The Large Conductance, Voltage-dependent, and Calcium-sensitive K⁺ Channel, Hslo, Is a Target of cGMP-dependent Protein Kinase Phosphorylation in Vivo*

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Abderrahmane Alioua‡‡§§, Yoshio Tanaka‡§, Martin Wallner‡, Franz Hofmann**, Peter Ruth**, Pratap Meera‡, and Ligia Toro‡ ‡‡§§

From the Departments of Anesthesiology and Molecular and Medical Pharmacology, and §§Brain Research Institute, UCLA, Los Angeles, CA 90095-1778 and **Institut für Pharmakologie und Toxikologie, Technische Universität München, 80802 München, Germany

Native large conductance, voltage-dependent, and Ca²⁺-sensitive K⁺ channels are activated by cGMP-dependent protein kinase. Two possible mechanisms of kinase action have been proposed: 1) direct phosphorylation of the channel and 2) indirect via PKG-dependent activation of a phosphatase. To scrutinize the first possibility, at the molecular level, we used the human pore-forming α-subunit of the Ca²⁺-sensitive K⁺ channel, Hslo, and the α isoform of cGMP-dependent protein kinase I. In cell-attached patches of oocytes co-expressing the Hslo channel and the kinase, 8-Br-cGMP significantly increased the macroscopic currents. This increase in current was due to an increase in the channel voltage sensitivity by ~20 mV and was reversed by alkaline phosphatase treatment after patch excision. In inside-out patches, however, the effect of purified kinase was negative in 12 of 13 patches. In contrast, and consistent with the intact cell experiments, purified kinase applied to the cytoplasmic side of reconstituted channels increased their open probability. This stimulatory effect was absent when heat-denatured kinase was used. Biochemical experiments show that the purified kinase incorporates γ-³²P into the immunopurified Hslo band of ~125 kDa. Furthermore, in vivo phosphorylation largely attenuates this labeling in back-phosphorylation experiments. These results demonstrate that the α-subunit of large conductance Ca²⁺-sensitive K⁺ channels is substrate for G-Iα kinase in vivo and support direct phosphorylation as a mechanism for PKG-Iα-induced activation of maxi-K channels.

Large conductance, voltage-dependent, and Ca²⁺-sensitive K⁺ (maxi-K)¹ channels are ubiquitously expressed, except in myocardial myocytes (1, 2). They play a key role in the control of neuronal firing (3, 4), cell secretion (5), and smooth muscle tone (6). Maxi-K channel activity is under a complex metabolic control that may involve G-proteins (7, 8), intracellular Ca²⁺ concentration (6), and a balance between phosphorylation/dephosphorylation mechanisms (9, 10).

Agents like nitrocompounds or atrial natriuretic peptide are potent vasodilators that act by increasing intracellular cGMP, resulting in cyclic GMP-dependent protein kinase (PKG) activation (11–13). Part of the response to these agents is antagonized by inhibitors of maxi-K channels, suggesting a key role of these channels in mediating their biological effects (12, 14, 15).

In agreement, maxi-K channels are activated by nitrocompounds (16), probably through a PKG-dependent phosphorylation mechanism (14, 17, 18). Two main mechanisms have been proposed to explain the activation of native maxi-K channels by PKG. One mechanism suggests that PKG activates maxi-K channels through a direct phosphorylation of the channel protein in a regulatory subunit (17, 19–21); this process can be reversed by phosphatase 2A (22). In contrast, the second mechanism proposes that PKG activates maxi-K channels indirectly via a PKG-dependent activation of protein phosphatase 2A (23, 24), which may directly dephosphorylate maxi-K channels. Alternatively, protein phosphatase 2A may exert its effect indirectly.

Recent biochemical experiments performed with purified channels (25, 26) and native membranes derived from tracheal smooth muscle show that PKG can incorporate ³²P from [γ-³²P]ATP into bands corresponding to the channel protein (21). Consistent with the view that maxi-K channels can be direct targets of PKG-dependent phosphorylation, sequence analysis of the pore-forming α-subunit shows one strong consensus sequence for PKG phosphorylation. This possible PKG phosphorylation site is conserved in all mammalian maxi-K (slo) channels and is localized within its C-terminal region (27).

