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Modulation of the Human Kv1.5 Channel by Protein Kinase C Activation: Role of the Kvβ1.2 Subunit

CHRISTINE P. WILLIAMS, NINGNING HU, WANGZHEN SHEN, AMY B. MASHBURN, and KATHERINE T. MURRAY

Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee

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ABSTRACT

Kv1.5 is the principal molecular component of I_{Kur}, an atrial-specific K^+ current in human myocytes that is suppressed by activation of protein kinase C (PKC). We examined the effect of phorbol 12-myristate 13-acetate (PMA), a direct activator of PKC, on Kv1.5 current. Although PMA had minimal effect when Kv1.5 was expressed alone, K^+ currents derived from coexpression of Kvβ1.2 (but not another closely related β subunit, Kvβ1.3) with Kv1.5 were markedly reduced by PMA, associated with a small depolarizing shift in the voltage dependence of channel activation. Additional experiments with an inactive stereoisomer, 4α-PMA, and the PKC inhibitor chelerythrine indicated that the effects of PMA were mediated by PKC activation. Assembly of Kv1.5 in vivo with both β subunits was demonstrated, and all three K^+ channel proteins were substrates for phosphorylation by PKC. These results demonstrate that coexpression of Kvβ1.2 enhances the response of Kv1.5 to PKC activation and that direct phosphorylation of K^+ channel subunits is a potential molecular basis for the effect. Furthermore, they suggest that Kvβ1.2 may be a component of the I_{Kur} complex in human atrium.

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia in the Western world (Prystowsky et al., 1996; Pelosi and Morady, 2000; Chugh et al., 2001; Go et al., 2001) and remains a major cause of stroke and death in the elderly in this country. Most antiarrhythmic drugs used to treat AF block K^+ channels as their primary mechanism of action. However, the efficacy of these drugs has been disappointing (Prystowsky et al., 1996; Eckman et al., 1998). In addition, all currently available K^+ channel blockers can cause marked prolongation of ventricular repolarization and the QT interval, predisposing patients to the development of the polymorphic ventricular tachycardia Torsades de Pointes, syncope, and sudden cardiac death (Roden, 1996; Nattel, 1999). In both atrium and ventricle, late or phase 3 repolarization of the action potential is mediated by noninactivating K^+ currents or delayed rectifiers (I_K). Both rapidly (I_{Kr}) and slowly (I_{Ks}) activating components with distinct pharmacologic profiles have been described (Nattel, 1999). Substantial evidence indicates that both cardiac and noncardiac drugs that cause Torsades de Pointes block I_{Kr} as a common mechanism of action (Nattel, 1999). Clearly, pharmacologic therapy directed specifically at atrial currents (rather than I_{Kr}, which resides in both atrium and ventricle) would reduce toxicity and likely improve efficacy.

A delayed rectifier with ultra-rapid activation (I_{Kur}) has been identified in atrial, but not ventricular, human cardiac myocytes (Fedida et al., 1998; Nattel et al., 1999). This K^+ current is distinct, because it is sensitive to block by low concentrations of 4-aminopyridine but resistant to tetraethylammonium, dendrotoxin, and charybdotoxin (Nattel et al., 1999). Given its atrial specificity, there is considerable interest in I_{Kur} as a pharmacologic target to treat AF. Kv1.5 encodes a rapidly activating delayed rectifier that is also sensitive to block by low micromolar concentrations of 4-aminopyridine but insensitive to tetraethylammonium, dendrotoxin, and charybdotoxin. These characteristics are very similar to those of human I_{Kur}, suggesting that Kv1.5 forms the principal molecular basis for the current (Paulmichl et al., 1991; Snyders et al., 1993; Coetzee et al., 1999). This concept is further supported by the fact that exposure of human atrial cells to Kv1.5 antisense oligonucleotides causes a major reduction in I_{Kur} (Feng et al., 1997).

Kvα subunits like Kv1.5 can coassemble as either homo- or heterotetramers to form a functional channel (Coetzee et al., 1999). Further diversity of K^+ currents arises from coassembly of auxiliary or accessory proteins, such as β subunits, with Kvα subunits (Rhodes et al., 1995; Shamotienko et al., 1997; Coleman et al., 1999). Two β subunits cloned from human heart, Kvβ1.2 and Kvβ1.3, represent splice variants

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ABBREVIATIONS: AF, atrial fibrillation; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PKA, cAMP-dependent protein kinase (protein kinase A); HA, hemagglutinin.

from the same gene, with identical carboxyl termini that mediate interaction with the α subunit and divergent amino termini. Multiple studies suggest that both Kvβ1.2 and Kvβ1.3 can coassemble with Kv1.5 (Sewing et al., 1996; Wang et al., 1996), as coexpression results in K^+ currents with partial, fast inactivation, a hyperpolarizing shift in the voltage dependence of activation, and slowing of deactivation compared with Kv1.5 alone (England et al., 1995a,b; Majumder et al., 1995). At present, it is not known whether β subunits coassemble with Kv1.5 in vivo to form I_{Kur}.

