Protein kinase C inhibits Kv1.1 potassium channel function

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Boland, Linda M., and Katharine A. Jackson. Protein kinase C inhibits Kv1.1 potassium channel function. Am. J. Physiol. 277 (Cell Physiol. 46):C100–C110, 1999.—The regulation by protein kinase C (PKC) of recombinant voltage-gated potassium (K) channels in frog oocytes was studied. Phorbol 12-myristate 13-acetate (PMA; 500 nM), an activator of PKC, caused persistent and large (up to 90%) inhibition of mouse, rat, and fly Kv1.1 currents. K current inhibition by PMA was blocked by inhibitors of PKC, and inhibition was not observed in control experiments with PMA analogs that do not activate PKC. However, site-directed substitution of potential PKC phosphorylation sites in the Kv1.1 protein did not prevent current inhibition by PMA. Kv1.1 current inhibition was also not accompanied by changes in macroscopic activation kinetics or in the conductance-voltage relationship. In Western blots, Kv1.1 membrane protein was not significantly reduced by PKC activation. The injection of oocytes with botulinum toxin C3 exoenzyme blocked the PMA inhibition of Kv1.1 currents. These data are consistent with the hypothesis that PKC-mediated inhibition of Kv1.1 channel function occurs by a novel mechanism that requires a C3 exoenzyme substrate but does not alter channel activation gating or promote internalization of the channel protein.

VOLTAGE-GATED K CHANNELS are a diverse family of proteins that function in the regulation of action potentials, cardiac pacemaking, signal integration, and neurotransmitter release in excitable cells. In nonexcitable cells, K channels help modulate hormone secretion, cell volume regulation, cell proliferation, and lymphocyte differentiation. The highly localized expression of the different molecular species of K channels (10) suggests that K channels may be specialized for different cellular functions. However, little comparative information is available regarding the second messenger regulation of the many different but related molecular forms of voltage-gated K channels. An understanding of how the different K channels are regulated by neurochemicals is critical to understanding the diverse and important functions of these channels.

The phosphorylation of K channels by protein kinase C (PKC) may regulate channel function. In several cell types, native K channel currents are affected by agents that directly activate PKC or by receptor systems that activate PKC through a second messenger cascade. For example, PKC activation inhibits a K current in cultured endothelial cells (38) and K currents in brain stem respiratory neurons (5) but enhances a K current in ventricular cells (36). In the Xenopus oocyte expression system, several types of recombinant, voltage-gated K channels have been shown to be modulated by PKC (18, 19, 21, 23, 29), and in some cases there is evidence that the K channel protein is the substrate for PKC phosphorylation (2, 3, 4, 8). However, the mechanisms and sites of kinase regulation of K channel activity are not known for many types of cells and K channels.

Xenopus oocytes are a useful system for studying the regulation of ion channel activity. The oocytes express a variety of native components of second messenger cascades including the G proteins Gα and Gβγ (25), phospholipase C (14), and PKC (26). Thus the molecular mechanisms of receptor-channel coupling can be studied by using the coexpression of foreign cell surface receptor and channel proteins linked by the native oocyte second messenger pathways.

In the present study, we tested for possible physiological differences in the modulation by PKC of recombinant voltage-gated K channels. We found that PKC activation had different consequences for K currents encoded by genes from the Shaker (Kv1), Shab (Kv2), Shaw (Kv3), and Shal (Kv4) subfamilies. PKC-mediated downregulation of Kv1.1 currents occurs by a novel mechanism that is inhibited by the C3 exoenzyme of botulinum toxin without an effect on channel gating or a significant reduction in K channel protein present at the membrane surface.

MATERIALS AND METHODS

Expression of K channels. Plasmids containing the cDNAs encoding voltage-gated K channels were linearized, and capped RNAs were synthesized in vitro with Ambion (Austin, TX) RNA polymerase kits. Similarly, plasmids containing turkey P2Y or human P2Y receptor cDNAs (gifts from T. K. Harden, Univ. of North Carolina, Chapel Hill, NC) were linearized and transcribed. RNA was purified by use of the RNAid kit (Bio 101, Vista, CA) and stored at −80°C in diethyl pyrocarbonate (DEPC)-treated water. Transcription reaction products were checked for size by agarose gel electrophoresis, and RNA concentrations were determined by spectrophotometry (Beckman DU-7000) by using the equation 1 A260 unit = 40 µg/ml RNA, where A260 is absorbance at 260 nm. K channel cDNAs were generous gifts from M. Covarrubias (mKv4.1), R. J. ohe (rKv2.1), O. Pongs (rKv1.1 and rKv3.4), L. Salkoff (rKv4.2), and B. Tempel (mKv1.1). We also used ShakerH4 from Drosophila melanogaster and a deletion mutant ShΔ (residues 6–46 deleted) that removes fast, N-type inactivation (13).