However, purified PKG has failed to activate cloned maxi-K channels in excised inside-out membrane patches (28).

We now show that the α-subunit of maxi-K channels (Hslo) when coexpressed with PKG-Iα is activated by a permeant analog of cGMP and that this activation is reversed by alkaline phosphatase treatment. Recombinant PKG-Iα activates Hslo channels after reconstitution into lipid bilayers. Furthermore, in vitro and in vivo phosphorylation assays show that the α-subunit of maxi-K channels is a direct target of PKG-Iα.

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† These authors contributed equally to this work.

‡ Recipient of an AHA-GLA Research Fellowship 1169-F11.

¶¶ Present address: Dept. of Pharmacology, Toho University School of Pharmaceutical Sciences, 2-2-1 Miyama, Funabashi-City, Chiba 274-8510, Japan.

§§ An Established Investigator of the American Heart Association.

1 The abbreviations used are: maxi-K, large conductance, voltage-dependent, and Ca²⁺-sensitive K⁺ channel; Hslo, α-subunit of maxi-K channels; HF1, c-Myc-tagged Hslo; MOPS, 3-(N-morpholino)propanesulfonic acid; TES, 3-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PKG, cGMP-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; 8-Br-cGMP, 8-bromo-cyclic GMP; 8-cPT-cGMP, guanosine-3′,5′-cyclic monophosphate, 8-4-chlorophenythiotriethyl-ammonium salt.

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Materials—Recombinant PKG-Iα was prepared as described previously (29). PKG-Iα/PKG-Iβ polyclonal antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY); alkaline phosphatase was from Boehringer Mannheim; [γ-32P]ATP (4000 Ci/mmol) was from NEN Life Science Products; L-[35S]methionine (~1300 Ci/mmol) was from ICN; lipids were from Avanti Polar Lipids; 8-pCPT-cGMP and e-Myc peptide were from Calbiochem; and okadaic acid was from RBI. Other materials were from Sigma. GenBank accession numbers were U11058 for Halo and Y08961 for PKG-Iα. e-Myc-tagged Halo (HF1) is as described by Meera et al. (30).

Patch Clamp—Xenopus oocytes expressing PKG-Iα and/or Halo channel were used. The pipette and bath solutions contained 110 mM potassium methanesulfonate, 10 mM HEPES, 2 mM MgCl2, pCa 5.3, pH 7.0. In the bath solution without MgCl2, A Ca2+-electrode was used to measure pCa (World Precision Instruments). In some batches of oocytes, control cell-attached currents showed a substantial current increase (~5–10-fold) with time (1–5 min); thus, cell-attached experiments were performed only in oocytes where currents were stable for ~10 min. To avoid misinterpretations due to possible uncontrolled Ca2+ increases near the channels in the cell-attached mode that could appear as 8-Br-cGMP-induced up-regulation, both control and 8-Br-cGMP-treated patches were excised and current half-activation potentials (V0.5) were determined at constant Ca2+. Drugs were applied in the same patch or in patches from the same oocyte to circumvent the large variation in V0.5 (at fixed Ca2+) of maxi-K currents in different oocytes (31). Patches from the same oocyte, injected with Halo or Halo plus PKG-Iα, have significantly less variation in V0.5 (S.D. of ~20 mV, n = 61) (31). Patches with Halo alone or coexpressed with PKG-Iα did not significantly alter the voltage sensitivity of Halo channels (32); at 5 μM Ca2+, V0.5 was 10 and 16 mV, respectively. Solid lines are the best fit to a Boltzmann equation: \( P_\text{on} = 1/(1 + \exp(-\Delta V/V_0)) \). Inset, currents (~150 to 50 mV pulses every 5 mV from a holding potential, Vh, of 0 mV) from oocytes expressing Halo alone (A) or coexpressed with PKG-Iα in Xenopus Oocytes—To address the possibility that the antibody-protein-A-bead complex was phosphorylated in vitro (back-phosphorylation) in the presence of 100 μCi of [γ-32P]ATP as indicated above. The reaction was terminated by washing three times with the washing buffer containing 1 μM cold MgATP. Proteins were resolved on SDSPAGE, and immunoblotted (see below). After washing for >8 h, the membrane was autoradiographed. Phosphorylation and protein content were quantitated by densitometric analysis (GS-670 Imaging Densitometer; Bio-Rad).

