

# Long QT2 Mutation on the Kv11.1 Ion Channel Inhibits Current Activity by Ablating a Protein Kinase C $\alpha$ Consensus Site

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## ABSTRACT

Mutations that inhibit Kv11.1 ion channel activity contribute to abnormalities of cardiac repolarization that can lead to long QT2 (LQT2) cardiac arrhythmias and sudden death. However, for most of these mutations, nothing is known about the molecular mechanism linking Kv11.1 malfunction to cardiac death. We have previously demonstrated that disease-related mutations that create consensus sites for kinases on ion channels can dramatically change ion channel activity. Here, we show that a LQT2-associated mutation can inhibit Kv11.1 ion channel activity by perturbing a consensus site for the Ser/Thr protein kinase C $\alpha$  (PKC $\alpha$ ). We first reveal by mass spectrometry analysis that Ser890 of the Kv11.1 ion channel is phosphorylated. Then, we demonstrate by a phospho-detection immunoassay

combined with genetic manipulation that PKC $\alpha$  phosphorylates Ser890. Furthermore, we show that Ser890 phosphorylation is associated with an increase in Kv11.1 membrane density with alteration of recovery from inactivation. In addition, a newly discovered and as yet uncharacterized LQT2-associated non-synonymous single nucleotide polymorphism 2660 G $\rightarrow$ A within the human *ether- $\alpha$ -go-go*-related gene 1 coding sequence, which replaces arginine 887 with a histidine residue (R887H), strongly inhibits PKC $\alpha$ -dependent phosphorylation of residue Ser890 on Kv11.1, and ultimately inhibits surface expression and current density. Taken together, our data provide a functional link between this channel mutation and LQT2.

## Introduction

Sudden cardiac death results from rapid arrest of cardiac contractions, most often occurring when electrical impulses in the heart become chaotic (Podrid and Myerburg, 2005). Sudden cardiac death affects 300,000 people each year in the United States alone (American Heart Association). These sudden deaths are often the result of a cardiac arrhythmia known as long QT syndrome (LQT), which is characterized by a prolongation of the QT interval of the electrocardiogram (Moss and Schwartz, 1979; Sanguinetti et al., 1995; Roden et al., 1996; Larsen et al., 2001). LQT is usually linked to an atypical polymorphic ventricular tachycardia known as *torsades de pointes*, which can occur without warning during physical exertion or psychosocial stress (Passman and Kadish, 2001) and can be lethal.

The human *ether- $\alpha$ -go-go*-related gene 1 (hERG1) encodes the Kv11.1 potassium ion channel that regulates the repo-

larization phase of the cardiac action potential (Sanguinetti et al., 1995). Many genetic variations of the hERG1 gene inhibit Kv11.1 ion channel activity. These variations are often associated with lengthening of the repolarization phase of the cardiac action potential, which results in prolongation of the QT interval (congenital LQT2) (Jervell and Lange-Nielsen, 1957; Sanguinetti et al., 1995; Larsen et al., 2001). It has also been established that clinically imperceptible genetic variations of the hERG1 gene could be responsible for adverse responses to medications (acquired LQT2) (Kannankeril and Roden, 2007). It has been established that phosphorylation of Kv11.1 can induce profound variations in its biophysical parameters, including activation, inactivation, and/or deactivation of the channel (Lemos et al., 1986; Levitan, 1994; Barros et al., 1998; Gentile et al., 2006, 2008; Cockerill et al., 2007). Kv11.1 phosphorylation may also influence channel trafficking and interactions with other proteins. Gentile et al. (2008) were the first to demonstrate that mutations that create consensus sites for kinases (termed "phosphorylopathy") can alter Kv11.1 current activity. Oth-

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**ABBREVIATIONS:** LQT, long QT syndrome; hERG-1, human *ether- $\alpha$ -go-go*-related gene 1; PKC, protein kinase C; PKC $\alpha$ , protein kinase C  $\alpha$  isozyme; CHO, Chinese hamster ovary; HA, hemagglutinin; WT, wild type; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MS/MS, tandem mass spectrometry; DAG, diacylglycerol; shRNA, small hairpin RNA; E4031, *N*-(2-[1-[2-(6-methyl-pyridin-2-yl)-ethyl]-piperidine-4-carbonyl]-phenyl)-methanesulfonamide; PMA, phorbol 12-myristate 13-acetate; CHX, cycloheximide.

ers have reported that activation of protein kinase C (PKC) can affect Kv11.1 ion channel activity (Thomas et al., 2003); however, the nature of these alterations and the specific PKC isozyme that contributes to these changes in Kv11.1 is still very controversial.

In the present study, we report that PKC $\alpha$  regulates Kv11.1 ion channel activity by augmenting its surface density. We show that the newly identified but as yet uncharacterized LQT2-associated nonsynonymous single nucleotide polymorphism within the hERG1 gene coding sequence, which replaces arginine 887 with histidine (Tester et al., 2005; Mank-Seymour et al., 2006), strongly inhibits PKC $\alpha$ -dependent regulation of Kv11.1 activity. We show that the mutant channel is resistant to phosphorylation of serine 890, because the mutation disrupts a putative PKC $\alpha$ -specific consensus site and is associated with reduced surface expression of the channel due to increased degradation compared with the wild-type channel.

## Materials and Methods

**Cells.** Chinese hamster ovary (CHO) cells were purchased from American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium (high glucose) with 10% fetal bovine serum. Cells were grown to 80% confluence, so that they remained spindle-shaped; then they were trypsinized and plated.

**Plasmids.** hERG1 plasmid encoding for Kv11.1 conjugated with HA localized in an extracellular domain of Kv11.1 (Kv11.1-HA-WT) was kindly provided by David Armstrong (National Institute Environmental Health of Sciences, Research Triangle Park, NC). Mutations in Kv11.1-HA were introduced with the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and confirmed by sequencing. The small hairpin RNA targeting PKC $\alpha$  type virus was generated in Phoenix-Ampho package cells as described previously (Jerome-Morais et al., 2009).

**Electrophysiology.** Current measurements were performed in cells grown on glass coverslips (Deutsche Spiegelglas; Carolina Biological Supply, Burlington, NC) transfected 48 h after plating with Kv11.1-HA-WT plasmid and a plasmid encoding green fluorescent protein (Clontech, Mountain View, CA) at a ratio of 10:1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twelve to 18 h after transfection, fire-polished 1.5-M $\Omega$  glass pipettes filled with 140 mM KCl, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, and 10 mM HEPES (pH 7.3) were used to make cell-attached patches on fluorescent cells that were bathed in 140 mM KCl, 0.1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose (pH 7.3). Transfected cells were identified on the basis of green fluorescent protein fluorescence. Only isolated spindle-shaped cells were selected for recording. High-resistance gigohm seals were obtained by releasing positive pressure on the pipette but without suction to ensure uniform patch size. The patches were voltage-clamped with a EPC-10 amplifier (HEKA Electronics, Lambricht/Pfalz, Germany).

**Activation and Voltage Dependence of Deactivation.** Kv11.1 currents were recorded from cell-attached patches on CHO cells that were held at 0 mV in symmetrical potassium by transiently repolarizing the patch to negative voltages for 500 ms at a 5-s interval. Only patches with stable peak currents of 100 to 400 pA at  $-120$  mV, with no response to perfusion with a control bath solution, and with leak currents after application of the selective Kv11.1 channel blocker E-4031 at the end of the experiment to within 10% of the control were analyzed further. The current at  $-120$  mV was measured by determining its peak amplitude and by integrating total current (Roden et al., 1996) during the first 100 ms after the peak. Tail currents measured by stepping from varying voltages to  $-120$  mV normalized to maximum currents measured at  $-120$  mV.  $I/I_{\max}$  at  $-120$  mV was plotted versus the preceding depolarization and fitted to a Boltz-

mann function to evaluate voltage dependence of activation. The time constant ( $\tau$ ) of deactivation during the test pulse is plotted versus the amplitude of the test pulse.