Oocytes were harvested from Xenopus laevis (Xenopus 1, Ann Arbor, MI), previously injected with human chorionic gonadotropin. Female frogs were anesthetized by immersion in 0.2% 3-aminobenzoic acid ethyl ester (Sigma Chemical).
Oocytes were released by gentle agitation for 2 h in 1 mg/ml collagenase D (Boehringer Mannheim) dissolved in a Ca-free OR-2 solution containing (in mM) 96 NaCl, 2 KCl, 1 MgCl₂, and 5 HEPES, pH 7.6, with NaOH. After the first hour, the solution was replaced with fresh enzyme. Subsequently, oocytes were extensively washed with Ca-free OR-2, and stage V and VI oocytes were collected with a dissecting microscope. Defolliculated oocytes were injected the same day or the following day with 50–100 nl of RNA (0.01–0.3 ng/ nl) dissolved in DEPC-treated water. Oocyte survival was best in a frog Ringer solution of (in mM) 96 NaCl, 1 KCl, 0.75 CaCl₂, 0.8 MgCl₂, and 10 HEPES, pH 7.4, with NaOH, 50 U/ml penicillin G, and 50 µg/ml streptomycin. Oocytes were maintained at 16–18°C in this solution (changed daily) for 1–6 days before electrophysiological recording.

Mutagenesis. Mutations were introduced into the mKv1.1 K channel cDNA in a Bluescript KS(+) vector (Stratagene) by oligonucleotide-directed mutagenesis. Miniprep DNA was prepared from isolated colonies with a DNA isolation kit (Promega). Mutant DNA was identified by dideoxy sequencing with Sequenase-modified DNA polymerase (United States Biochemical) and electrophoresis on 6% polyacrylamide urea sequencing gels. For each site studied, two mutant colonies were sequenced and prepared and tested separately.

Electrophysiology. Macroscopic K currents from oocytes were recorded with a two-electrode voltage clamp equipped with an OC-725B amplifier (Warner Instrument, Hamden, CT). Voltage-measuring and current-passing electrodes were filled with 3 M KCl and had resistances between 0.3 and 1.1 MΩ. Currents were sampled at 5 kHz and filtered at 2 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). All recordings were done at room temperature (−20°C). Oocytes were clamped at −70 or −80 mV (as noted in figure legends) and pulsed every 1–5 s to various test potentials to activate K channels.

Oocytes were perfused continuously with an external solution containing (in mM) 96 NaCl, 2 KCl, 1.8–2 CaCl₂, 1 MgCl₂, and 5 HEPES, pH 7.4, with NaOH. External solution changes were made by manually switching a valve that controlled gravity-driven solution flow across the oocyte recording chamber. Data were recorded on 80586-based computers equipped with National Instruments AT-MIO boards and interfaced with the experimental setup. Data acquisition software was written and generously donated by G. Yellen. Data were transferred to commercially available software programs for analysis and the production of figures. Data are expressed as means ± SE.

Biotinylation and recovery of surface proteins. Kv1.1-expressing oocytes (54–60/treatment) were washed twice with 2 ml of frog Ringer solution and then incubated in a labeling solution of 1 mg/ml EZ-Link NHS-SS-biotin [sulfosuccinimidyl 2-(biotinamido) ethyl-1,3-dithiopropionate; Pierce, Rockford, IL] dissolved in frog Ringer solution, pH 7.4. We used modifications to the methods of Stern-Bach et al. (28). Cells were labeled for 2 h at 21°C with gentle mixing on a laboratory shaker and then washed twice for 10 min each in frog Ringer solution with gentle mixing. Biotin-labeled oocytes were then washed once with 1 ml ice-cold homogenization buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7.4) containing a protease inhibitor cocktail: 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml aproatin, 1 µM pepstatin (all from Boehringer Mannheim), and 2 µg/ml antipain and 1 µg/ml leupeptin (both from Sigma). Oocytes were then homogenized by several passages through a pipette tip in 200 µl ice-cold homogenization buffer with protease inhibitors. Membrane-bound proteins were solubilized by 1.5% Triton X-100 (Sigma). Samples were incubated for 1 h on ice and centrifuged for 30 min (16,000 g at 8°C). The supernatant was carefully removed to a fresh tube. A 5-µl aliquot was removed and represented the total protein (T) fraction. The remaining supernatant was incubated for 2 h with a 20-µl bed volume of UltraLink immobilized monomeric avidin beads (Pierce; 50% slurry equilibrated in homogenization buffer with 1.5% Triton X-100) with gentle mixing on a laboratory rotator at 8°C. The resulting biotin-avidin complex was washed five times by suspension in 1 ml homogenization buffer (with the protease inhibitor cocktail and 1.5% Triton X-100). The samples were centrifuged in a PicoFuge (Stratagene) (1 min, 325 g), and the supernatant was carefully removed. Membrane proteins were released from the biotin-avidin complex by adding 25–50 µl loading buffer with reducing agent [0.13 M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 150 mM dithiothreitol, 0.01% bromphenol blue] and gently mixed for 15 min at 21°C. Beads were removed by centrifugation for 2 min at 10,000 g. The resulting supernatant was collected as the membrane-bound protein (M) fraction. In some experiments, protein concentration was determined by the bicinchoninic acid protein assay (Pierce), according to the manufacturer’s instructions.