Immunoblotting—PKG-Iα and HF1 were immunoblotted according to the manufacturer’s instructions or as described previously (26). First antibodies (1:2000) were anti-PKG raised against CDEPPPDNSGW-DIDF or anti-Halo raised against VNDTQVFQLQDD in the C terminus (26). The secondary antibody was anti-rabbit peroxidase-conjugated IgG (1:4000). The immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Data are expressed as mean values ± S.D. A two-tailed Student’s t test for paired data was used. Results were considered significantly different with a p < 0.05.

RESULTS

Expression of PKG-Iα in Xenopus Oocytes—To address the possible functional coupling between Halo channels and PKG-Iα, we first tested if Xenopus oocytes express any “endogenous” PKG using Western blot analysis. As shown in Fig. 1A, the antibody raised against the mammalian PKG-Iα/PKG-Iβ only labels a band (~70 kDa) in oocytes injected with mammalian PKG-Iα cRNA (lane 1), but not in oocytes expressing e-Myc-tagged Halo (HF1, see Fig. 5) alone (lane 2). These results indicate that in Xenopus oocytes PKG content is below the detection limit and/or its primary sequence differs from that in room temperature. The channel proteins were immunoprecipitated in the presence of phosphatase inhibitors (300 nM okadaic acid, 10 μM NaF, and 10 μM Na-HPO4). The Halo channels attached to the antibody-protein-A-bead complex were phosphorylated in vitro (back-phosphorylation) in the presence of 100 μCi of [γ-32P]ATP as indicated above. The reaction was terminated by washing three times with the washing buffer containing 1 μM cold MgATP. Proteins were resolved on SDS-PAGE, and immunoblotted (see below). After washing for >8 h, the membrane was autoradiographed. Phosphorylation and protein content were quantitated by densitometric analysis (GS-670 Imaging Densitometer; Bio-Rad).

Halo channels were expressed in oocytes by injecting mRNA encoding Halo with or without PKG-Iα cRNA (300 nM okadaic acid, 10 μM NaF, and 10 μM Na-HPO4). The Halo channels attached to the antibody-protein-A-bead complex were phosphorylated in vitro (back-phosphorylation) in the presence of 100 μCi of [γ-32P]ATP as indicated above. The reaction was terminated by washing three times with the washing buffer containing 1 μM cold MgATP. Proteins were resolved on SDS-PAGE, and immunoblotted (see below). After washing for >8 h, the membrane was autoradiographed. Phosphorylation and protein content were quantitated by densitometric analysis (GS-670 Imaging Densitometer; Bio-Rad).

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mammalian isoforms. Therefore, to study PKG modulation of human maxi-K channels at the molecular level, we coexpressed both Hslo and PKG-Iα cRNAs. Currents from oocytes expressing Hslo with or without PKG-Iα had similar voltage activation curves when measured in inside-out patches with 5 mM Ca\(^{2+}\), and the FP\(_{50} \sim V\) curve was obtained. V\(_{50}\) was 34 nM. Inset, currents from −150 to 100 mM every 5 mM; bars, 2 nA and 20 ms. C, cell-attached currents at 0 and 10 min after patch attainment. 1 mM 8-Br-cGMP in the pipette increased current amplitude (1.3–5.6-fold after 10 min and at 150 mV; n = 6). Pulses were from −50 to 150 mM every 5 mM. D, FP\(_{50} \sim V\) curve after the patch in C was excised in 5 mM Ca\(^{2+}\). V\(_{50}\) was −4 mV. Inset, currents are from −150 to 100 mM in 5-mV steps; bars, 4 nA and 20 ms. FP\(_{50} \sim V\) curves were obtained as described previously (31). All traces are without leak subtraction.