**Inactivation and Recovery from Inactivation.** Inactivating current was calculated in macro-patch measurements by a double-pulse protocol on CHO cells that were held at 0 mV, stepped to  $-120$  mV for 20 ms (recovery from inactivation), and then, in a second step, to several test pulses from  $-40$  to  $40$  mV in 20-mV steps.  $\tau$  was calculated by fitting test pulses at each potential with a single exponential function. Recovery from inactivation was calculated from the protocol used to measure deactivation.  $\tau$  of recovery from inactivation was measured by fitting the initial fraction of the tail current to a single exponential. Values are reported as mean  $\pm$  S.E. Differences between groups were evaluated with the Student's *t* test; significance ( $P < 0.05$ ) is indicated with an asterisk.

**Cell Surface ELISA.** Twenty-four-well plates were plated with approximately 200,000 CHO cells/well 48 h before transfection with Lipofectamine and DNA constructs encoding Kv11.1-HA-WT or mutant channels. Kv11.1 surface expression was detected 12 to 18 h after transfection by means of a whole-cell ELISA colorimetric assay (AnaSpec, Fremont, CA). This assay makes use of absorbance detection (405 nm, Synergy HT Multi-Mode Microplate Reader; BioTek Instruments, Winooski, VT) of an alkaline phosphatase-conjugated secondary antibody to detect the primary antibody directed against HA (anti-HA high-affinity clone 3F10; Roche Diagnostics, Indianapolis, IN). To determine the signal/noise ratio, the assay was performed on plates with CHO cells transfected with empty vector and compared with an assay performed with CHO cells expressing Kv11.1 channels.

**Western Blot Analysis.** Cells were lysed 24 h after transfection with radioimmunoprecipitation assay buffer containing 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% Tergitol (Sigma-Aldrich, St. Louis, MO), 0.5% Na<sup>+</sup>-deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 $\times$  ProteaseArrest (G-Biosciences, St. Louis, MO). After cell lysis and/or immunoprecipitation, 4 $\times$  Laemmli buffer was added to the protein samples, which were subsequently heated for 5 min at 95°C. The samples were then subjected to SDS-polyacrylamide gel electrophoresis using 6.5% Tris-glycine homemade gels or 4 to 12% precast NuPAGE gels (Invitrogen) and electrophoretically transferred onto nitrocellulose membranes at 90 V for 90 min. To ensure effective transfer, the nitrocellulose membranes were stained with 0.1% Ponceau S in acetic acid. The Ponceau S was removed by washing with PBS, and the nitrocellulose membranes were blocked in 3% bovine serum albumin-Tris-buffered saline for 1 h at room temperature with gentle shaking. The membranes were incubated with Roche high-affinity rat anti-HA antibody (clone 3F10), rabbit anti-phosphoserine PKC substrate antibody (Cell Signaling Technology, Danvers, MA), mouse anti-PKC $\alpha$  (AbD-Serotec, Oxford, UK), or mouse anti- $\beta$ -actin antibody (Sigma-Aldrich) (primary antibodies) and visualized with the ECL detection kit (Thermo Fisher Scientific, Waltham, MA).

**Western Blot Analysis of Fractionated Cellular Proteins.** CHO cells expressing Kv11.1-HA-WT or Kv11.1-HA-R887H channels were fractionated using the Subcellular Protein Fractionation Kit. Normalized portions of each extract (16  $\mu$ g) were analyzed by Western blotting using anti-HA high-affinity antibody (clone 3F10) from the cytoplasmic or plasma membrane fraction. The blots were probed with goat anti-rat horseradish peroxidase and detected with Thermo Fisher Scientific SuperSignal West Pico Chemiluminescent Substrate.

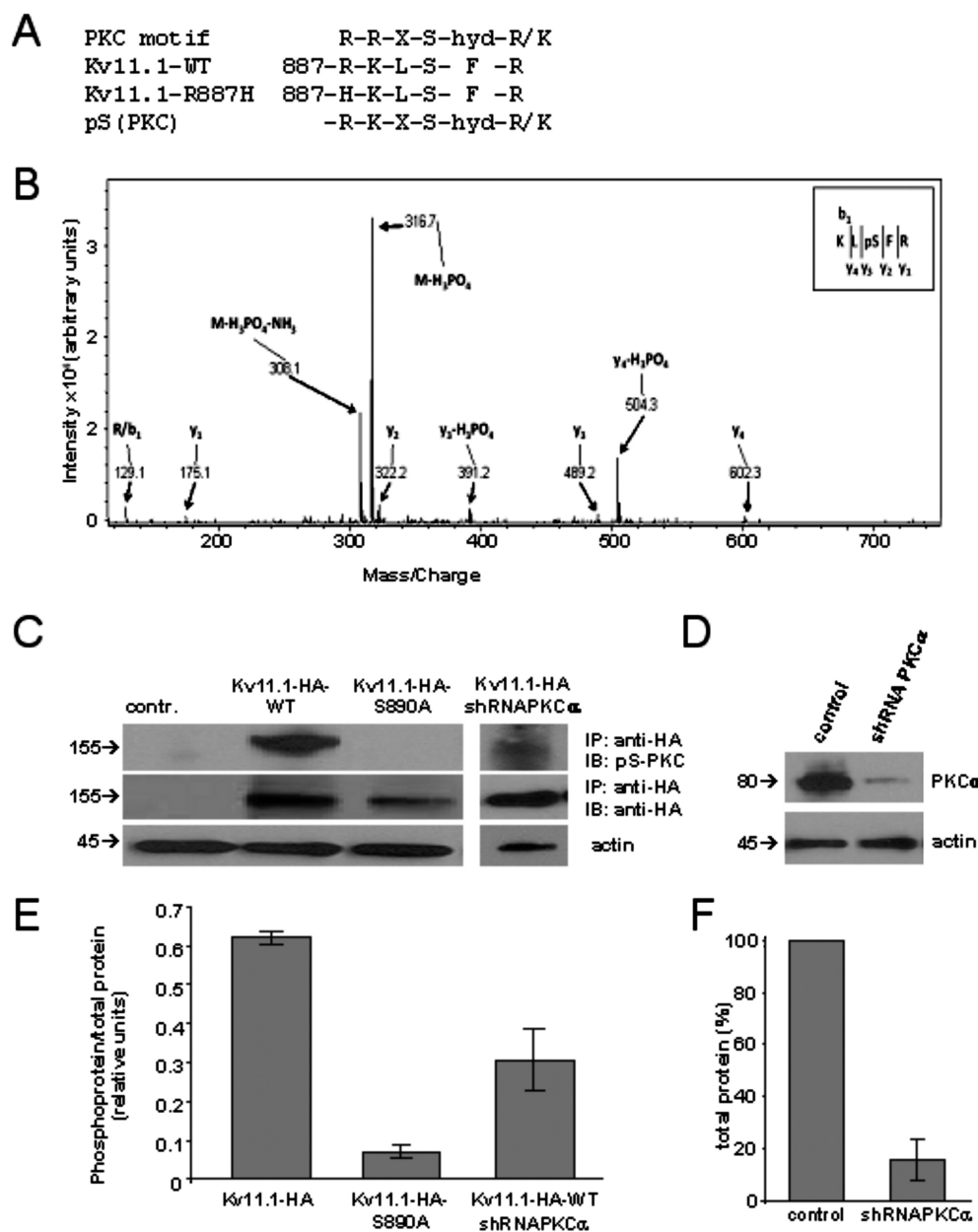
**Immunoprecipitation.** After determination of protein concentrations, 30 ng of high-affinity rat anti-HA antibody (clone 3F10) was added to 800  $\mu$ g of total cell lysate and incubated at 4°C overnight on a horizontal shaker. The next day, 20  $\mu$ l of Dynabeads protein G (Invitrogen) was washed three times at room temperature with PBS. The lysate-antibody mixture was then incubated with the protein G beads for 2 h at 4°C with gentle shaking. After incubation, the

supernatant was removed, and the beads were washed three times with PBS before being subjected to Western blot analysis.