SDS-PAGE and Western blots. The oocyte lysates were denatured in Laemmli sample buffer (161–0737; Bio-Rad) for 5 min at 100°C and electrophoresed on an SDS-7.5 or 10% polyacrylamide gel. To obtain a sufficient signal on the Western blot, we generally loaded the gel with 1–2 µl of the T fraction (represents total Kv1.1 protein in 0.25–0.5 oocytes) and the full volume of the M fraction (represents membrane Kv1.1 protein in 50 oocytes). After electrophoresis, separated proteins were transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) by electroblotting. Rainbow colored protein molecular weight markers (Amer sham Life Sciences) were used.

The PVDF membrane was rinsed in distilled water and blocked with 4% Blotto (4% nonfat dried milk in Tris-buffered saline, pH 7.6, with 0.1% Tween 20 (TBS-T)) for 1 h at room temperature or overnight at 4°C. The blots were incubated with primary antibodies in sealable pouches. We used 1 µg/ml anti-rat Kv1.1 monoclonal antibody (Upstate Biotech or gift from J. Trimmer), diluted in 4% Blotto, for 1 h at room temperature on a shaking platform. The anti-Kv1.1 antibody was raised against residues 458–476, part of the putative intracellular COOH terminus of rat brain Kv1.1. After primary antibody incubation, the blot was washed five times in TBS-T for 5 min each. The blot was then incubated with the secondary antibody for 1 h on a shaking platform in a Pyrex dish. We used a 1:80,000 dilution of goat anti-rat IgG conjugated with horseradish peroxidase (Sigma) and diluted in 4% Blotto. Finally, the blot was washed three times in TBS-T for 10 min each. Immunolabeled proteins were detected by the ECL Plus enhanced chemiluminescence detection system (A mer sham Life Sciences). X-ray films were scanned and analyzed with a model GS-700 scanning densitometer and Molecular Analyst software (Bio-Rad).

A crude synapticosomal membrane fraction was prepared from freshly dissected adult mouse brains as described by Trimmer (33), and an aliquot (6 µg protein/lane) was included as a positive control for Kv1.1 expression. Extracts from uninjected oocytes do not react with the Kv1.1 antibody (data not shown).

We probed the M and T fractions with an anti-actin primary antibody (Sigma) that specifically recognized an ~46-kDa band present in the mouse brain extract and in purified actin, run as a positive control for the antibody. By Western blotting, native oocyte actin was detected only in the T fractions, not in the M fractions prepared from either uninjected oocytes or Kv1.1-expressing oocytes (data not
Because of the selectively large inhibitory effect of PMA (for inactivating currents) or at the end of a 60- to 120-min inhibition were compared, either at the peak current indicated in the legend for Fig. 1, levels of current inhibition remained the same regardless of the Shaker subfamily K channel currents expressed in frog oocytes (Fig. 1A). PMA applied to oocytes expressing mouse Kv1.1 (n = 8), rat Kv1.1 (n = 7), Drosophila Shal and ShakerH4 (n = 4), and mouse Kv1.3 (n = 3) caused nearly complete inhibition in some cases, with an average of 73% current inhibition. The time courses and magnitudes of the inhibition were the same regardless of the Shaker channel species. Voltage-gated K channels from the Shab (Fig. 1B) and Shal (Fig. 1C) subfamilies were much less affected by PMA application than were K channels from the Shaker subfamily (Fig. 1D). At the membrane potential indicated in the legend for Fig. 1, levels of current inhibition were compared, either at the peak current (for inactivating currents) or at the end of a 60- to 80-ms pulse if currents lacked N-type inactivation. Because of the selectively large inhibitory effect of PMA on Shaker subfamily K currents, we further studied the inhibition of mKv1.1 K channels.