**Fig. 2. 8-Br-cGMP activates expressed Hslo channels in cell-attached patches.** Oocytes expressing Hslo and PKG-Iα were used. Vh was 0 mV. A, cell-attached currents at 0 and 10 min after patch attainment; pulses were from −50 to 195 mV every 5 mV. The pipette solution was without 8-Br-cGMP. B, after 10 min in the cell-attached mode, the patch in A was excised in 5 mM Ca\(^{2+}\), and the FP\(_{50} \sim V\) curve was obtained. V\(_{50}\) was 34 nM. Inset, currents from −150 to 100 mM every 5 mM; bars, 2 nA and 20 ms. C, cell-attached currents at 0 and 10 min after patch attainment. 1 mM 8-Br-cGMP in the pipette increased current amplitude (1.3–5.6-fold after 10 min and at 150 mV; n = 6). Pulses were from −50 to 150 mM every 5 mM. D, FP\(_{50} \sim V\) curve after the patch in C was excised in 5 mM Ca\(^{2+}\). V\(_{50}\) was −4 mV. Inset, currents are from −150 to 100 mM in 5-mV steps; bars, 4 nA and 20 ms. FP\(_{50} \sim V\) curves were obtained as described previously (31). All traces are without leak subtraction.

8-Br-cGMP Activates Hslo Channels Coexpressed with PKG-Iα—To determine if PKG-Iα modulates Hslo channel activity, we stimulated expressed PKG-Iα with 8-Br-cGMP in the pipette. Control experiments (without 8-Br-cGMP) show that currents elicited by depolarizing pulses (−50 to 195 mV) were practically the same at time 0 (upon patch attainment) and after 10 min in the cell-attached configuration (Fig. 2A) (see “Experimental Procedures”). In this example, currents are slightly faster after 10 min; this small change in kinetics could be due to a small change in Ca\(^{2+}\) near the vicinity of the channels in the cell-attached patch. After excision of the patch in 5 mM Ca\(^{2+}\), the half-activation potential (V\(_{50}\)) was 34 mV.

**Fig. 3. Alkaline phosphatase reverses the stimulatory effect induced by 8-Br-cGMP.** Macroscopic currents in inside-out patches of oocytes expressing Hslo and PKG-Iα, at 5 mM [Ca\(^{2+}\)]. Patches were held for 10 min in the cell-attached mode prior to excision. Vh was 0 mV. A, control currents without 8-Br-cGMP in the pipette. B, currents in another patch of the same oocyte as in A but with 1 mM 8-Br-cGMP in the pipette. C, same patch following treatment with 100 units/ml alkaline phosphatase. Traces in A–C are from −150 to 40 mV every 5 mV. D, corresponding FP\(_{50} \sim V\) curves. 8-Br-cGMP shifted the voltage activation curve from a V\(_{50}\) of 4 mV (○) to −22 mV (△); the shift was reversed to 1 mV (□) following alkaline phosphatase perfusion. Mean values are as follows: for control, 25 ± 18 mV; stimulated with 8-Br-cGMP, 4 ± 22 mV (p = 0.03); and after phosphatase treatment, 30 ± 24 mV (p = 0.005) n = 3.

(Fig. 2B). In contrast, Fig. 2C shows that in another cell-attached patch of the same oocyte as in Fig. 2A, but with 1 mM 8-Br-cGMP in the pipette, macroscopic cell-attached currents underwent an −3-fold increase in their amplitudes after 10 min of drug treatment. To determine if the 8-Br-cGMP-induced Hslo activation was due to an increase in the voltage sensitivity of the channels and not to a large spontaneous increase in intracellular Ca\(^{2+}\), we excised the patch in the same 5 mM Ca\(^{2+}\) solution as in Fig. 2B and constructed a voltage activation curve (Fig. 2D). The half-activation potential shifted to negative potentials to a value of −4 mV. In six out of eight similar paired experiments, V\(_{50}\) significantly changed from 17 ± 20 mV in untreated oocytes to −4 ± 17 mV after 8-Br-cGMP or 8-pCPT-cGMP treatment (p = 0.007). These results show that 8-Br-cGMP treatment induces an increase in Hslo voltage sensitivity of about −20 mV (21 ± 12 mV) at constant Ca\(^{2+}\).

