. As shown in Figs. 3B and 4B, co-expression of BK$_{Ca}$ channels with dead cGKI does in fact preclude augmentation of channel activity by either SNP or db-cGMP when compared with BK$_{Ca}$ channels expressed alone. This observation thus suggests that the $\alpha$ isoform of cGKI alone is sufficient to support the regulation of BK$_{Ca}$ channel activity by the NO/cGMP signaling pathway in cells expressing a type I cGMP-dependent protein kinase. Based on these novel results, along with the previous observations of others (9, 14, 15, 43), we conclude that cGKI acts directly on BK$_{Ca}$ channels in situ, resulting in enhanced channel activity. Our conclusion thus agrees with that of a recent study by Sausbier et al. (47), who used the cGKI-deficient mouse to demonstrate the important role of cGKI in both the activation of BK$_{Ca}$ channels and nitric oxide/cGMP-dependent vasodilation. Recent studies from Han et al. (48) and White et al. (49) further demonstrate that cGKI is the primary protein kinase involved in the activation of BK$_{Ca}$ channels in coronary smooth muscle myocytes by vasodilatory, cAMP-elevating agents such as dopamine or forskolin.

Several groups have already reported that protein kinases may physically associate with membrane ion channels (50–54), presumably as part of a phosphorylation-dependent regulatory mechanism. Our findings (see Fig. 6, A and D) that cGKI can associate with mammalian BK$_{Ca}$ channels are analogous to recent results showing that endogenous cAMP-dependent protein kinase and Src tyrosine kinase can independently associate with native BK$_{Ca}$ channels immunoprecipitated from Drosophila head (44). In the context of phosphorylation-dependent regulation, the stronger association observed for catalytically inactive cGKI with BK$_{Ca}$ channels compared with wild-type kinase may serve to explain how the dead kinase acts in a dominant negative fashion to suppress enhancement of channel activity via the NO/cGMP signaling pathway. This stronger interaction of dead kinase with the channel could thus account for the observed suppression of channel activity in situ by (a) binding and depletion of the channel as a phosphorylation substrate and/or (b) displacement of active cGKI from anchoring proteins that localize the kinase near the BK$_{Ca}$ channel complex (23, 24).

In summary, the findings of our study using a dominant negative suppression strategy implicate an important role for cGKI in the enhancement of BK$_{Ca}$ channel activity by the NO/cGMP signaling pathway in intact cells. These results are further consistent with the observed phenotype of cGKI-deficient knockout mice, which display impaired endothelium and NO-dependent relaxation of smooth muscle, resulting in vascular and intestinal dysfunction (45, 47). The question of whether NO and NO donors may also be able to directly activate BK$_{Ca}$ channels is not supported by our data and remains controversial (55–58); it is possible that such a phenomenon may depend upon the specific preparation in use, along with the types and concentrations of agents under study.