**Mass Spectrometry.** For mass spectrometry analysis, CHO cells were solubilized in a glycerol lysis buffer containing cocktails of protease (Complete Mini, EDTA-free; Roche) and phosphatase (SetII, Calbiochem, San Diego, CA) inhibitors. HA-tagged hERG1 was immunoprecipitated overnight using HA antibody (1:100; Covance Research Products, Princeton, NJ) and resolved by SDS-polyacrylamide gel electrophoresis. Bands corresponding to hERG1 were visualized with Simply Blue Safe stain (Invitrogen), manually excised, and digested with trypsin (Promega, Madison, WI) for 8 h in a ProGest robotic digester (Genomic Solutions (Holliston, MA). Samples were lyophilized and then resuspended in 35 nl of 0.1% formic acid. Nano-liquid chromatography-electrospray ionization-MS/MS analyses were then performed using an Agilent 1100 nanoLC system on-line with an XCT Ultra ion-trap mass spectrometer with the ChipCube Interface (Agilent Technologies). MS/MS data were processed and searched against the NCBI database using the Spectrum Mill software suite (Agilent Technologies). Peptide identifications were validated manually.

## Results

**Ser890 of Kv11.1 Is a PKC $\alpha$  Target.** Regulation of Kv11.1 channel activity as a consequence of stimulated Ser/Thr PKC activity has been reported previously (Thomas et al., 2003; Cockerill et al., 2007), but the mechanism by which PKC regulates the channel is still unclear. Our bioinformatic analysis of the Kv11.1 amino acid sequence indicates that Ser890 is a putative phosphoacceptor because it falls within a canonical PKC consensus phosphorylation site (Fig. 1A). We used mass spectrometry to confirm that Ser890 is constitutively phosphorylated (Fig. 1B). To obtain direct evidence that this event is mediated by PKC, we first transfected Kv11.1-HA-WT and its mutated form in which Ser890 was substituted with alanine (Kv11.1-HA-S890A) with the purpose of disrupting the PKC phosphorylation site, and then we immunoprecipitated from transfected CHO cell lysates using an antibody specific for phosphorylated serine in typical PKC



**Fig. 1.** A, sequence alignment of predicted PKC site on wild-type Kv11.1, Kv11.1-R887H, and consensus sequence for phosphorylation by PKC recognized by the pS(PKC) antibody. X indicates any amino acid, and hyd indicates an amino acid with a hydrophobic side chain. B, electrospray ionization-MS/MS spectrum of the ion  $m/z$  365.7. The  $m/z$  365.7 precursor ion corresponds in mass to the doubly protonated peptide containing residues 887 to 891 with the addition of a single phosphorylation (mass 729.4). The base peak in the spectrum at  $m/z$  316.7 corresponds to the neutral loss of  $H_3PO_4$  from the precursor. The extensive y-series fragment ions, with and without loss of  $H_3PO_4$ , localize the site of phosphorylation to Ser890. C, analysis of Kv11.1 or Kv11.1-HA-S890A channel phosphorylation in CHO cells. D, analysis of Kv11.1 phosphorylation in CHO cells expressing shRNA targeting PKC $\alpha$ . Blots shown are representative of three experiments. E, densitometry analysis of phosphorylated Kv11.1-HA-WT, Kv11.1-HA-S890A, or Kv11.1-HA-WT in cells expressing shRNA (shRNAPKA $\alpha$ ) targeting PKC $\alpha$  relative to total protein F, densitometry analysis of PKC $\alpha$  protein in cells expressing shRNA PKA $\alpha$  compared with control cells. contr., control; IP, immunoprecipitation; IB, immunoblot.

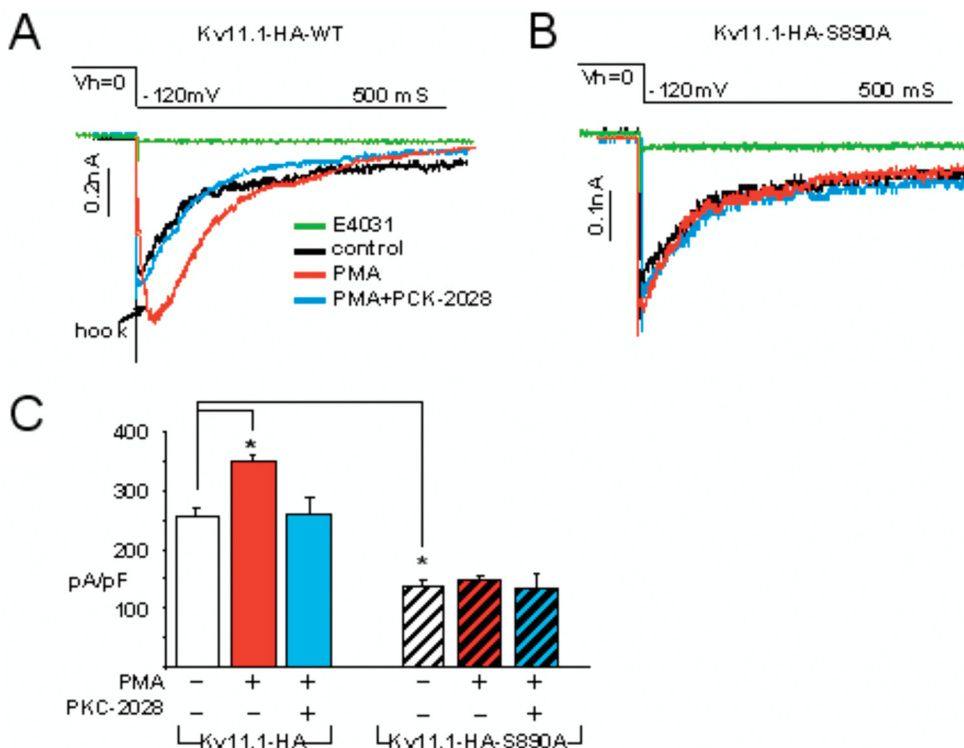


consensus sequences (Fig. 1C). We discovered that, although the phospho-specific antibody reacted strongly with wild-type channels, mutation of Ser890 to alanine completely abolished this signal, supporting our hypothesis that Ser890 on Kv11.1 is the target of a member of the mammalian family of PKC kinases. The mammalian family of Ser/Thr protein kinase C comprises at least nine isozymes shown to play distinctive regulatory roles in cells. They are subdivided according to their mechanism of activation: conventional PKCs ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) that are activated by  $\text{Ca}^{2+}$  and diacylglycerol (DAG), novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ) that are activated by DAG, and atypical PKCs ( $\zeta$ ,  $\iota$ , and  $\gamma$ ) that are  $\text{Ca}^{2+}$ - and DAG-independent (Spitaler and Cantrell, 2004; Griner and Kazanietz, 2007). The human heart expresses six PKC isoforms, namely PKC $\alpha$ , PKC $\beta$ , PKC $\delta$ , PKC $\epsilon$ , PKC $\lambda$ , and PKC $\zeta$  (Simonis et al., 2007). However, during ontogenesis, PKC $\beta$  and PKC $\delta$  expression completely disappears, suggesting that the PKC $\alpha$  isozyme is the major contributor to constitutive PKC activity in the adult human heart (see *Discussion*). Therefore, we tested the hypothesis that PKC $\alpha$  is the specific conventional PKC regulating Kv11.1 ion channel activity by measuring phosphorylation status of Kv11.1 expressed in CHO cells in which PKC $\alpha$  has been down-regulated using shRNA in retroviral infection. We found that in these cells phosphorylation of the wild-type Kv11.1 ion channel was strongly inhibited (Fig. 1, C and D), thus confirming the role of this specific PKC isozyme in phosphorylating Ser890 of the Kv11.1.