**Alkaline Phosphatase Reverses the Effect of 8-Br-cGMP Treatment**—To investigate if the 8-Br-cGMP-induced activation of Hslo (coexpressed with PKG-Iα) involves a phosphorylation mechanism, we determined if phosphatase treatment could reverse this effect. Fig. 3A shows control currents in an excised patch at 5 mM Ca\(^{2+}\) that was previously held for 10 min in the cell-attached mode under nonstimulating conditions (no 8-Br-cGMP in the pipette). In this patch, the Hslo normalized voltage activation curve had a V\(_{50}\) of 4 mV (Fig. 3D, ○). As expected, 8-Br-cGMP treatment of another cell-attached patch on the same oocyte caused a leftward shift of the channel voltage activation curve as measured after excision in 5 mM
Ca\textsuperscript{2+} (V\textsubscript{m} = -22 mV; Fig. 3, B and D, A). This is reflected by larger inward currents at negative potentials and faster activation kinetics in Fig. 3B. Treatment of the same excised patch with alkaline phosphatase (100 units/ml) for ~10 min induced a clear reduction in steady-state inward currents and activation kinetics (Fig. 3C) when compared with traces in Fig. 3B. The latter supports a change in open probability of the channels rather than a diminution of the number of channels in the patch due to “run-down.” Indeed, the voltage activation curves normalized to the maximum conductance at each condition showed that treatment with alkaline phosphatase (Fig. 3D, □) shifts the curve back to control values (V\textsubscript{m} = 1 mV). In three such paired experiments, stimulation by 8-Br-cGMP caused a leftward shift of 20 ± 5 mV (p = 0.03) from control values; subsequent phosphatase treatment reversed this action and caused a slight rightward shift (~9 mV) above control values in two experiments. This shift above control values may be explained by phosphorylation of Hslo channels by endogenous kinases (but not by expressed PKG-Iα; see Fig. 1), since in three out of seven control inside-out patches expressing Hslo and PKG-Iα, alkaline phosphatase induced a 14 ± 4 mV (p < 0.05) rightward shift of the channel voltage activation curve. However, in the other four control patches phosphatase treatment did not significantly change the channel voltage sensitivity (shift was 1 ± 3 mV, p = 0.6). Taken together, these results show that alkaline phosphatase can reverse the stimulatory effect induced by 8-Br-cGMP, and thus, the activation of Hslo induced by 8-Br-cGMP in cell-attached patches is probably due to a phosphorylation mechanism.

Effect of Purified PKG-Iα on Hslo Channels after Patch Excision—To test the hypothesis that the activation of Hslo induced by 8-Br-cGMP involves phosphorylation of Hslo channels, we directly tested the effect of purified PKG-Iα on Hslo channels expressed alone using inside-out patches. Control records were taken after perfusing PKG-Iα buffer and MgATP plus cGMP. These compounds had negligible effects on channel activity as measured by voltage activation curves. PKG-Iα buffer (at the final concentration used in the experiments) produced a mean leftward shift of 1 ± 4 mV (n = 12), and MgATP plus cGMP (0.1–0.5 mM) induced a leftward shift of 3 ± 6 mV (n = 13). In only one out of 13 experiments did further perfusion of PKG-Iα (plus MgATP, cGMP, 0.01% bovine serum albumin) produce a stimulatory effect (10 mV leftward shift of the voltage activation curve). To rule out the possibility that PKG-Iα-induced channel activation was masked by endogenous phosphorylation or by an associated phosphatase, patches were treated with alkaline phosphatase or with okadaic acid. Pretreatment with 100 units/ml alkaline phosphatase (n = 7) or the addition of 3 μM okadaic acid after PKG-Iα (n = 2) did not lead to stimulation of channel activity by the purified kinase. Alkaline phosphatase had varied effects on Hslo activity. From 18 patches, alkaline phosphatase produced no significant effect in nine cases (change of 0.8 ± 3 mV; p = 0.5), a rightward shift of 16 ± 4 mV in 7 cases (p < 0.001), and a mean leftward shift of −16 mV in two experiments. Nevertheless, as stated above, none of the predephosphorylated channels were activated by purified PKG-Iα.