A notable feature of I_{Kur} is its modulation by adrenergic stimulation (Li et al., 1996). Activation of protein kinase A (PKA) causes an increase in K^+ current amplitude for I_{Kur} (Li et al., 1996). We have previously shown that coexpression of Kvβ1.3, but not Kvβ1.2, with Kv1.5 results in a reproducible increase in K^+ current in response to PKA stimulation (Kwak et al., 1999), suggesting that β subunit coassembly with Kv1.5 is required to recapitulate the complete I_{Kur} phenotype. Stimulation of α-adrenergic receptors causes suppression of I_{Kur} in human atrial myocytes, an effect mediated by PKC (Li et al., 1996). To test the hypothesis that the response of Kv1.5 to PKC activation also requires β subunit coassembly, we have examined the effects of PKC activation on K^+ currents derived from Kv1.5 in the absence and presence of β subunit coexpression. Our results indicate that coassembly of Kvβ1.2 in the Kv1.5 complex enhances the response of the K^+ current to PKC activation. Taken together with our previous findings, these results suggest that one or more β subunits may coassemble with Kv1.5 to recapitulate the response of I_{Kur} to adrenergic stimulation.

Experimental Procedures

Materials and Antibodies. Reagent grade chemicals and PMA were obtained from Sigma-Aldrich (St. Louis, MO), and 4α-PMA and chelerythrine were obtained from LC Laboratories (Woburn, MA). Enzymes and buffers were from Roche Diagnostics (Indianapolis, IN) and Promega (Madison, WI). The antibody directed against the influenza hemagglutinin (HA) epitope (anti-HA) was purchased from CRP Inc. (Princeton, NJ), the anti-FLAG M2 and anti-FLAG horseradish peroxidase-conjugated antibodies were obtained from Sigma-Aldrich, and a polyclonal antibody directed against the amino terminus of Kv1.5 (anti-Kv1.5) was kindly provided by Dr. Mike Tamkun (Colorado State University, Ft. Collins, CO). The source of other reagents is specified below.

K^+ Channel Expression. DNA constructs of Kv1.5, Kvβ1.2, and Kvβ1.3 (each in a modified pSP64T vector) (England et al., 1995a,b) were linearized with *Eco*RI and cRNA transcribed using the SP6 RNA polymerase (SP6 Cap-Scribe; Roche Diagnostics). Defolliculated *Xenopus laevis* oocytes were prepared as described previously and injected with approximately 40 nl of RNA (Kwak et al., 1999). Kv1.5 cRNA was diluted using RNase-free water so that currents for experimentation did not exceed 8 μA. This was combined with an excess of undiluted β cRNA in ratios that achieved maximal effect, as assessed by K^+ current characteristics during electrophysiologic recordings (England et al., 1995a,b; Uebele et al., 1996). Use of animals was performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

Kvβ1.2-HA and Kvβ1.3-HA were constructed by fusing, in frame, three copies of the HA epitope to the 3′ end of the cDNA. The human Kv1.5 cDNA was inserted into p3XFLAG-CMV 7.1 expression vector (Sigma-Aldrich), resulting in amino-terminal insertion of the FLAG epitope. HEK 293 (human embryonic kidney) cells that were approximately 70 to 80% confluent were transiently transfected with Kv1.5-FLAG + Kvβ1.2-HA or Kvβ1.3-HA using LipofectAMINE Plus (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were harvested 48 h later for immunoprecipitation experiments.

Electrophysiologic Recordings and Data Analysis. Electrophysiologic recordings were performed 24 to 48 h following injection of oocytes, using the two-microelectrode voltage-clamp technique as described previously (Kwak et al., 1999). The holding potential was −80 mV, and the cycle time for all pulse protocols was 10 s or slower to allow full recovery from inactivation between pulses. To calculate cell membrane electrical capacitance, the capacitive transient was recorded during a small voltage step (−80 to −70 mV) during which K^+ currents were not activated. Integration of the leak-corrected transient yielded the charge (Q) transferred during the voltage step (V) from which capacitance (C) was calculated: $C = Q/V$. All experiments were conducted at room temperature (22 ± 2°C).