Inhibition of K current by PMA always began after a short delay (Fig. 2A), probably limited by the permeation of PMA through the plasma membrane and the accumulation to an unknown concentration within the cell. Drug application was always marked when the drug solution was switched on. For comparison of the rates of PMA effect, in this perfusion system, K channel block by 30 mM external tetraethylammonium (TEA) is maximal within 10 s [time constant for block (τon) = 3.1 ± 1.1 s; n = 3]. The time course of current inhibition by PMA followed a characteristic pattern with a half time to maximal effect of 8.2 ± 0.6 min (n = 8 representative cells) and a maximal effect after ~15 min of external washing of the oocyte. Short applications of PMA were sufficient to produce this long-lasting inhibition of K current (Fig. 2A; see also Fig. 6A, curve labeled + C3, 16°C), which did not recover in up to 2 h, the longest time period recorded. Over the course of hour-long recordings, K current inhibition was never seen in control oocytes not treated with PMA. The PMA application (Fig. 2A) suggests that K channel activity was steadily and slowly decreasing, and the pattern was not indicative of sudden oocyte death or perfusion artifacts. K current inhibition was also observed with shorter applications of higher concentrations of PMA or longer applications of lower concentrations. For comparison and analyses, we standardized the PMA applications at 500 nM for 1 min and measured the steady-state current inhibition 15 min after the PMA application, with test pulses to 0 or +20 mV. We were careful to perform all experiments on defolliculated oocytes because, in early experiments, we noticed that 1-min applications of 500 nM PMA were without effect on mKv1.1 channels expressed in oocytes with intact follicle cells.

Pharmacological specificity. Phorbol esters such as PMA are membrane-permeant drugs that bind with high affinity to the diacylglycerol site of PKC. To test whether the inhibition of mKv1.1 K channel currents by PMA was due to the activation of native oocyte PKC, we used inactive phorbols and inhibitors of PKC activation. Two structural analogs of PMA that do not activate PKC in in vitro phosphorylation assays (for review see Ref. 22), 4α-phorbol and PMM, were ineffective inhibitors of K channel current (Fig. 2, B, C, and F). The analog 4α-phorbol always caused a small and reversible inhibition of current (Fig. 2B), unlike the persistent inhibition produced by PMA (Fig. 2A). PMM caused a small but insignificant (unpaired t-test; P < 0.05) inhibition of mKv1.1 K current 15 min after a 1-min application of 500 nM drug (Fig. 2, C and F).

Prior and concurrent bath application of the specific PKC inhibitor bisindolylmaleimide (31) prevented the large and persistent inhibition of K current by PMA (Fig. 2F). In some oocytes treated with the inhibitor, we also tested PMA applications longer than 1 min (Fig. 2D) and still did not observe a significant inhibition, as seen for PMA application to oocytes not treated with PKC inhibitor (compare with Fig. 2A). Staurosporine, a nonspecific kinase inhibitor, is also a potent inhibitor of PKC (30) and was injected into channel-expressing oocytes 1–2 h before the experiment to achieve an estimated concentration of 0.9 µM in the oocyte (see MATERIALS AND METHODS). Injection of staurosporine also blocked the K current inhibition by PMA (Fig. 2E). Taken together (Fig. 2F), these pharmacological data support the idea that the inhibition of K current by PMA application is due to the activation of PKC.

Receptor-dependent coupling of PKC to K currents. The inhibition of Shaker subfamily K channel currents by PMA persisted with up to 2 h of washing. This might be explained by either the persistent activation of PKC...
by PMA that accumulates and cannot be washed from the oocyte membrane or the presence of a stable phosphorylation event. To distinguish between these ideas, we studied K channel modulation by receptor-stimulated PKC activation. We coinjected oocytes with RNA encoding the mKv1.1 K channel and a P2Y purinergic receptor, previously shown to activate phospholipase C in stably transfected astrocytoma cells (11). In coinjected oocytes (Fig. 3A), the P2Y agonist ATP (100 µM) mimicked the effect of PMA inhibition of mKv1.1 K current (n = 6). ATP had no persistent effect on mKv1.1 K channels in oocytes injected only with channel RNA (Fig. 3B; n = 4 cells).

An unexpected finding was that the inhibition of K current by activation of P2Y receptors required ~10 min to reach maximal effect, was nearly complete, and persisted after ATP was washed off, similar to the time course and long-lasting effects of the PMA-induced inhibition. This was true even for short applications of ATP that were followed by up to 30 min of washing. Unlike PMA, which could accumulate in the oocyte, water-soluble compounds such as ATP should wash completely from the perfusion chamber. G protein-coupled phospholipase C activation would be expected to be a transient event, dependent on agonist-receptor binding. The K channel block by TEA, in comparison, is

Fig. 1. PMA inhibition (>PMA) of different K channels. Acute application of phorbol 12-myristate 13-acetate (PMA) largely inhibits K channel currents from Shaker subfamily (A). K channel currents from mouse, rat, and fly clones are shown. ShΔ is deletion mutant of ShakerH4 (ShH4) with N-type inactivation removed. For comparison, the effect of PMA on K channels from Shab (B) and Shal (C) subfamilies are shown. D: comparison of percent inhibition of peak K channel current for the three subfamilies. Data are means ± SE for n cells. PMA inhibition was measured 15 min after a 1-min bath perfusion with 500 nM PMA. Oocytes were clamped at −80 mV and stepped to 0 or +20 mV.
fully reversible within 30 s in the same recording system [time constant for recovery from block (τoff) = 8.0 ± 1.2 s; n = 3]. So the ongoing K current inhibition in the absence of ATP might be explained by the initiation of a PKC-dependent event(s) that cannot be reversed or halted under these experimental conditions. The stable event could be phosphorylation in the absence of an appropriate phosphatase required to dephosphorylate the protein substrate. These data suggest that the delayed time to reach steady-state inhibition and the persistence of the inhibition are not simply explained by a prolonged accumulation of hydrophobic PMA in the oocyte membrane.