Purified PKG-Iα Induces an Increase in the Open Probability of Hslo Channels Reconstituted into Lipid Bilayers—In contrast to experiments in inside-out patches, Hslo channels reconstituted into lipid bilayers could be consistently activated by treatment with purified PKG-Iα (10 out of 12 experiments). Purified PKG-Iα, cGMP, and ATP were added directly to the cytoplasmic side of the channels together with 0.01% bovine serum albumin. In most experiments, okadaic acid (100 nM) was present to prevent a possible dephosphorylation mechanism. Fig. 4, A and B, show typical examples of channel activation after treatment with PKG-Iα. In Fig. 4A, PKG-Iα was tested at constant voltage (~30 mV). Representative traces are illustrated above the P\textsubscript{o} versus time plots. In this experiment, okadaic acid (100 nM) added prior to PKG-Iα treatment caused no significant variation in channel activity (n = 5). In contrast, the addition of 80 nM PKG-Iα significantly increased channel activity from a mean P\textsubscript{o} of 0.04 to 1.0. In most cases, the effect of the kinase was observed within 5–10 min after PKG-Iα application and produced a 3 ± 1.8-fold increase (n = 10) in channel activity. Note that neither channel amplitude nor number of channels were affected by PKG-Iα treatment, but open probability was affected. Fig. 4B shows the effect of PKG-Iα at different voltages obtained from ramp experiments. PKG-Iα treatment caused a 15 mV leftward shift in the voltage activation curve of Hslo channels, V\textsubscript{m} changed from +11 mV to −4 mV. The mean leftward shift induced by PKG-Iα for three experiments was 15 ± 0.7 mV. This modulatory effect of
PKG-Iα on the open probability of Hslo channels accounts for the increase in their macroscopic current amplitudes and is similar to the shift of the activation curves recorded in intact cells (Fig. 2). The specificity of PKG-Iα action was tested using boiled kinase (Fig. 4C). In four out of four experiments, boiled PKG-Iα had no effect in channel activity. In two experiments where active PKG-Iα was further tested, the active enzyme was able to induce a 2- and 8-fold increase in channel activity. Furthermore, in one case washout of active PKG-Iα in the continuous presence of okadaic acid failed to reverse channel activation. Taken together, these results indicate that Hslo channels or a closely associated protein are targets of PKG-Iα-induced phosphorylation.

**Direct Phosphorylation of Hslo Channels by PKG-Iα**—To determine if Hslo channels are indeed directly phosphorylated by PKG-Iα, we examined 1) the incorporation of γ-^32P into immunoprecipitated channels and 2) the degree of back-phosphorylation in intact cells stimulated with 8-pCPT-cGMP a specific and potent activator of PKG-Iα (37). For efficient immunoprecipitation, we used an Hslo construct tagged with the c-Myc epitope at its amino terminus (Fig. 5B). Anti-c-Myc antibody specifically immunoprecipitated a ~125-kDa band (c-Myc-tagged Halo) with a purity > 90%.

**Fig. 5. Immunoprecipitation of epitope-tagged Halo channels.**

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Solubilization and immunoprecipitation may expose a site(s) that is not normally available for phosphorylation; in addition, in vitro phosphorylation conditions may not reflect the native cell environment. Therefore, to investigate an in vivo phosphorylation, we utilized the back-phosphorylation assay (38) (Fig. 6B). In this procedure, endogenous phosphate is incorporated in vivo into the channel protein after stimulation of expressed PKG-Iα with 8-pCPT-cGMP in intact oocytes. Thereafter, stimulated and nonstimulated oocytes are in vitro phosphorylated (back-phosphorylated) with purified PKG-Iα to incorporate ^32P to the Halo sites that were left unphosphorylated after treatment with 8-pCPT-cGMP. In this protocol, a decrease in back-phosphorylation (incorporated ^32P) reflects an increase in the in vivo phosphorylation of Hslo channels by expressed PKG-Iα.