**Ser890 Phosphorylation of the Kv11.1 Protein Determines Increased Current Activity.** To test the functional effects of PKC-dependent phosphorylation of Ser890 on Kv11.1, we first recorded Kv11.1 channel activity in CHO cells treated with the PKC activator phorbol 12-myristate 13-acetate (PMA). We found that a 2-h treatment of the cells with 200 pM PMA significantly increased current density

through the Kv11.1-HA-WT channel compared with that in vehicle-treated cells. Remarkably, PMA had no effect on current density in CHO cells expressing the mutant Kv11.1-HA-S890A channel (Fig. 2, A and C), suggesting that phosphorylation of Ser890 by PKC is important for regulating Kv11.1 current density. Of interest, the mutant channel also exhibited a significantly smaller basal current density than the wild-type channel (Fig. 2, A–C). To investigate the role of the specific PKC $\alpha$  isozyme in regulating Kv11.1 channel activity we repeated the experiments described above in CHO cells pretreated with a selective PKC $\alpha$  blocker. It has been reported previously that Kv11.1 ion channel activity can be directly inhibited by several commonly used PKC blockers including bisindolylmaleimides or staurosporine (Thomas et al., 2004). The effects of these drugs on the channel are rapid and include altered membrane trafficking and reduced current density, thus making their use in evaluating the role of PKC in channel regulation impractical. To circumvent this problem, we took advantage of the commercially available membrane-permeable myristoylated PKC $\alpha$  selective blocker (PKC 20-28; Calbiochem) (Eichholtz et al., 1993; Ward and O'Brian, 1993; Zhang et al., 2006). We used this reagent to evaluate the contribution of PKC $\alpha$  activity to the phosphorylation of wild-type or mutated Kv11.1 channels expressed in cells treated with PMA. Pretreatment of cells with PKC 20-28 for 2 h had no effect on Kv11.1-HA-S890A currents (Fig. 2B); however, the effects of PMA on Kv11.1 wild-type current density were completely inhibited (Fig. 2, A and C), suggesting that this event was mediated by PKC $\alpha$ .

**Ser890 Phosphorylation on Kv11.1 Delays Recovery from Inactivation.** Under PMA stimulation, a rightward shift of WT Kv11.1 peak current can be observed (hook in Fig. 2A). This shift is abolished after exposure to PMA plus PKC 20-28 and absent when channels incorporate the S890A mu-



**Fig. 2.** A and B, voltage protocol and resulting currents elicited from CHO cells expressing Kv11.1-HA-WT or Kv11.1-HA-S890A (black trace), Kv11.1-HA-WT or Kv11.1-HA-S890A in cells treated for 2 h with PMA (red trace), and Kv11.1-HA-WT or Kv11.1-HA-S890A in cells pretreated for 2 h with PKC 20-28 and for 2 h with PMA (blue trace). Ensemble averages of eight traces of Kv11.1-HA-WT, Kv11.1-HA-WT + PMA, or Kv11.1-HA-S890A mutant channel are represented. To confirm that the electrical activities were specifically recorded from the Kv11.1 channel, currents were isolated by application of the Kv11.1 selective class III antiarrhythmic agent *N*-(2-[2-(6-methyl-pyridin-2-yl)-ethyl]-piperidine-4-carbonyl)-phenyl-methanesulfonamide (E4031), which completely blocks Kv11.1 ion channel currents at micromolar (5–10  $\mu\text{M}$ ) concentrations (green traces). C, amplitude histograms of the basal peak current activity recorded from CHO cells 12 to 18 h after transfection with Kv11.1-HA-WT ( $n = 8$ ) or Kv11.1-HA-S890A ( $n = 8$ ) channels in CHO cells pharmacologically treated as indicated. Data are means  $\pm$  S.E.,  $P < 0.05$ .

tation (Fig. 2B), which suggests that PKC $\alpha$ -dependent phosphorylation of Ser890 may affect channel gating.

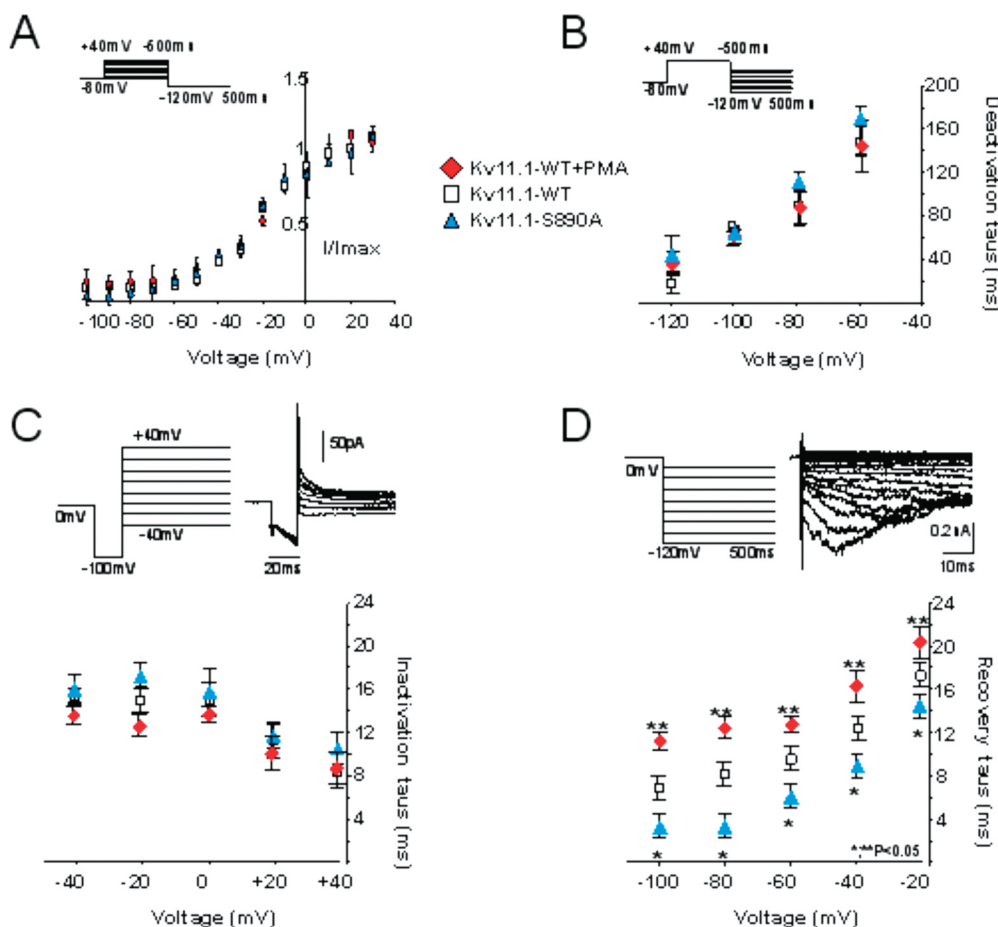
Currents through Kv11.1-HA-WT or Kv11.1-HA-S890A channels in cells treated with PMA did not differ in voltage dependence of activation (Fig. 3A) or time constant of deactivation (Fig. 3B) or inactivation (Fig. 3C). However, the time constant of recovery from inactivation was significantly delayed at all potentials in cells expressing Kv11.1-HA-WT that were treated with PMA compared with that in untreated cells (Fig. 3D). In contrast, recovery from inactivation was faster in cells expressing Kv11.1-HA-S890A than in wild-type Kv11.1 (Fig. 3D). Together these data indicate that phosphorylation of Ser890 alters the Kv11.1 rate of recovery from inactivation.