Current gating after inhibition by PKC. We plotted conductance-voltage (G-V) relationships and scaled current traces during the inhibition of K current to study the impact of PKC activation on current gating. We observed overlap of the control and inhibited-K-current G-V profiles. The large inhibition of the K current by PMA (Fig. 4A) or ATP application to cells coexpressing K channels and P2Y receptors (Fig. 4B) was not accompanied by a significant change in the voltage range of activation or the slope of the activation curve. Furthermore, mKv1.1 K channel currents were inhibited by PKC activation without a significant effect on current kinetics (Fig. 4C). Scaling the inhibited current to match the steady-state control current showed that inhibition by PMA did not alter macroscopic activation kinetics (Fig. 4D). This demonstrates that the large inhibition measured cannot be simply explained by an effect on K channel activation gating. This is in contrast to other reports that PKC phosphorylation may change the voltage dependence of ion channel activation gating, possibly through direct phosphorylation of the channel protein (1, 24). With activating pulses up to 10 s long, PMA inhibition also did not alter C-type
inactivation rates (data not shown). In Shaker H4 channels with intact N-type inactivation (Fig. 1A) and in fast-inactivating channels of the Shal subfamily (Fig. 1C; rKv4.2), PMA inhibition also did not change the rate of inactivation (see also Ref. 21).

PKC activation does not inhibit all K channel subfamilies. PKC activation does not inhibit to the same degree all recombinant voltage-gated K channels expressed in oocytes. As reported previously for a human Shaw subfamily K channel (8), we confirmed that PMA application largely removed the N-type inactivation of rKv3.4 (in 5 of 5 cells; data not shown). In coinjected oocytes, we found that functional coupling to purinergic receptor activation largely removed the N-type inactivation of rKv3.4 (in 3 of 3 cells; data not shown). Because this PKC-mediated pathway has been well studied (2, 8), it provided an interesting comparison for the time course of the PKC-mediated event we studied. We observed that the inhibition of inactivation of rKv3.4 by either PMA or purinergic receptor activation required 5–10 min to reach maximal effect and was a relatively stable event, similar to the PKC-dependent inhibition of Shaker subfamily K channels. Thus the time courses and levels of stability of the PKC phosphorylation events for the different groups of oocytes studied may be similar even though PKC activation may have different consequences for the function of different K channel proteins. In cells expressing multiple types of K channels, PKC activation could be an important and complex process for regulating cellular excitability and signaling.

Site-directed mutagenesis of PKC phosphorylation sites. We identified only three potential PKC phosphorylation sites on mKv1.1 channels, based on the consensus sequence identified by Woodgett et al. (37): Ser/Thr-X-Lys/Arg, where X is generally an uncharged residue. Residues 318 and 322 in mKv1.1 channels are present in the S4-S5 linker, and residue 442 is in the S6 region, all thought to be intracellular in current working models of the channel. The PKC phosphorylation sequence at Thr-318 is conserved in most voltage-gated K channels (except Kv1.5 and Kv1.6), whereas the phosphorylation sequence at Ser-322 is conserved throughout the Shaker subfamily and in Drosophila Shab and Shaw. The phosphorylation sequence at Ser-442 in mKv1.1 is shared only by Kv1.1 channels of the Shaker subfamily.

Using mutagenesis experiments, we tested the hypothesis that PKC inhibition of mKv1.1 K channel current is due to phosphorylation of one of these three consensus PKC sites, present in four-fold symmetry in the homotetrameric K channel. We replaced, one at a time, the native serine or threonine residue at position 318, 322, or 442 in mKv1.1 with alanine or valine. These conservative substitutions remove the PKC phosphorylation sequence. The mutant channels were sequenced, expressed, and tested for inhibition by PMA. Channel mRNAs for T318A and S442A mutants gave rise to K currents with no functional differences in macroscopic gating or current stability recorded. In addition, both mutants demonstrated K current inhibition after PMA application, with effects not significantly different (unpaired t-test) from those found in control experiments. K currents from the T318A mutant were inhibited by 83.3 ± 6.8% (n = 6), and currents from the S442A mutant were inhibited by 69.5 ± 6.3% (n = 5). In addition, channels expressing both the T318A and S442A mutations were also inhibited by PKC activation (76.8 ± 10%; n = 5). For comparison, wild-type mKv1.1 currents were inhibited by 73.4 ± 5.2% (n = 8). The only difference in these mutant channels was a current amplitude smaller than that for wild-type channels, after injections of the same quantity of RNA (generally 2- to 3-fold-smaller currents). The mutant S322A did not express functional K channel currents, even though all three of the mutant
RNAs directed membrane protein expression that could be identified on a Western blot with a Kv1.1-specific primary antibody (not shown). We conclude that at least two of the three consensus PKC phosphorylation sites are not required for the PKC-mediated inhibition of mKv1.1 K current. We cannot rule out the possibility that additional, unidentified phosphorylation sites exist in the native, folded protein.