As expected for a direct phosphorylation mechanism on the channel protein, the amount of ^32P incorporated during back-phosphorylation diminished when intact channels coexpressed with PKG-Iα were stimulated in vivo with 8-pCPT-cGMP (Fig. 6B, top). Immunoblots (Fig. 6B, bottom) using antibodies against the carboxyl terminus of Hslo corroborate the identity of the phosphorylated band (~125 kDa) as Hslo channels and served to normalize back-phosphorylation to the amount of Hslo protein. Quantitative analysis of four experiments showed that back-phosphorylation was significantly diminished to 38 ± 16% of the control value (p < 0.005) (Fig. 6C). These
results demonstrate that Hslo channels are direct targets of PKG-Iα-dependent phosphorylation in vivo.

**DISCUSSION**

We have investigated the possibility that activation of the maxi-K channel by PKG involves a direct phosphorylation of its α-subunit (Hslo). We studied changes in their electrical activity induced by expressed or purified PKG-Iα and biochemical incorporation of phosphate into the channel protein.

PKG and maxi-K channels are abundant proteins in smooth muscles (39), and their activities exert a tight control of vascular reactivity. We have explored their functional coupling in *Xenopus* oocytes, since these cells express none, or insignificant amounts, of these two proteins (31, 40) (Fig. 1). Heterologous expression of PKG-Iα in oocytes did not alter the voltage or apparent calcium sensitivity of co-expressed Hslo channels unless the kinase was activated by cGMP (Figs. 1 and 2). This is in contrast with the constitutive activity of expressed PKG-Iα in Chinese hamster ovary cells (24) that enhances the calcium sensitivity of an endogenous 50 picosiemens K⁺ channel. Thus, *Xenopus* oocytes seem to be a good system to study PKG-induced phosphorylation of Hslo channels, since both expression and activity of PKG can be controlled.

Upon stimulation of PKG-Iα with 8-Br-cGMP, the macroscopic current amplitudes of Hslo channels were increased (Fig. 2). These results are in agreement with those obtained by other investigators using native maxi-K channels (14, 16, 17, 17–20, 24). We demonstrate for the first time that the increase in current amplitude in intact cells is due to a leftward shift in the voltage activation curve of Hslo channels. This was accomplished by first stimulating the cells with 8-Br-cGMP in the cell-attached mode, followed by patch excision at a Ca²⁺ concentration (5 μM), where accurate voltage-activation curves can be obtained (41). A leftward shift of the voltage sensitivity of ~20 mV was observed (Fig. 2). This value is somewhat smaller than the one obtained in native tissues (14, 17). This difference may be due to the level of expression of the regulatory β-subunit (25, 42) or of splice variants of Hslo in native cells. The regulatory β-subunit is known to modify the biophysical, pharmacological, and modulatory properties of Hslo (31, 41, 43–45). Future studies are necessary to address the question of whether the β-subunit affects the PKG-induced activation of the α-subunit (Halo) of maxi-K channels.

A standard procedure to determine if maxi-K channels or a closely related protein are targets of PKG-dependent modulation is to apply purified kinase to inside-out patches. In native tissues, purified PKG activates maxi-K channels using this patch configuration (14, 17, 18, 20, 22, 46). It is remarkable that cloned maxi-K channels seem not to respond to purified PKG after patch excision. PKG failed to activate Cslo (canine maxi-K channel) (28), which has an amino acid sequence almost identical to that of Hslo and has a conserved strong consensus site for PKG phosphorylation (27). In our experiments, purified PKG-Iα failed to activate Hslo channels in 12 out of 13 inside-out patches, including patches pretreated with alkaline phosphatase (seven out of seven). In only one experiment there was a 10-mV increase in the voltage sensitivity. At present, these results are difficult to explain. However, they stress the point that maintenance of a native intracellular architecture may be important for specific channel responses. In fact, other kinases like cAMP-dependent protein kinase and PKG-II are targeted to ion channels via anchoring proteins or anchoring domain(s) enhancing channel regulation (47, 48). In line with this view, PKG-Iα has been found to interact with the intermediate filament protein vimentin (49). It is also possible that the phosphorylation site(s), probably localized in the carboxyl terminus of Hslo channels (Fig. 5A), becomes inaccessible to the kinase after patch excision or that these inside-out patches had a restricted access, slowing PKG diffusion and preventing it from reaching its target. Another possibility is that PKG phosphorylates a soluble, loosely bound intracellular component responsible for Hslo activation, lost in inside-out patches. However, this explanation is difficult to reconcile with the reconstitution experiments where PKG could activate Hslo channels (Fig. 4), although it is known that reconstituted vesicles maintain functional protein complexes (50).