**Ser890 Phosphorylation of the Kv11.1 Protein Determines Increased Surface Expression.** Because Kv11.1 currents are electrophysiologically detectable only when the channels are expressed on the cell surface, increases in Kv11.1 current density data are consistent with the hypothesis that PKC-dependent phosphorylation of Ser890 of Kv11.1 regulates the surface density of the channel. To test whether Ser890 phosphorylation regulates membrane localization of the channel protein, we examined surface expression of HA-tagged Kv11.1 wild-type or S890A mutant channels expressed in CHO cells using a whole-cell ELISA colorimetric assay before and after pharmacological treatment with PMA. The HA epitope is located extracellularly, which allowed us to selectively label the surface pool of the channel and monitor cell surface channels under various

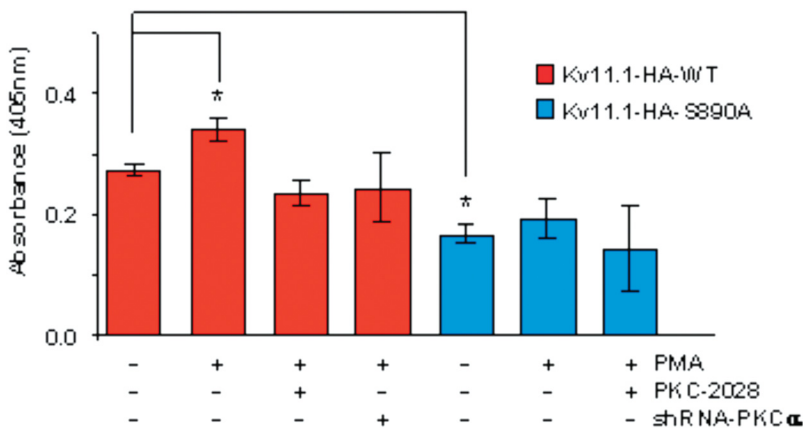
treatment conditions. In CHO cells expressing Kv11.1-HA-WT or Kv11.1-HA-S890A, signals are apparent for cell surface receptors (mock-transfected values have been subtracted). As shown in Fig. 4, the surface expression of Kv11.1-HA-WT was significantly increased ( $20 \pm 2\%$ ) after 2 h of treatment with PMA compared with that in untreated cells (control; 100% surface expression). This effect was strongly inhibited by pretreatment of the cells with PKC 20-28 or by transfection with shRNA PKC $\alpha$  (Fig. 4). In contrast, surface membrane density of the mutant channel Kv11.1-HA-S890A did not change in response to PMA or to the PKC inhibitor compared with that in untreated cells (Fig. 4). Taken together, these data indicate that PKC $\alpha$ -dependent phosphorylation of the Kv11.1 protein at Ser890 plays a major role in mediating surface membrane expression of the channel.

**LQT2-Related Mutation R887H on Kv11.1 Disrupts Phosphorylation of Ser890.** Mutation-dependent malfunction of the Kv11.1 ion channel leads to a delay in repolarization of the cardiac action potential associated with LQT2 syndrome. It has been reported that the hERG1 gene non-synonymous single nucleotide polymorphism 2660 G→A replaces arginine in position 887 with histidine (R887H) (Tester et al., 2005; Mank-Seymour et al., 2006). This mutation is associated with LQT2 in patients, although the mechanism linking this ion channel mutation to the disease has not been elucidated.

Ca<sup>2+</sup>-activated PKC isozymes phosphorylate serines or threonines within consensus sites containing arginine or lysine at the -3, -2, and +2 positions and hydrophobic



**Fig. 3.** A and B, activation curve and kinetics of deactivation for Kv11.1-HA-WT ( $n = 8$ ; open square), Kv11.1-HA-WT in cells treated for 2 h with 200 pM PMA ( $n = 8$ ; red diamond), or Kv11.1-HA-S890A ( $n = 8$ ; blue triangle). C, time course of the Kv11.1-HA-WT inactivation response in cells treated for 2 h with PMA or Kv11.1-HA-S890A. D, time course of recovery from inactivation of Kv11.1-HA-WT treated for 2 h with PMA or Kv11.1-HA-S890A. Data are means  $\pm$  S.E. \*, \*\*,  $P < 0.05$ .



**Fig. 4.** Quantification of Kv11.1-HA-WT (red bars) or Kv11.1-HA-S890A (blue bars) surface expression in CHO cells in response to 200 pM PMA for 2 h or PMA + PKC 20-28 or in cells expressing shRNA PKC $\alpha$  and compared with untreated cells. Each value represents the mean  $\pm$  S.E. of at least six determinants in eight independent experiments. \*, significant difference from the control (untreated) ( $P < 0.001$ ,  $t$  test).

amino acids at position +1 (R-K/R-K-X-S-hyd-K/R) (Nishikawa et al., 1997). The amino acid residue arginine 887 is located in position -3 of the amino acid sequence forming the PKC consensus site in which Ser890 is the phosphoacceptor (Fig. 1A). Therefore, we hypothesized that the LQT2-associated mutation R887H on Kv11.1 protein might disrupt the PKC site by removing an important flanking residue forming the motif, thereby altering PKC $\alpha$ -dependent regulation of ion channel activity.

To test this hypothesis, we first measured phosphorylation of Ser890 in the HA-tagged Kv11.1 potassium channel in the LQT2 R887H mutant (Kv11.1-HA-R887H). Strikingly, we observed that phosphorylation of Kv11.1-HA-R887H was markedly reduced compared with phosphorylation of the WT channel (Fig. 5, A and B), suggesting that the LQT2-associated R887H mutation of Kv11.1 disrupts PKC $\alpha$ -dependent phosphorylation of Ser890.

**R887H Mutation of Kv11.1 Inhibits Ion Channel Current Activity by Decreasing Its Surface Density.** To investigate the possible functional effects of the LQT2-related mutation R887H on Kv11.1, we recorded and analyzed currents of the mutant channel Kv11.1-HA-R887H expressed in CHO cells. We found that the basal current activity of Kv11.1-HA-R887H was significantly reduced compared with that for the WT channel (Fig. 5, B and C), suggesting that the R887H mutation affects the surface membrane density of the channel, as observed with the Kv11.1-HA-S890A channel. To test this hypothesis, we measured cell surface expression of the Kv11.1-HA-R887H ion channel expressed in CHO cells before and after treatment with PMA or PMA plus PKC 20-28. We found that the PKC-dependent increase in the Kv11.1-HA-R887H surface membrane abundance was significantly inhibited compared with that in the wild-type channel (Fig. 5D) because PMA only increased the Kv11.1-HA-R887H abundance by  $8 \pm 2\%$  compared with the  $22 \pm 2\%$  increase in the WT channel. In addition, the small stimulatory effect of PMA on the surface membrane density of Kv11.1-HA-R887H was completely inhibited by pretreatment of the cells with PKC 20-28.

Further membrane fractionation studies were performed on CHO cells expressing Kv11.1-HA-WT or Kv11.1-HA-R887H channels (Fig. 5, F and G). These studies revealed that approximately 60% of the wild-type channel was localized in the cytoplasm and the remaining 40% was localized at the membrane. However, substitution of R887 with H changed the ratio of distribution because the Kv11.1-HA-

R887H membrane fraction was only 18% of the total ion channel expressed. These data are consistent with the hypothesis that the LQT2-associated R887H mutation of the Kv11.1 ion channel affects channel function by inhibiting its PKC $\alpha$ -dependent surface expression.

Furthermore, the voltage dependence of activation and time course of deactivation and inactivation were comparable in Kv11.1-HA-R887H and wild-type channels (Fig. 6, A–C). However, recovery from inactivation was significantly faster, similar to that for the Kv11.1-HA-S890A mutant.