Data analysis was performed using custom programs designed to read and analyze files generated by pClamp (Axon Instruments, Inc., Union City, CA). Activation curves were constructed from deactivating tail currents. A paired t test was used to compare voltage-dependent and kinetic properties of K^+ currents before and after drug exposure. Comparison of normalized K^+ current density after different interventions was performed using one-way analysis of variance with the Scheffe multiple comparison procedure. Results are presented as mean ± S.E.M.

Immunoprecipitation of K^+ Channel Subunits. Following transfection with K^+ channel subunits, HEK 293 cells were harvested and lysed in 20 mM Tris (pH 7.5), 20 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, and protease inhibitors (complete mini; Roche Diagnostics). Cellular debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C. Two milligrams of whole cell lysate were incubated with 4 μg of anti-HA or anti-FLAG antibody overnight at 4°C. Immunocomplexes were precipitated by the addition of 30 μl of packed protein A-Sepharose affinity resin. The resin was washed with lysis buffer, and proteins were eluted at 100°C in Laemmli sample buffer. Samples were size-fractionated on a denaturing polyacrylamide gel and transferred to nitrocellulose (Hybond-ECL; Amersham Biosciences, Piscataway, NJ) via semidry transfer. Membranes were blocked in phosphate-buffered saline containing 2% bovine serum albumin. To detect HA-tagged proteins, membranes were incubated with primary antibody (anti-HA, 1:8,000) in blocking solution. Horseradish peroxidase-conjugated secondary antibodies in phosphate-buffered saline allowed for subsequent detection of protein with the enhanced chemiluminescence system (Amersham Biosciences). To detect FLAG-tagged proteins, a horseradish peroxidase-conjugated anti-FLAG antibody was used (1:10,000).

Phosphorylation of K^+ Channel Subunits. cDNAs for Kvβ1.2-HA, Kvβ1.3-HA, and Kv1.5 were amplified using the polymerase chain reaction and subsequently ligated into pGEX2T-KG (Guan and Dixon, 1991) to create glutathione *S*-transferase fusion proteins. The recombinant plasmids were sequenced to confirm that the reading frames were not disrupted and that there were no errors arising from the amplification. The plasmids were transformed into JM109 bacteria for growth and induction of expression with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The bacteria were lysed by passing twice through a French press in the presence of 100 mM Tris, pH 7.5, and 1 mM EDTA, and cellular debris was pelleted by centrifugation at 58,000*g*. The fusion protein was purified with glutathione-agarose affinity resin and eluted with 10 mM glutathione. The glutathione *S*-transferase moiety was cleaved from the channel subunit by incubation with four units of thrombin protease at room temperature for 20 min (Guan and Dixon, 1991). Ten micrograms of purified K^+ channel subunit were incubated at 30°C for 30 min in kinase reaction buffer [50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 100 μM ATP, and 2 μCi of γ-[^{32}P]ATP] in the absence or presence of 4 ng of the PKC catalytic subunit (Calbiochem, San Diego, CA). Following electrophoretic separation and transfer to nitrocellulose, phosphorylated proteins were visualized by autoradiography.

Results

The Role of Kvβ1.2 in the Response to PKC Activation. To investigate the effects of PKC activation, K^+ currents derived from expression of Kv1.5 alone were studied initially. PKC was activated directly using the phorbol ester PMA. In Fig. 1A, K^+ currents elicited by depolarizing voltage steps are shown under control conditions (left) and following exposure to PMA (right). Bath superfusion of PMA had little effect on Kv1.5 current, as also highlighted by the summary data in Fig. 3 (−9 ± 5% at +50 mV after 30 min of PMA).

As discussed above, Kvβ1.2 or Kvβ1.3 can modify Kv1.5 currents. In addition, their amino acid sequences contain multiple consensus sites for phosphorylation by PKC (Kennelly and Krebs, 1991; Pinna and Ruzzene, 1996). To explore the role of α-β subunit interaction in the response to PKC, the effects of PMA were determined following coexpression of either Kvβ1.2 or Kvβ1.3 with Kv1.5. As previously reported, coexpression of either β subunit produced K^+ currents that demonstrated partial, fast inactivation and slowed deactivation compared with Kv1.5 alone (Fig. 1, B and C, left panels), with a negative shift in the voltage dependence of channel activation (e.g., midpoint or $V_{1/2}$ of the activation curve was −9 ± 2 mV for Kv1.5 alone and −19 ± 3 mV for Kv1.5 + Kvβ1.2). We have previously shown that coexpression of Kvβ1.3 is necessary for the PKA-mediated increase in Kv1.5 current (Kwak et al., 1999). However, PMA had no effect on K^+ currents derived from Kv1.5 + Kvβ1.3 (−1 ± 2% after 30 min; Figs. 1B and 3). On the other hand, the phorbol ester markedly reduced K^+ current when Kvβ1.2 was coexpressed with Kv1.5 (−45 ± 8% after 30 min; Figs. 1C and 3). Thus, coexpression of Kvβ1.2, but not Kvβ1.3, with Kv1.5 enhanced the response of the channel to PMA.