Assay for channel internalization. As shown in Fig. 4, the inhibition of mKv1.1 K channel current was not accompanied by changes in the channels’ G-V relationship or kinetics of activation, suggesting that the channels that contribute the remaining current are not functionally different from the channels that account for the inhibited current. Instead, the inhibition of K current may reflect a reduction in the number of functional K channels by, for example, downregulating the K channels from the membrane surface. Indeed, several pumps, transporters and ion channels (7, 9, 34), and G protein-coupled receptors (12, 35) are thought to be up- or downregulated by insertion or internalization from the plasma membrane, and these processes may also require a phosphorylation event.

The possible movement of biotin-labeled Kv1.1 K channels from the membrane surface to a nonmembrane (cytosolic) compartment was determined by avidin recovery of the biotinylated surface proteins and subsequent immunoblotting for Kv1.1 protein. Groups of oocytes were placed in single wells of a 24-well plate and exposed for 2 min to one of the following, dissolved in extracellular recording solution: 0.01% DMSO, 500 nM 4α-phorbol, or 500 nM PMA. The pharmacological treatments paralleled the physiological experiments, although we used a 2-min drug application because the oocytes were in a static bath rather than a perfusion stream. Over the next 30 min, oocytes were washed a minimum of 7 times by exchanging the drug solution for normal recording solution. Thirty minutes after beginning the washing, 4–7 oocytes from each drug treatment and untreated controls were randomly selected and tested for current magnitude. The remaining intact oocytes were incubated with NHS-SS-biotin, a membrane-impermeant reagent, to label Kv1.1 channels and other proteins also present at the cell surface. After being labeled, 50 intact oocytes were washed, and total protein extracts or membrane extracts were prepared (see MATERIALS AND METHODS). Samples were electrophoresed, blotted to nitrocellulose, and probed with an antibody specific for Kv1.1 protein. The antibody recognizes a sequence present in the COOH-terminal region that is thought to be intracellular.

No detectable differences were found in comparing the densities of Kv1.1 protein in membrane fractions from oocytes treated with DMSO alone, 4α-phorbol, or PMA (Fig. 5A), even though oocytes from the very same treatment wells showed a >90% inhibition of Kv1.1 K current by PMA (Fig. 5B). To confirm that the membrane extracts can be used to detect different levels of
METHODS. T lanes represent 0.5 oocytes, and M lanes represent 50 biotin. T and M extracts were prepared as described in MATERIALS AND METHODS. A: each lane represents extract from oocytes from same frog injected with same volume of same mRNA preparation and allowed to express channels for 2 days. Oocytes were treated for 2 min with either vehicle (0.01% DMSO), 500 nM 4α-phorbol (4α), or 500 nM PMA and then washed for 30 min before being labeled with NHS-SS-biotin. T and M extracts were prepared as described in MATERIALS AND METHODS. T lanes represent 0.5 oocytes, and M lanes represent 50 oocytes. Positive control is 6 µg of crude mouse brain (br) extract and shows a single band migrating at ~83 kDa. Extracts from un.injected oocytes show no signal (data not shown on this gel). The Kv1.1 protein in the T fraction runs ~58–63 kDa (at lower intensity this is resolved into two bands), and Kv1.1 protein in M fraction shows 2 bands at ~59 and ~64 kDa; these are approximations because Rainbow molecular mass markers (MW) are not linearly related to migration distance (see manufacturer’s literature). B: densitometric data showing fraction of total signal that is represented by membrane-bound channel protein (M/T) are compared with amplitudes of K channel currents in drug-treated oocytes relative to control (un-injected oocytes), 4α, or PMA treatments, the C3 exoenzyme was heated to 110°C for 2 h, required for K current inhibition. In control experiments, the C3 exoenzyme was heated to >110°C for 2 h, allowed to cool, and then injected into oocytes. This heat-inactivated preparation did not inhibit PKC-mediated inhibition of K channel current (Fig. 6, A and B). Alone, none of these treatments altered the K channel current kinetics, gating, or amplitude, compared with injection of heat-inactivated C3 toxin or no injections.