**R887H Mutation of Kv11.1 Accelerates Protein Degradation.** We next examined the total complement of Kv11.1 channels by Western blot analysis. These experiments revealed that stimulation of PKC $\alpha$  activity leads to a significant increase in the total protein channel abundance (39%) (Fig. 7, A and B), which was inhibited by down-regulation of PKC $\alpha$  activity by using PKC 20-28 or in cells infected with shRNA PKC $\alpha$  (Fig. 7, A and B). Furthermore, a significant reduction in channel total protein was also observed in cells expressing Kv11.1-HA-R887H. In addition, substitution of R887 with H inhibited the PMA effects on the channels because PMA only increased channel abundance by 18%. These data raised the possibility that the R887H mutation of Kv11.1 affects degradation of the channel by disrupting the PKC site.

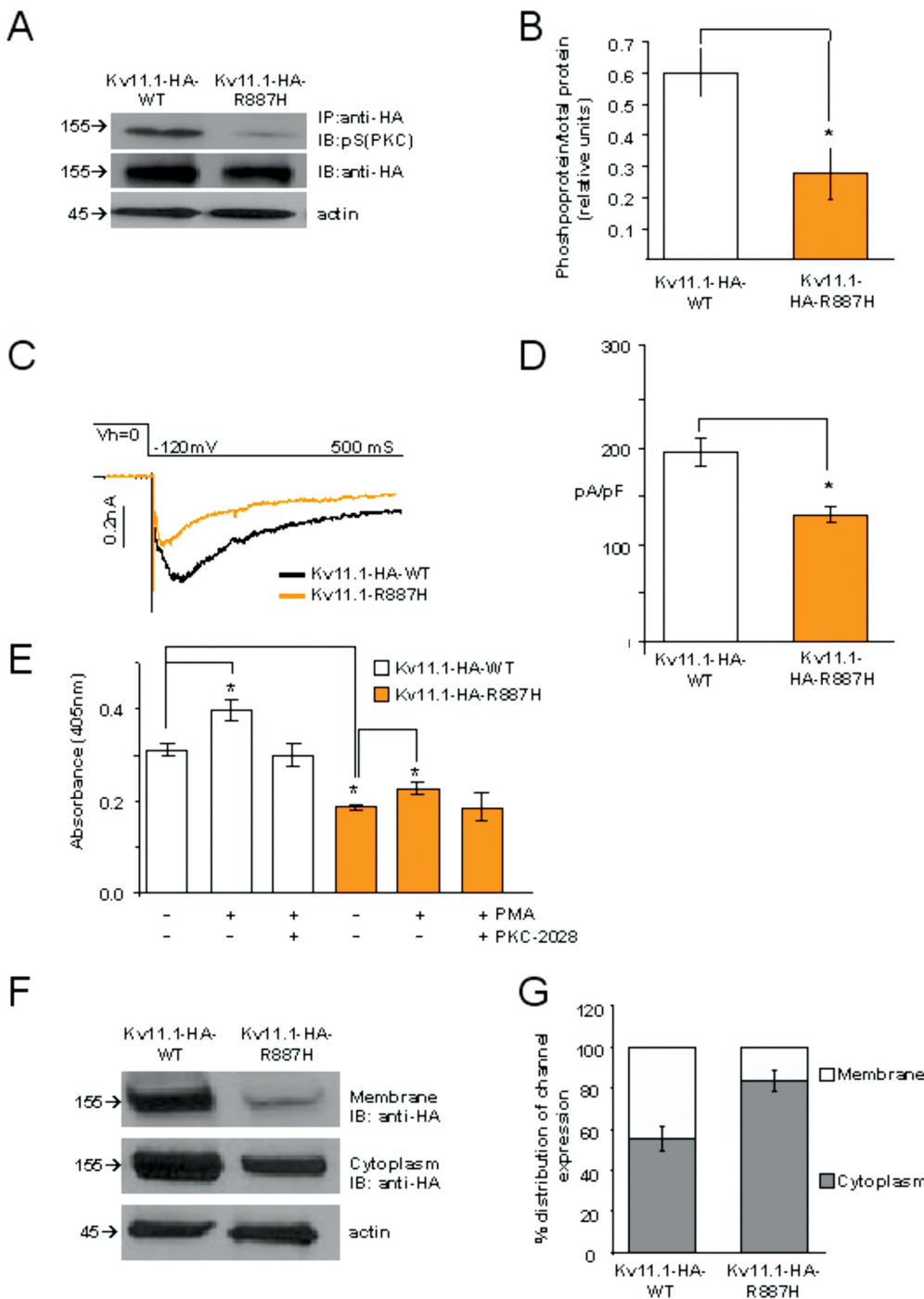
To test this hypothesis, CHO cells expressing the wild-type or the Kv11.1-HA-R887H mutant channels were treated with the protein synthesis inhibitor cycloheximide (CHX) (Wu et al., 2008) for 4, 12, or 24 h (Fig. 7, C and D), and channel protein levels were measured by Western blot analysis. Data were analyzed and compared with data from untreated cells (0 h).

We found that after 4 h of 40  $\mu$ M CHX treatment, Kv11.1-HA-WT protein density was reduced by approximately 40% (Fig. 7, C and D) in contrast with that of Kv11.1-HA-R887H, which was reduced by approximately 80%. These data indicate that phosphorylation of Ser890 may contribute to stability of the Kv11.1 channel protein; therefore, the LQT2-associated R887H mutation of Kv11.1 produces augmented degradation of the channel, which ultimately leads to reduced current density.

## Discussion

This study demonstrates that Kv11.1 potassium ion channel activity can be regulated by a PKC $\alpha$ -dependent direct