The time course of the PMA effect is illustrated in Fig. 2. Because phorbol esters can cause a concentration- and time-dependent reduction in cell membrane surface area due to internalization, capacitance was also measured as in indicator of cell membrane surface area, and data are shown as current density (current normalized for capacitance). For a representative group of cells, there was no significant change in capacitance over the time course of experiments (−6–7 ± 2–3% after 30 min of PMA; data not shown).

Additional experiments were performed to determine whether the effects of PMA resulted from PKC stimulation rather than a nonspecific effect of the phorbol ester. The inactive stereoisomer 4α-PMA had essentially no effect on K^+ current derived from Kv1.5 + Kvβ1.2, as demonstrated in Fig. 3 (+4 ± 3% after 30 min of PMA). If the effects of PMA are mediated by PKC activation, they should be reduced or eliminated by inhibition of the kinase. Cells expressing Kv1.5 + Kvβ1.2 were preincubated with the PKC inhibitor chelerythrine (20 μM) for 20 to 30 min (Herbert et al., 1990). Under these conditions, as shown in Fig. 3, chelerythrine significantly blunted the effect of PMA (−14 ± 9% after 30 min). Taken together, these findings indicate that the effects of PMA on K^+ currents derived from Kv1.5 + Kvβ1.2 are mediated by activation of PKC.

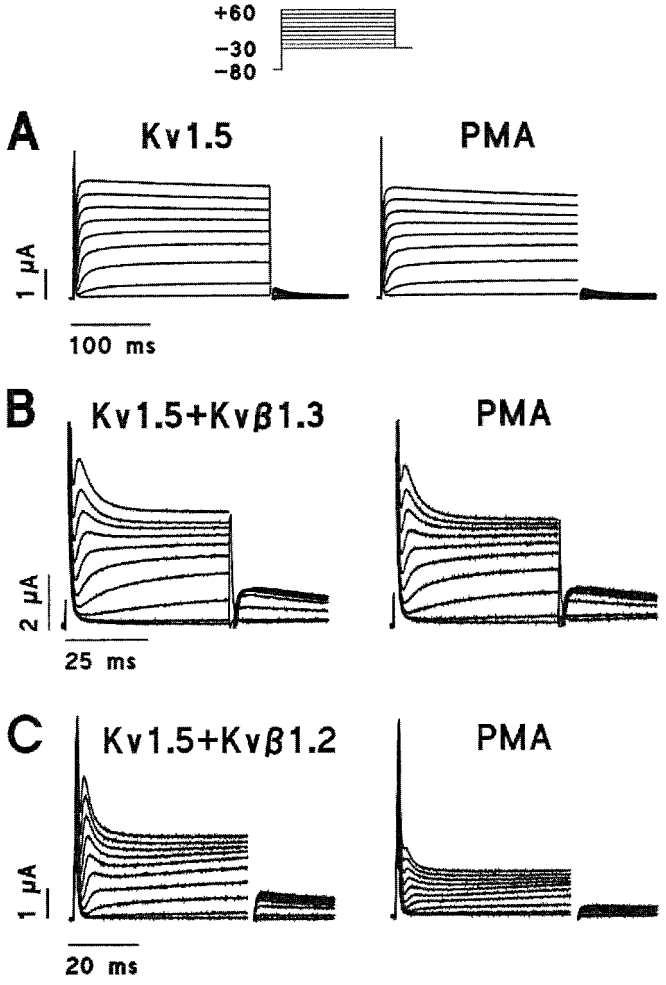

Fig. 1. Effect of PMA on Kv1.5 currents in the absence and presence of Kvβ subunit coexpression. Outward K^+ currents were elicited by step depolarizations in *X. laevis* oocytes injected with cRNA from Kv1.5 alone (A), Kv1.5 + Kvβ1.3 (B), or Kv1.5 + Kvβ1.2 (C). Currents on the left were recorded under control conditions (pulse protocol shown in inset; voltage is stepped from −80 mV to a maximal potential of +60 mV, with repolarization to −30 mV), whereas currents recorded on the right were obtained following bath superfusion of PMA (10 nM), representing an exposure time of approximately 30 min for each experiment.