DISCUSSION

A membrane-permeant activator of PKC inhibits cloned Shaker subfamily K channel currents expressed in Xenopus oocytes. The inhibition of K current is specific for a phorbol ester known to activate PKC, and the effects of PMA application are prevented by two inhibitors of PKC. Agonist stimulation of coexpressed purinergic receptors also inhibits the K channel currents, mimicking the effect of PKC activation. Together, these data strongly suggest that direct activation of PKC through membrane-permeant activators, or indirect activation through cell-surface receptors coupled to phospholipase C, inhibits K channel currents of the Shaker subfamily.

Cell surface receptors that couple to PLC may also inhibit K channel currents in native cells. PKC is a ubiquitous enzyme, present in high concentrations in brain and other tissues (16), and many neurochemicals activate phosphatidylinositol 4,5-bisphosphate hydrolysis, including ATP, histamine, thrombin, and bradykinin. The regulation of neuronal and glial K channels by transmitter- or hormone-activated PKC may be an important physiological mechanism for the modulation of membrane excitability and K homeostasis. Likewise, there may be important roles for PKC regulation of

channel protein that correlate with different magnitudes of K channel current, we compared the Kv1.1 protein signals with the K current amplitudes at 0.5–3 days postinjection. Importantly, as the current amplitude increased with time of expression, so did the density of the protein signal on the Western blot (data not shown). However, in our experiments, the Kv1.1 current decreases by 90% on activation of PKC, without a significant change in the membrane protein level. In addition, large inhibition of Kv1.1 current by PMA was not accompanied by a change in the capacitative transient induced in the oocyte membrane by the voltage step (data not shown).

Botulinum toxin sensitivity. The specific components of the regulatory pathway of the PKC-mediated inhibition of Kv1.1 K channels are not known. Mechanisms ascribed to other channel regulatory pathways, such as phosphorylation of the channel protein and either modulation of the voltage sensor or translocation from the membrane to the cytosol, seem unlikely to explain this large inhibition of K current by PKC activation. In a search for alternative mechanisms, we discovered that the C3 exoenzyme of botulinum toxin blocked the inhibition of Kv1.1 by PKC activation. The known targets of C3 toxin are low-molecular mass (20–25 kDa) G proteins, specifically RhoA and RhoC, which are selectively modified by ADP-ribosyltransferase activity, preventing their interaction with effector proteins (6, 27, 31).

Two days after injection with Kv1.1 channel RNA, we injected oocytes with C3 exoenzyme and then incubated the oocytes at different temperatures for 1–2 h. Figure 6 shows the effect of PMA application on C3-injected oocytes. C3 inhibited PKC-mediated K channel inhibition in a temperature-sensitive fashion. The temperature dependence is, perhaps, not surprising if C3 exoenzyme mediates ADP-ribosylation of a substrate required for K current inhibition. In control experiments, the C3 exoenzyme was heated to >110°C for 2 h, allowed to cool, and then injected into oocytes. This heat-inactivated preparation did not inhibit PKC-mediated inhibition of K channel current (Fig. 6, A and B). Alone, none of these treatments altered the K channel current kinetics, gating, or amplitude, compared with injection of heat-inactivated C3 toxin or no injections.

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Cell surface receptors that couple to PLC may also inhibit K channel currents in native cells. PKC is a ubiquitous enzyme, present in high concentrations in brain and other tissues (16), and many neurochemicals activate phosphatidylinositol 4,5-bisphosphate hydrolysis, including ATP, histamine, thrombin, and bradykinin. The regulation of neuronal and glial K channels by transmitter- or hormone-activated PKC may be an important physiological mechanism for the modulation of membrane excitability and K homeostasis. Likewise, there may be important roles for PKC regulation of

K CHANNEL INHIBITION BY PKC
nonneuronal K currents by endogenous hormones and messenger molecules (see, e.g., Ref. 29).

The molecular mechanisms of PKC regulation of K channels are not completely understood. In some cases, phosphorylation of channel proteins themselves may be the structural basis for PKC-mediated events. Consensus protein phosphorylation sites are prevalent in the deduced amino acid sequences of cloned ion channel proteins. Commonly located in regions thought to be intracellular or near the cytoplasmic face are sequences targeted by PKC, cAMP-dependent protein kinase, and other posttranslational modification sites. Busch et al. (3) showed that a specific Ser-to-Ala mutation in a cloned voltage-dependent K channel subunit (I_{SK}) from rat kidney prevented K current inhibition by PKC activators. Also, Cai and Douglass (4) reported biochemical evidence that PKC phosphorylates a voltage-gated K channel (hKv1.3) in T lymphocytes. And Covarrubias et al. (8) suggested that inactivation of a Shaw subfamily K channel (Kv3.4) is regulated by direct phosphorylation of the channel protein. In the present study, however, site-directed substitution of the consensus PKC phosphorylation sites on mKv1.1 channels did not prevent the inhibition of K current by PMA. A similar result was reported for the inhibition by PKC activation of rKv4.2 K currents (21). Although we cannot rule out the possibility of phosphorylation at novel or dormant sites in the folded channel protein, at least two consensus PKC sites in Shaker subfamily K channels are not likely to be directly phosphorylated by PKC, on the basis of mutagenesis data. This suggests that the substrate for phosphorylation may be a protein that associates with or indirectly regulates the Kv1.1 channel protein.