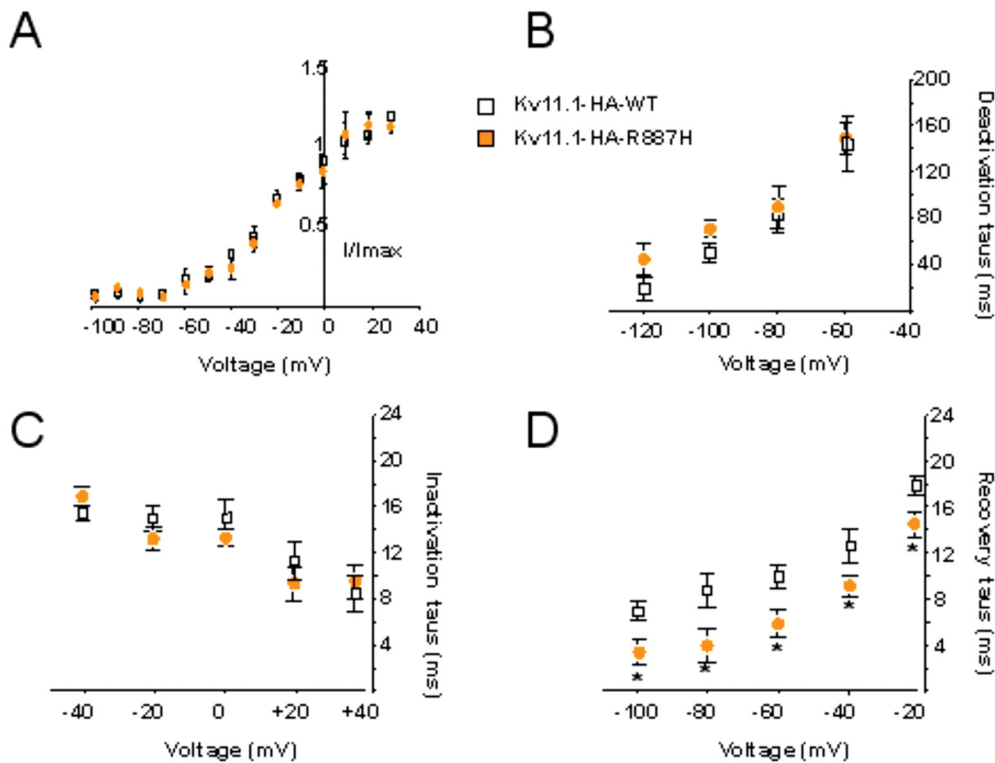


**Fig. 5.** A, Western blot analyses indicating the phosphorylation status of Kv11.1-HA-R887H compared with that of the wild-type channel and effects of stimulation of PKCs with 200 pM PMA + 10  $\mu$ M PKC 20-28. B, densitometry analysis of phosphorylated Kv11.1-HA-WT or Kv11.1-HA-S890A relative to total protein. C and D, representative tail currents and amplitude histograms of the basal current activity from Kv11.1-HA-WT ( $n = 8$ ) or Kv11.1-HA-R887H ( $n = 8$ ) channels in CHO cells. E, quantification of Kv11.1-HA-WT (open bars) or Kv11.1-HA-S890A (orange bars) surface expression in CHO cells in response to 200 pM PMA for 2 h or PMA + PKC 20-28 or in cells expressing shRNA PKC $\alpha$  and compared with untreated cells. Each value represents the mean  $\pm$  S.E. of at least six determinants in eight independent experiments. \*, significant difference from the control (untreated) ( $P < 0.05$ ,  $t$  test). F, Western blot analysis of Kv11.1-HA-WT or Kv11.1-HA-R887H in cytoplasm and plasma membrane fractions of CHO cells 18 h after transfection. Actin was detected only in the cytoplasmic fraction and was used as a loading control. G, amount of Kv11.1-HA-WT or Kv11.1-HA-R887H in the plasma membrane- or cytoplasm-containing fraction (relative to total = cytosol + membrane). The graphs represent consolidated data for each mutant from a representative of three experiments performed in triplicate, and the error bars show the S.D.s of triplicates. All the experiments were done in parallel. IP, immunoprecipitation; IB, immunoblot.

phosphorylation of Ser890 on the channel protein. Furthermore, the naturally occurring LQT2 mutation substituting R887 with H affects Kv11.1 ion channel activity by disrupting the PKC $\alpha$  recognition site.

Regulation of the Kv11.1 ion channel by stimulated PKC activity has been found in other expressing systems. However, data are very controversial because treatments with the PKC activator PMA can either stimulate the activity of the Kv11.1 channel expressed in human embryonic kidney cells (Chen et al., 2010) or can inhibit activity of the Kv11.1 channel expressed in *Xenopus laevis* oocytes (Thomas et al., 2003). It has also been reported that no changes were seen in Kv11.1 channel activity in human embryonic kidney cells

treated with PKC activators phorbol-12,13-didecanoate (Thomas et al., 1999) or 1-stearoyl-2-arachidonyl-glycerol (Kiehn et al., 1998). We believe that this discrepancy in PKC-dependent changes of Kv11.1 channel activity can depend on the type and/or concentration of PKC activators, expression systems, and different technical methodologies used. We intentionally recorded Kv11.1 currents in metabolically intact mammalian cells to minimize the effects of dilution of cellular components caused by dialysis during conventional whole-cell patch clamp. We also used a low concentration of the PKC activator, PMA, to lessen the possible effects of this compound on other kinases (Gentile et al., 2008). This amount of PMA did not exert any appar-



**Fig. 6.** A, activation curves and kinetics of deactivation for Kv11.1-HA-WT ( $n = 8$ ; open square) or for Kv11.1-HA-R887H ( $n = 8$ ; orange circle).  $I/V_{max}$  at  $-120$  mV is plotted versus the preceding depolarization. Data are means  $\pm$  S.E. B, amplitude histograms of the basal Kv11.1-HA-WT ( $n = 8$ ) or Kv11.1-HA-R887H ( $n = 8$ ) channels. C, time course of the Kv11.1-HA-WT inactivation response in cells treated for 2 h with PMA or Kv11.1-HA-S890A. D, time course of recovery from inactivation of Kv11.1-HA-R887H compared with that for the WT channel. Data are means  $\pm$  S.E. \*,  $P < 0.05$ .

ent effects on viability or proliferation during the time of application.

Of interest, human heart malfunction has been associated with increased levels and activity of conventional PKC isoforms, especially PKC $\alpha$  (Braz et al., 2004; Liu et al., 2009; Palaniyandi et al., 2009), raising the possibility that this PKC isoform plays an important role in human heart pathophysiology. Furthermore, studies in rats on the role of PKC $\alpha$  in heart physiology have demonstrated that this kinase acts as a sensor for intracellular calcium homeostasis, and activation of PKC $\alpha$  by adrenergic stimulation causes phosphorylation of a number of ionic channels, which ultimately regulate cardiac contractility (Barros et al., 1998; Braz et al., 2004; Aydin et al., 2007; Churchill et al., 2008). More recent studies (Belmonte and Blaxall, 2011) have shown that pharmacological manipulation of PKC $\alpha$  represents a tenable therapeutic approach for treating human heart failure. However, very little is known about the possible targets for this kinase. We found that PMA caused phosphorylation of Ser890 on Kv11.1 protein and an increased surface membrane density of this channel, which corresponded to a functional effect of augmented Kv11.1 current. Preincubation with the selective PKC $\alpha$  inhibitor PKC 20-28 inhibited the PMA effects similarly as was also observed in cells in which PKC $\alpha$  was targeted by shRNA. Our data indicate that PKC $\alpha$  plays an important role in regulating the Kv11.1 ion channel and may be of physiological relevance.

Although it might seem in this model that PKC $\alpha$  would be the only isozyme responsible for Ser890 phosphorylation, the possible role of other kinases cannot be excluded. For example, the finding (Fig. 1) that shRNA PKC $\alpha$  was less effective than abolishing the Ser890 site in preventing phosphorylation argues for additional kinase activity. To the best of our knowledge, there is indeed at least one study showing a possible role of Ser890 on Kv11.1 in modulating the effects of