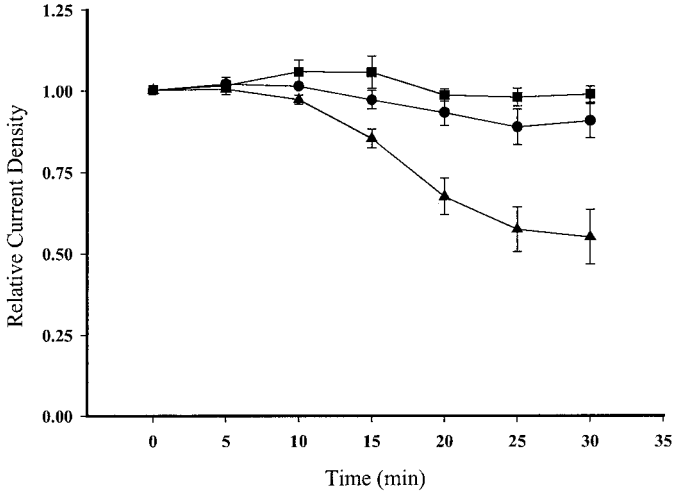

Fig. 2. Time course of PMA effect on K^+ current density. K^+ currents were recorded using repeated step depolarizations from −80 to +50 mV, and peak current at +50 mV was plotted as a function of experimental time. Data are expressed normalized to predrug values, and time 0 corresponds to the start of drug infusion into the bath. Averaged values are shown for Kv1.5 (●; n = 12), Kv1.5 + Kvβ1.3 (■; n = 5), and Kv1.5 + Kvβ1.2 (▲; n = 12).

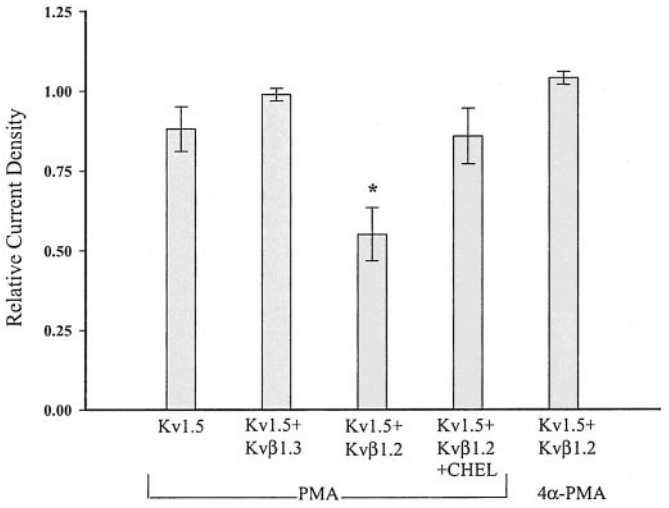

Fig. 3. Effect of interventions on K^+ current density. Bar graphs (±S.E.M.) display normalized K^+ current density after a 30-min exposure to PMA for Kv1.5, Kv1.5 + Kvβ1.3, and Kv1.5 + Kvβ1.2 (⋆ indicates a significant difference from Kv1.5, $p < 0.05$). Similar results are shown for Kv1.5 + Kvβ1.2 with preincubation in chelerythrine (20 μM) prior to exposure to PMA and following bath superfusion of 4α-PMA.