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**REFERENCES**

1. Furchgott, R. F. (1984) *Annu. Rev. Physiol.* 46, 175–197.
2. McDonald, L. J., and Murad, F. (1995) *Adv. Pharmacol.* 31, 263–275.
3. Corbin, J. D., and Francis, S. H. (1999) *J. Biol. Chem.* 274, 13729–13732.
4. Pfeifer, A., Ruth, P., Dostmann, W., Sausbier, M., Klatt, P., and Hofmann, F. (1999) *Rev. Physiol. Biochem. Pharmacol.* 135, 105–149.
5. Surks, H. K., Mochizuki, N., Kasai, H., Georgescu, S. P., Tang, K. M., Ito, M., Lincoln, T. M., and Mendelsohn, M. E. (1999) *Science* 286, 1583–1587.
6. Sperelakis, N., Tohse, N., Ohy, Y., and Masuda, H. (1994) *Adv. Pharmacol.* 26, 217–252.
7. Yamakage, M., Hirshman, C. A., and Croxton, T. L. (1996) *Am. J. Physiol.* 270, L328–L345.
8. Stockand, J. D., and Sansom, S. C. (1996) *Am. J. Physiol.* 270, C1773–C1779.
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9. Taniguchi, J., Furukawa, K.-I., and Shigekawa, M. (1993) Pflugers Arch. 423, 167–172
10. Lu, G., Mazet, B., Sarr, M. G., and Szurszewski, J. H. (1998) Am. J. Physiol. 274, G488–G496
11. Peng, W., Hoidal, J. R., and Farrukh, I. M. (1996) J. Appl. Physiol. 81, 1264–1272
12. Archer, S. L., Huang, J. M., Hampl, V., Nelson, D. P., Shultz, P. J., and Weir, E. K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7983–7987
13. George, M. J., and Shibata, E. F. (1995) J. Invest. Med. 43, 451–458
14. Alioua, A., Tanaka, Y., Wallner, M., Hofmann, F., Ruth, P., Meera, P., and Toro, L. (1998) J. Biol. Chem. 273, 32850–32856
15. Fukao, M., Mason, H. S., Britton, F. C., Kenyon, J. L., Horowitz, B., and Keef, K. D. (1999) J. Biol. Chem. 274, 10927–10935
16. Bialecki, R. A., and Stinson-Fisher, C. (1995) Am. J. Physiol. 268, L152–L159
17. Zanzinger, J., Czachurski, J., and Seller, H. (1996) Adv. Pharmacol. 274, L1057–L1063
13. George, M. J., and Shibata, E. F. (1995) J. Invest. Med. 43, 451–458
14. Alioua, A., Tanaka, Y., Wallner, M., Hofmann, F., Ruth, P., Meera, P., and Toro, L. (1998) J. Biol. Chem. 273, 32850–32856
15. Fukao, M., Mason, H. S., Britton, F. C., Kenyon, J. L., Horowitz, B., and Keef, K. D. (1999) J. Biol. Chem. 274, 10927–10935
16. Bialecki, R. A., and Stinson-Fisher, C. (1995) Am. J. Physiol. 268, L152–L159
17. Zanzinger, J., Czachurski, J., and Seller, H. (1996) Adv. Pharmacol. 274, L1057–L1063
18. Li, P.-L., Jin, M.-W., and Campbell, W. B. (1998) Hypertension 31, 303–308
19. Francis, S. H., and Corbin, J. D. (1994) Adv. Pharmacol. 26, 115–170
20. Smolenski, A., Burkhard, A. M., Eigenthaler, M., Butler, E., Gambaryan, S., Lohmann, S. M., and Walter, U. (1998) Pflugers Arch. 438, 134–139
21. Lohmann, S. M., Vaandrager, A. B., Smolenski, A., Walter, U., and De Jonge, H. R. (1997) Trends Biochem. Sci. 22, 307–312
22. Keilbach, A., Ruth, P., and Hofmann, F. (1992) Eur. J. Biochem. 208, 467–473
23. Hedgeson, I. (1997) Nature 391, 219–222
24. A. (1999) Pflugers Arch. 438, 671–677
25. Li, P.-L., Jin, M.-W., and Campbell, W. B. (1998) Hypertension 31, 303–308
26. Francis, S. H., and Corbin, J. D. (1994) Adv. Pharmacol. 26, 115–170
27. Smolenski, A., Burkhard, A. M., Eigenthaler, M., Butler, E., Gambaryan, S., Lohmann, S. M., and Walter, U. (1998) Pflugers Arch. 438, 134–139
28. Deng, W. P., and Nickoloff, J. A. (1994) Anal. Biochem. 209, 81–88
29. Lefkowitz, I. (1987) Nature 329, 219–222
30. Sheppard, D. N. (1994) Am. J. Respir. Cell Mol. Biol. 5, 2514–2523
31. Pallanck, L., and Ganetzky, B. (1994) Hum. Mol. Genet. 3, 1239–1243
32. Laemmli, U. K. (1970) Nature 227, 680–685
33. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
34. Wolfe, L., Francis, S. H., Laidiss, L. R., and Corbin, J. D. (1987) J. Biol. Chem. 262, 16906–16913
35. Glass, D. B., and Krebs, E. G. (1982) J. Biol. Chem. 257, 1198–1200
36. Dinerman, J. L., Steiner, J. P., Dawson, T. M., Dawson, V., and Snyder, S. H. (1994) Neuropharmacology 33, 1245–1251
37. Ramamoorthy, S., Giovanetti, E., Qian, Y., and Blakely, R. D. (1998) J. Biol. Chem. 273, 2458–2466
38. Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971–1005
39. Hanks, S. K., and Hunter, T. (1995) FASEB J. 9, 576–596
40. Robertson, B. E., Schubert, R., Hescheler, J., and Nelson, M. T. (1993) Am. J. Physiol. 265, C299–C303
41. Wang, J., Zhou, Y., Wen, H., and Levitan, I. B. (1999) J. Neurosci. 19, 1–7
42. Pfeifer, A., Klatz, P., Massberg, S., Nuber, S., Schubert, R., Hirnese, C., Wang, G.-X., Korth, M., Assodi, A., Andersson, R.-E., Krenbach, P., Mayerhofer, A., Ruth, P., Fassler, R., and Hofmann, F. (1998) EMBO J. 17, 3045–3051
43. Stockand, J. D., and Sansom, S. C. (1996) Am. J. Physiol. 271, C1669–C1677
44. Schaubert, M., Schubert, R., Voigt, V., Hirnese, C., Pfeifer, A., Korth, M., Kleppisch, T., Ruth, P., and Hofmann, F. (2000) Circ. Res. 87, 825–830
45. Han, G., Kryman, J. P., McMillan, P. J., White, R. E., and Carrier, G. O. (1999) J. Cardiovasc. Pharmacol. 34, 819–827
46. White, R. E., Kryman, J. P., El-Mowafy, A. M., Han, G., and Carrier, G. O. (2000) Circ. Res. 86, 897–905
47. Swayne, S. L., Mose, S. J., Blackstone, C. D., and Huganir, R. L. (1992) FASEB J. 6, 2514–2523
48. Esquerra, M., Wang, J., Foster, C. D., Adelman, J. P., North, R. A., and Levitan, I. B. (1994) Nature 369, 563–565
49. Holmes, T. C., Fadool, A. A., Dorn, R., and Levitan, I. B. (1996) Science 274, 2089–2091
50. Yu, X.-M., Askalan, R., Keil, G. J., and Salter, M. W. (1997) Science 275, 674–678
51. Hu, X.-Q., Singh, N., Muckhospadhyay, D., and Akbarali, H. I. (1998) J. Biol. Chem. 273, 5357–5342
52. Boletina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., and Cohen, R. A. (1994) Nature 368, 850–853
53. Mistry, D. R., and Garland, C. J. (1998) Br. J. Pharmacol. 124, 1131–1140
54. Abderrahmane, A., Salvail, D., Dumoulin, M., Garon, J., Cadieux, A., and Rousseau, E. (1998) Am. J. Respir. Cell Mol. Biol. 19, 485–497
55. Haburcak, M., Wei, L., Viana, F., Peenen, J., Droogmans, G., and Nilius, B. (1997) Cell Calcium 21, 291–300