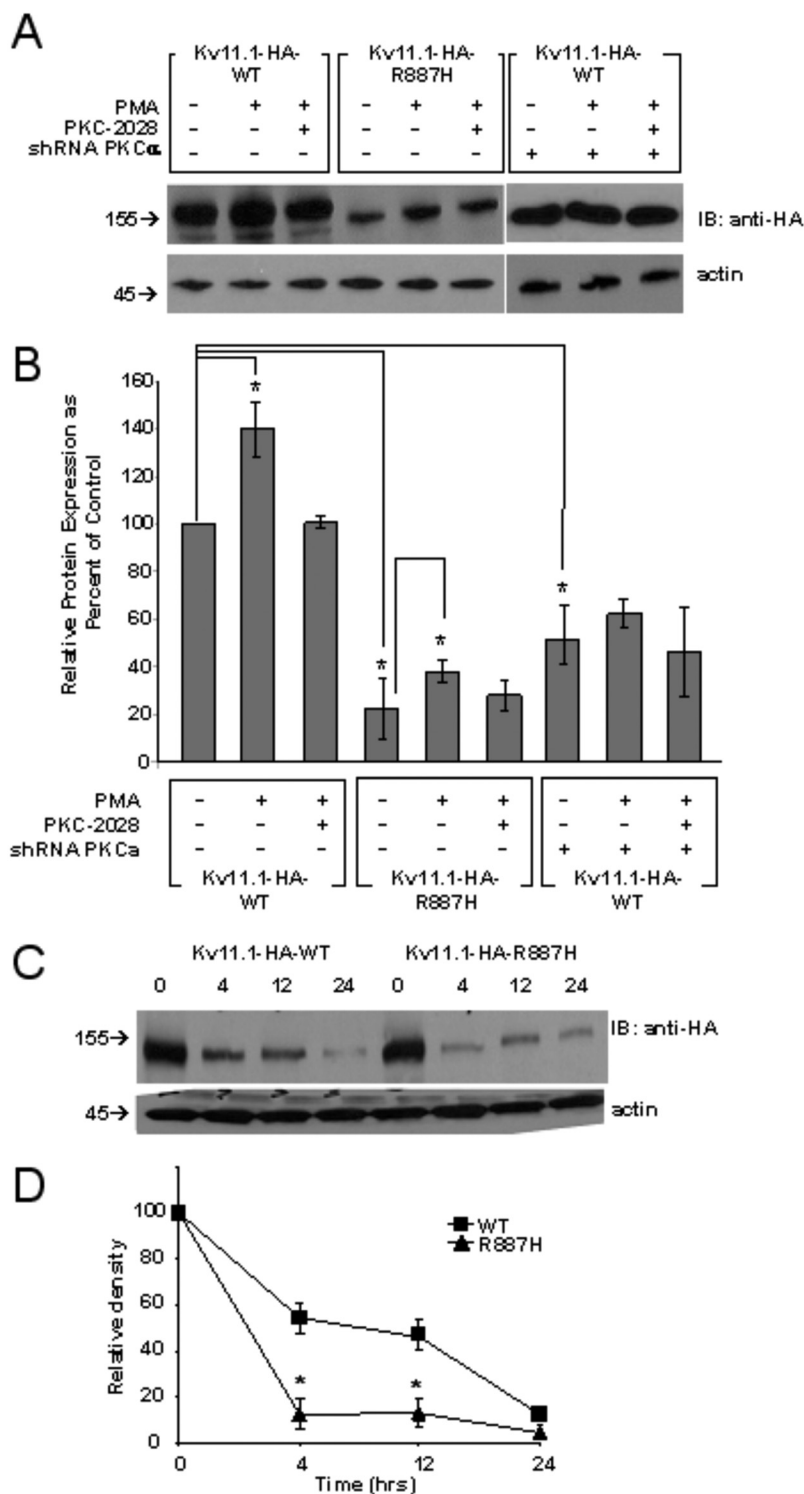
PKA on the channel (Thomas et al., 1999); therefore, we might speculate that PKC $\alpha$  and PKA share Ser890 on Kv11.1 as a target. However, several researchers have demonstrated that PKC kinase can be considered as a downstream effector of PKA because stimulation of PKA activity can lead to activation of PKC in several cells or tissues including heart (Wooten et al., 1996; Chio et al., 2004).

We also found that PKC $\alpha$ -dependent phosphorylation of Ser890 caused delayed recovery from inactivation and no other biophysical parameters were altered including activation, inactivation, or deactivation of the channel. We believe that this finding cannot account for the increase in current density; however, it might provide evidence for the hypothesis that each gating step of the Kv11.1 channel is expected to involve different conformational changes.

Overall, it seems clear that phosphorylation of the Kv11.1 ion channel has a profound effect on channel activity (Lemos et al., 1986; Levitan, 1994; Barros et al., 1998; Gentile et al., 2006, 2008; Cockerill et al., 2007), raising the question of whether dysregulation of this event affects the function of specific organs expressing the Kv11.1 channel including the heart, brain, and pancreas (Rosati et al., 2000). We have previously reported that other LQT-associated mutations create new consensus sites for the kinases on ion channels and that aberrant phosphorylation of the channel can change its biophysical properties including kinetics of deactivation (Gentile et al., 2008). We called this phenomenon ion channel phosphorylopathy. Other mutations have been found to inhibit Kv11.1 protein trafficking, and, for the vast majority of LQT2-related mutations occurring in the Kv11.1 cytoplasmic domain, the mechanism linking genetic variation to ion channel malfunction is not yet understood.

We show here that the naturally occurring LQT2-associated mutation R887H on Kv11.1 protein affects Kv11.1 protein abundance and current density by disrupting the PKC $\alpha$ -





**Fig. 7.** A, Western blot indicating the expression level of Kv11.1-HA-WT and Kv11.1-HA-R887H in CHO cells treated with or without 200 pM PMA or 200 pM PMA + 10  $\mu$ M PKC 20-28 or Kv11.1-HA-WT in CHO cells expressing shRNA targeting PKC $\alpha$  treated with 200 pM PMA + 10  $\mu$ M PKC 20-28. B, Kv11.1-HA-WT and Kv11.1-HA-R887H protein expression is represented graphically upon treatment with 200 pM PMA or 200 pM PMA + 10  $\mu$ M PKC 20-28 for 2 h. The chart shows the average total optical density of the channel band at 155 kDa from the densitometric analysis of immunoblots (IB) from three independent experiments expressed as percentage change of control. Data were normalized to Kv11.1-HA-WT expression levels present in cells (immunoprecipitates) with no drug added. C, representative immunoblot showing the total protein density of Kv11.1-HA-WT and Kv11.1-HA-R887H after treatment with 40  $\mu$ M CHX for 0, 4, 8, and 24 h.  $\beta$ -Actin is used as an internal control. D, graphical representation of Kv11.1-HA-WT and Kv11.1-HA-R887H protein density after treatment with CHX. The chart shows the relative optical density of the hERG1 band at 155 kDa from the densitometric analysis of immunoblots from three independent experiments. The half-lives for Kv11.1-HA-WT and Kv11.1-HA-R887H were estimated to be 4.5 and 1.9 h. Data are means  $\pm$  S.E. \*,  $P < 0.05$ .

dependent regulation of Kv11.1 ion channel activity. Disruption of the PKC-dependent phosphorylation of Ser890 of Kv11.1 profoundly reduces the surface density of the channel by accelerating its degradation and recovery from inactivation. Ultimately, these events lead to inhibition of the total potassium current compared with that in wild-type channels. Therefore, prolongation of the QT interval of the electrocardiogram in patients carrying the Kv11.1 R887H mutation could be explained by abatement of Kv11.1 current density caused by dysregulation of PKC-dependent modulation of

Kv11.1 ion channel activity. In view of this possibility, PKC $\alpha$ -dependent regulatory processes may be considered as pharmacological targets for developing a strategic therapeutic approach in antiarrhythmia therapy.

Biochemical pathways leading to degradation of ion channels including Kv11.1 are largely unknown. However, studies (Cha et al., 2008) have underlined the role of PKC in increasing transient receptor potential V5 ion channel abundance by inhibiting caveolae-promoted endocytosis. Our finding that PKC-dependent phosphorylation of Kv11.1 limits degrada-

tion of the channel might suggest that more studies on the degradation process of Kv11.1 are needed.

It is also important to note that investigations have revealed that both PKC and Kv11.1 activities play an important role in regulating cellular functions such as secretion from the cells in the pituitary gland and pancreas (Rosati et al., 2000). For example, modulation of Kv11.1 ion channel activity causes changes in action potential frequency in pancreatic  $\beta$  cells. Furthermore, the latest studies in cancer research have shown that Kv11.1 overexpression occurs in several cancer cell types. Although little is known about the role and regulation of Kv11.1 in cancer cells, this ion channel may be involved in stimulation of proliferation of cancer cells (Pardo, 2004). It has also been reported that PKC $\alpha$  plays an important role in proliferation of cancer cells (Lin et al., 2006); however, the signal transduction pathways downstream of PKC $\alpha$  are not clearly understood. It will be important in the future to determine whether PKC $\alpha$ -dependent regulation of Kv11.1 ion channels plays a role in disease states, including cancer.

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#### Authorship Contributions

*Participated in research design:* Donovan, Denning, and Gentile.  
*Conducted experiments:* Donovan, Lansu, Williams, and Gentile.  
*Contributed new reagents or analytic tools:* Denning and Williams.  
*Performed data analysis:* Donovan, Lansu, Williams, and Gentile.  
*Wrote or contributed to the writing of the manuscript:* Donovan, Denning, and Gentile.

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