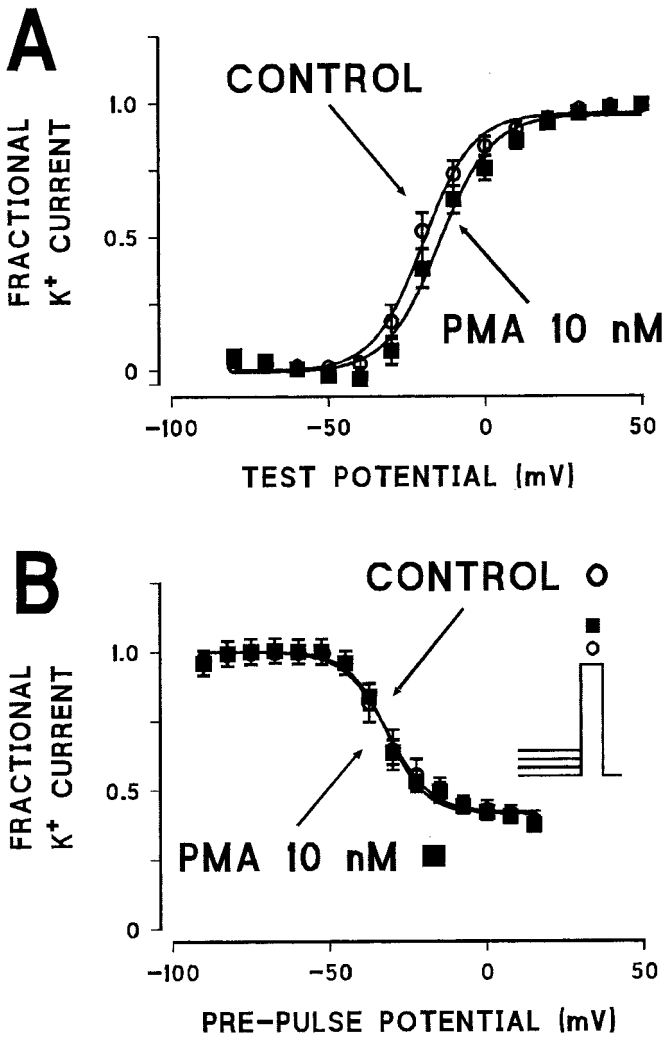

Fig. 4. Activation and inactivation of K^+ current derived from Kv1.5 + Kvβ1.2. Using the voltage-clamp protocol shown in Fig. 1, activation curves were constructed (deactivating tail currents were plotted as a function of prior test step potential, with the data normalized to average peak current amplitude at baseline) (A). Averaged values (mean ± S.E.M.) for the group of experiments are presented (current after PMA is scaled to control values). A standard two-pulse protocol (inset) was used to assess the voltage dependence of steady-state channel availability (inactivation curve; an initial 5-s prepulse varying from −90 to +50 mV was followed by a test pulse to a voltage-producing maximal K^+ current at +50 mV) (B). Averaged values for the normalized data are shown (current after PMA is scaled to control values). The line through the data represents the best nonlinear least-squares fit of a Boltzmann function.

Effect of PKC Activation on K^+ Current Gating. The effect of PMA on the biophysical properties of Kv1.5 + Kvβ1.2 currents was also examined. As illustrated in Fig. 4A, a small depolarizing shift in the activation curve occurred with bath application of PMA (−19 ± 3 mV at baseline and −14 ± 3 mV following PMA; $p < 0.001$). Although this effect was statistically significant, it was not sufficient to account for the reduction observed in K^+ current. There was no change in either the extent of macroscopic K^+ current inactivation (ratio of steady state to peak current was 61 ± 5% at baseline and 63 ± 5% following PMA) or the voltage dependence of this process following PMA ($V_{1/2}$ of the inactivation curve was −31 ± 3 mV before and −32 ± 3 mV after PMA; Fig. 4B). Of note, PMA did not alter the voltage dependence of channel activation in cells expressing Kv1.5 alone ($V_{1/2}$ of the activation curve was −9 ± 2 mV at baseline and −9 ± 2 mV following PMA).

Kvβ1.2 and Kvβ1.3 Coassemble with Kv1.5. Using the yeast two-hybrid assay, Kv1.5 has been shown to bind Kvβ1.2 and Kvβ1.3 in vitro (Wang et al., 1996), and coexpression studies have also suggested α-β coassembly. To confirm that these α and β subunits associate in vivo, reciprocal coimmunoprecipitation experiments were performed. Using a Kv1.5 construct that was tagged with the FLAG epitope and Kvβ subunits tagged with the HA epitope, immunoprecipitation was performed using anti-FLAG and anti-HA antibodies following α-β coexpression. Following electrophoretic separation and transfer to nitrocellulose, Western blot analyses were performed. When anti-FLAG is used as the precipitating antibody, a doublet at ~75 and 85 kDa is detected (Fig. 5A, lanes 5 and 6), and this signal comigrates with bands representing Kv1.5 in the absence of precipitating antibody (Fig. 5A, lanes 2 and 3). Furthermore, Kvβ1.2 and Kvβ1.3 are also detected under these conditions (Fig. 5B, lanes 5 and 6), indicating that the α and β subunits are associated within the cell. As well, immunoprecipitation with anti-HA results in detection of the β subunits (Fig. 5B, lanes 8 and 9) as well as Kv1.5 (Fig. 5A, lanes 8 and 9). No signals were detected in nontransfected cell lysate subjected to immunoprecipitation by either anti-HA or anti-FLAG (Fig. 5, A and B, lanes 1, 4, and 7). Taken together, these data indicate that Kv1.5 is associated with the Kvβ subunits in vivo.