Hslo (with or without PKG-Iα) seems not to be substantially phosphorylated by endogenous oocyte kinases, since under basal conditions phosphatase treatment changed channel activity in only 12 out of 25 inside-out patches. In 10 cases, it induced a positive shift of about 15 mV, whereas in two patches it caused a negative shift of about the same magnitude. These results reveal phosphorylation states of Hslo channels that activate or inhibit their basal activity. As stated above, their dephosphorylation does not allow PKG channel modulation in inside-out patches.

Activation of intact Hslo channels by 8-Br-cGMP and its reversal by alkaline phosphatase treatment (Fig. 3) after patch excision indicates that in this system a phosphorylation process is involved with the substrate(s) being Hslo channels or a closely associated protein. Additional evidence supporting this view is the bilayer experiments, where treatment with purified PKG-Iα induced an increase in channel open probability (Fig. 4). Okadaic acid, a phosphatase inhibitor did not cause any change in the basal activity of reconstituted channels (Fig. 3) (24). This can be explained by a lack of both reconstituted phosphatases or basal activity of coexpressed PKG-Iα (Fig. 1). However, in three cases reconstitution of Hslo and phosphatase activity seemed to occur, since okadaic acid enhanced the stimulatory effect of purified PKG-Iα or its washout reversed the stimulatory effect (not shown). These results are in agreement with those obtained in human pulmonary arterial myocytes (46) and human mesangial cells (20, 22, 46) and support the idea that PKG-Iα activates Hslo channels via a direct phosphorylation mechanism. Our results are in contrast with those observed in maxi-K channels from bovine tracheal smooth muscle (24) and from a rat pituitary tumor cell line (23), where okadaic acid attenuated the stimulatory effect of PKG, suggesting a dephosphorylation-mediated mechanism. This difference may be explained by the presence of different isoforms of maxi-K channels, differential expression of phosphatases, and/or different colocalization of maxi-K channels, kinases, and phosphatases in these tissues.

Direct evidence showing that Hslo channels are targets of PKG-induced phosphorylation was obtained by measuring incorporated phosphate to the channel protein. Biochemical experiments were performed using a c-Myc-tagged maxi-K channel (HF1) (30) that allowed its specific immunoprecipitation (Fig. 5). In *vitro* phosphorylation of HF1 channels after immunoprecipitation clearly labeled a band corresponding to HF1. This is in agreement with studies showing that PKG directly phosphorylates purified maxi-K channels from tracheal smooth muscle (21). However, both types of experiments have the disadvantage that they involve channels that may have lost their native conformation during the different biochemical steps. A change in conformation may facilitate or improve their accessibility to phosphorylation, leading to false positive results. Therefore, we performed *in vivo* phosphorylation experiments using intact oocytes where channels are in their native conformation. The back-phosphorylation results show that in vivo phosphorylation of Hslo channels are targets of PKG-Iα. Additional experiments are required to determine if unknown proteins could also be phosphorylated and
contribute to the increase in channel activity.

In conclusion, the present study demonstrates that 1) PKG-1α activates the α-subunit of maxi-K channels (Hslo) by increasing its open probability without affecting its unitary conductance, and 2) PKG-1α directly phosphorylates Hslo channels in vivo. Since maxi-K channels and cGMP-dependent pathways are important regulators of smooth muscle function, the establishment of how both proteins interact provides important tools to modify smooth muscle function.

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