We found that the C3 exoenzyme of botulinum toxin blocks the inhibitory effect of PKC activation on Shaker subfamily K currents. The C3 exoenzyme of botulinum toxin ADP-ribosylates small-molecular weight G proteins of the ras superfamily. A major target of C3 exoenzyme is thought to be RhoC (27), which has a known role in the regulation of actin microfilament assembly. For example, C3 exoenzyme causes morphological changes in mammalian cells due to disassembly of actin microfilaments (6). Thus one hypothesis is that small GTP-binding proteins, downstream from PKC activation, mediate the inhibition of Shaker subfamily K channel currents through interactions with actin microfilaments.

In most cases, protein kinases are thought to be "modulators" or modifiers of ion channel gating. Perozo and Bezanilla (24) found that PKC activation shifts the voltage dependence of activation gating of a squid giant-axon delayed rectifier K channel. They suggested that phosphorylation of the channel protein modulates the voltage-sensing capabilities of the channel through an electrostatic interaction of added phosphate groups with the voltage sensor. Also for squid axon, Augustine and Bezanilla (1) found that MgATP modulates K conductance through an endogenous, unidentified protein kinase. Similarly, PKC activation caused a rightward shift in the voltage dependence of activation gating of a rat kidney K channel, and mutagenesis experiments were used to identify the serine substrate for the phosphorylation event (3). In addition, phosphorylation of Kv2.1 was reported to cause a depolarizing shift in activation gating (20). In all of these studies, the inhibitory effects of PKC may be explained by a phosphorylation-mediated positive shift in the voltage dependence of K channel gating. The addition of phosphate groups to the K channel protein is proposed to increase the negative surface charge on the cytoplasmic site...
side of the membrane, thus changing the local electric field, rendering the channels less sensitive to activating stimuli.

However, gating changes are not the only mechanism by which PKC activation alters channel function. In our experiments and those of others on Kv4.2 and Kv4.3 K channels (21), there was no evidence for a PKC-induced change in K channel gating. Moran et al. (18), however, noted a small change in the voltage dependence of activation and inactivation gating of Shaker H4 channels. Together with our results that the consensus PKC phosphorylation sites are not required for the PKC-induced inhibition of mKv1.1 K channels, our data suggest that PKC inhibits K channel function. This could occur by reducing the number of functional channels so that after PKC inhibition of current, the remaining channels are normal, or by reducing the single-channel conductance of each channel.

One mechanism for a change in the number of functional channels is a selective endocytotic mechanism, as has been reported for hormone-activated endocytosis of K channels in starfish oocyte plasma membranes (17). Such a process has also been implicated in the receptor and second messenger regulation of the plasma membrane stability of adrenergic receptors (12, 35). Na+ channels (9), and Na+–K+–ATPase (34). Interestingly, the time course of agonist-induced β-adrenergic receptor or activity-induced Na+ channel internalization parallels the time course of the inhibition of K channel current in our studies. However, we assayed for changes in the density of Kv1.1 channel protein present at the cell surface before and after PKC stimulation in oocytes. Although the K channel current is 90% inhibited by PKC activation, there is no concomitant reduction in the amount of Kv1.1 protein detected in membrane extracts, indicating that PKC stimulation does not cause internalization of the K channel protein. This is consistent with the lack of observable change in the oocyte membrane capacitance after PKC activation. We conclude therefore, that PKC activation causes K channels present at the cell surface to become nonfunctional or significantly reduces the average single-channel conductance.

Interestingly, the regulation of K channel function by PKC is clearly different for the different molecular species of K channel. The large, nonmodulatory, PKC-dependent or significantly reduces the average single-channel conductance. Functional channels is a selective endocytotic mechanism. PKC-induced change in K channel gating. Moran et al. (18), however, noted a small change in the voltage dependence of activation and inactivation gating of Shaker H4 channels. Together with our results that the consensus PKC phosphorylation sites are not required for the PKC-induced inhibition of mKv1.1 K channels, our data suggest that PKC inhibits K channel function. This could occur by reducing the number of functional channels so that after PKC inhibition of current, the remaining channels are normal, or by reducing the single-channel conductance of each channel.

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