Phosphorylation of K^+ Channel Subunits. The response to PKC activation suggests that direct phosphorylation of the α and/or β subunit may underlie the reduction in current. The amino acid sequences of Kv1.5 and Kvβ1.2 contain numerous consensus sequence sites for phosphorylation by PKC. Therefore, in vitro phosphorylation studies were carried out to determine whether the subunits are substrates for PKC. Ten micrograms of bacterially expressed, purified Kvβ1.2 protein were incubated with ATP in the presence or absence of the catalytic subunit of PKC. As shown in Fig. 6A (lanes 1 and 2), a ~46 kDa protein was

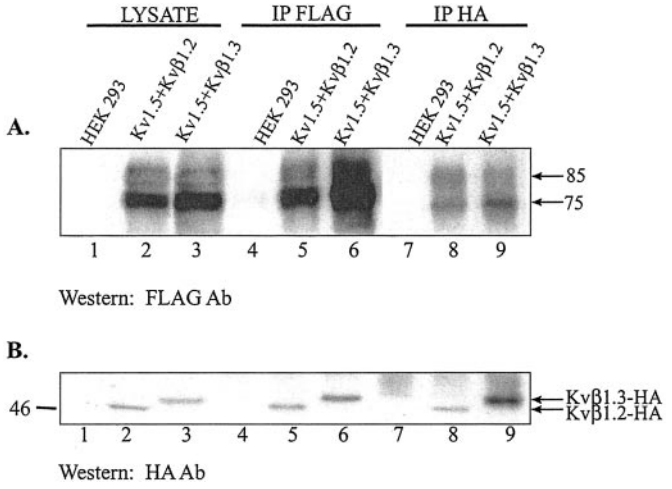

Fig. 5. Coassembly of Kv1.5 with Kvβ subunits in vivo. Kv1.5-FLAG (Kv1.5) + Kvβ1.2-HA (Kvβ1.2) or Kvβ1.3-HA (Kvβ1.3) were transiently transfected into HEK 293 cells. Whole-cell lysate from these and nontransfected cells were subjected to reciprocal coimmunoprecipitation using antibodies directed against the FLAG or HA epitopes (A). Western blot analysis using anti-FLAG to detect Kv1.5 epitope-tagged protein. Lysate represents extract in the absence of precipitating antibody, and IP FLAG and IP HA indicate precipitation by anti-FLAG or anti-HA, respectively (B). Western blot analysis using anti-HA to detect epitope-tagged Kvβ1.2-HA and Kvβ1.3-HA. The lanes in panel B correspond to those in panel A. Molecular weight standards are shown on the left-hand side of the panel.

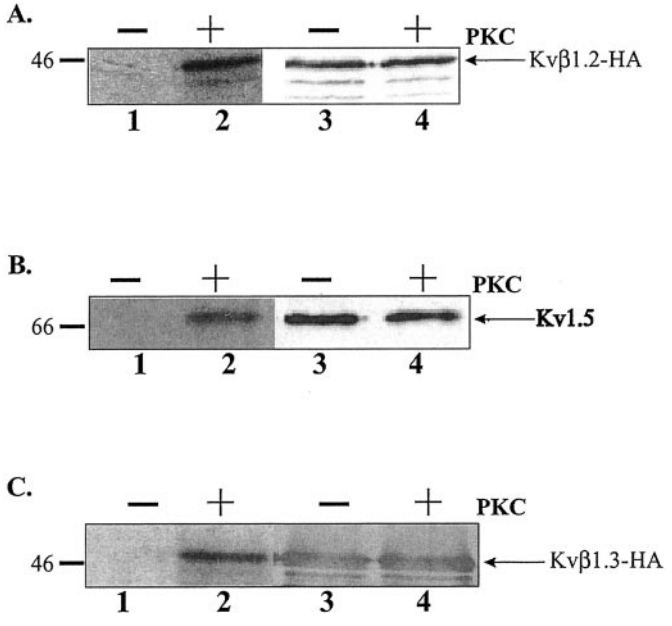

Fig. 6. In vitro phosphorylation of K^+ channel subunits. Ten micrograms of bacterially expressed, purified, and thrombin protease-treated protein were incubated in the absence (−) and presence (+) of purified PKC catalytic subunit and size-fractionated on SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2 in each panel represent an autoradiograph of the phosphorylated signal. The same membranes were subjected to Western blot analysis using an antibody directed against the HA epitope (A and C, lanes 3 and 4) or Kv1.5 (B, lanes 3 and 4). Molecular weight standards are denoted to the left of each panel, and the arrows indicate the position of the protein.

phosphorylated in the presence of the kinase. Subsequent Western analysis with an antibody directed against the HA epitope was performed on the same membrane, confirming that the phosphorylation signal comigrated with Kvβ1.2 and that there was equal loading of protein (lanes 3 and 4). A similar approach was used to determine whether Kv1.5 is a substrate for PKC. A ~72 kDa protein is specifically modified in the presence of the kinase (Fig. 6B, lanes 1 and 2), and this signal comigrated with a protein that was detected by an anti-Kv1.5 antibody (lanes 3 and 4). Note that Kv1.5 migrated as a single band. This is likely due to lack of posttranslational modification of mammalian proteins in bacteria. Thus, both channel subunits are substrates for PKC in vitro.

There are a number of putative phosphorylation sites in Kvβ1.2 that are present in the conserved carboxyl terminus shared by the β subunits. Consequently, we also examined whether Kvβ1.3 could be modified by PKC. Like Kvβ1.2, Kvβ1.3 is a substrate for PKC phosphorylation in vitro (Fig. 6C, lanes 1 and 2).

Discussion

Our results demonstrate that coexpression of the Kvβ1.2 subunit with Kv1.5 enhances the response of the channel to PKC activation. The resultant suppression of K^+ current resembles that seen for I_{Kur} (Li et al., 1996), an atrial-specific K^+ current in human myocytes for which Kv1.5 is known to be the principal molecular correlate (Paulmichl et al., 1991; Snyders et al., 1993; Coetzee et al., 1999). I_{Kur} is one of multiple cardiovascular K^+ currents inhibited by activation of the α-adrenergic receptors and PKC. The resulting prolongation in action potential duration will increase atrial refractoriness and thus could potentially help protect against the development of atrial fibrillation (Li et al., 1996; Nattel et al., 1999). Although the K^+ channel subunit composition of I_{Kur} is currently unknown, our results indicate that Kv1.5 can coassemble with both β subunits studied in vivo, and they suggest a requirement for Kvβ1.2 to recapitulate the response of I_{Kur} to PKC activation. It was recently shown that Kvβ1.2 is associated with Kv1.5 in the formation of Kv channel complexes in vascular smooth muscle (Thorneloe et al., 2001) and human atrium (Kuryshev et al., 2001), providing additional supporting evidence that the two K^+ channel subunits might coassemble in the I_{Kur} complex.

Previous studies using mouse (Attali et al., 1993), rat (Timpe and Fantl, 1994), and canine (Vogalis et al., 1995) homologs of Kv1.5 have suggested that the α subunit is sensitive to activation of PKC. However, in some cases, K^+ current suppression was variable (Timpe and Fantl, 1994). In addition, for some studies, a reduction in K^+ current due to significant internalization of plasma membrane cannot be ruled out given the concentration of phorbol ester used (Vasilets et al., 1990) and the fact that capacitance, and thus K^+ current density, was not monitored (Attali et al., 1993; Vogalis et al., 1995). The response of the human Kv1.5 isoform to PKC activation has not been examined previously.

Our experimental results indicate that all three K^+ channel subunits under study are substrates for phosphorylation by PKC. Consensus sequences for PKC phosphorylation include (R/K_{1-3}, X_{2-0})-S/T-(X_{2-0}, R/K_{1-3}) > S/T-(X_{2-0}, R/K_{1-3}) ≥ (R/K_{1-3}, X_{2-0})-S/T (Kennelly and Krebs, 1991; Pinna and Ruzzene, 1996). Twenty potential PKC phosphorylation sites are present within the intracellular regions of the Kv1.5 amino acid sequence. For the Kvβ subunits, 10 consensus sites for PKC phosphorylation are present in the unique amino terminus of Kvβ1.2, whereas there are 27 sites present in the shared

carboxyl terminus. Given the multiplicity of potential unique sites within Kvβ1.2, we did not attempt to identify the site(s) responsible.

We have previously shown that coexpression of Kvβ1.3 with Kv1.5 is required for the PKA-mediated increase in K^+ current (Kwak et al., 1999). I_{Kur} is similarly modulated by β-adrenergic stimulation and activation of PKA. Taken together with the results of our present study, these data suggest that coassembly of more than one β subunit with Kv1.5 may be required to recapitulate the complete phenotype of I_{Kur} and its modulation by adrenergic stimulation. The possibility that more than one Kvβ subunit might be required is not surprising, given that immunoprecipitation experiments in brain have revealed that often two β subunits can be found coassembled with a single Kv1α subunit (Xu and Li, 1998; Coleman et al., 1999). However, it is stressed that Kv1.5 + Kvβ1.2 + Kvβ1.3 cannot by itself represent the cell molecular components of I_{Kur} due to the fast inactivating phenotype of the recombinant K^+ currents, which is not a property of I_{Kur}.

In summary, we have shown that coassembly of Kvβ1.2 with Kv1.5 enhances response of the channel to PKC activation, with a reduction in K^+ current. These data lend further support to the concept that the Kv1.5α subunit alone may not form the sole molecular basis of I_{Kur} but that additional K^+ channel subunits are required.

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Address correspondence to: Dr. Katherine T. Murray, Department of Pharmacology, Room 559, Preston Research Building, Vanderbilt University School of Medicine, 23rd Avenue South at Pierce Avenue, Nashville, TN 37232-6602. E-mail: kathy.murray@mcmail.vanderbilt.edu