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Kv4.2 is a locus for PKC and ERK/MAPK cross-talk

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Abstract

Transient outward K⁺ currents are particularly important for the regulation of membrane excitability of neurons and repolarization of action potentials in cardiac myocytes. These currents are modulated by protein kinase C (PKC) activation, and the K⁺ channel subunit, Kv4.2, is a major contributor to these currents. Furthermore, the current recorded from Kv4.2 channels expressed in oocytes is reduced by PKC activation. The mechanism underlying PKC regulation of Kv4.2 currents is unknown. In this study, we determined that PKC directly phosphorylates the Kv4.2 channel protein. *In vitro* phosphorylation of the intracellular amino (N)- and carboxyl (C)-termini of Kv4.2 glutathione *S*-transferase (GST) fusion protein revealed that the Kv4.2 C-terminal was phosphorylated by PKC, while the N-terminal was not. Amino acid mapping and site-directed mutagenesis revealed that the phosphorylated residues on the Kv4.2 C-terminal were Serine (Ser) 447 and Ser537. A phospho-site specific antibody showed that phosphorylation at the Ser537 site increased in the hippocampus in response to PKC activation. Surface biotinylation experiments revealed that alanine mutation to block phosphorylation at both of the PKC sites increased surface expression compared to wildtype Kv4.2. Electrophysiological recordings of the wildtype and both the alanine and aspartate mutant Kv4.2 channels expressed with KChIP3 revealed no significant difference in the half activation or inactivation voltage of the channel. Interestingly, the Ser537 site lies within a possible extracellular regulated kinase (ERK)/mitogen activated protein kinase (MAPK) recognition (docking) domain in the Kv4.2 C-terminal sequence. We found that phosphorylation of Kv4.2 by PKC enhanced ERK phosphorylation of the channel *in vitro*. These findings suggest the possibility that Kv4.2 is a locus for PKC and ERK cross-talk.

Keywords

Protein Kinase C; phosphorylation; potassium channel; extracellular regulated kinase; mitogen activated protein kinase; cross talk

INTRODUCTION

Regulation of transient outward K⁺ currents in cardiac myocytes and in neurons can have profound effects on membrane excitability. Kv4 family members are the primary subunits underlying the transient outward K⁺ currents (*I*_{to}) in dog and human ventricular myocytes [1]

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and Kv4.2 is the main subunit underlying this current in the rat [2,3] and mouse ventricle myocytes [3,4]. Moreover in neurons, Kv4.2 is the primary pore-forming subunit of the transient outward K⁺ currents (I_A) in the dendrites of hippocampal pyramidal cells [5], visual cortical pyramidal neurons [6] and spinal cord dorsal horn neurons [7]. These currents are regulated by kinase activation. I_A in the dendrites of pyramidal and dorsal horn neurons is modulated by PKC activation [8,9]. In many cell types, including neurons, PKC activates ERK/MAPK [10], and studies have shown that the effect of PKC on I_A in area CA1 dendrites is mediated by ERK activation [9,11]. This modulation of I_A amplitude can regulate the peak of back-propagating action potentials and excitability of dendrites [5,11,12]. In addition, activation of a number of cell surface receptors regulate I_{to} in the heart, possibly through the common effector, PKC [13,14]. For example, in lower mammals I_{to} of ventricular and atrial myocytes is regulated by alpha adrenergic receptor activation most likely through PKC activation [15-18].

PKC activation modulates other voltage-gated ion channels, including Na⁺ channels [19] and K⁺ channels [20-23]. These PKC effects on K⁺ channels include the conversion of a rapidly inactivating human Kv3.4 (h-Kv3.4) current to a non-inactivating current [22]. An additional PKC effect is a reduction in currents mediated by both Kv4.2 [23] and long-Kv4.3 (L-Kv4.3), a splice-variant of Kv4.3 that has a 19 amino acid insert that contains a PKC consensus sequence compared to short-Kv4.3 [13,14]. In the case of h-Kv3.4 and L-Kv4.3, these effects are due to direct phosphorylation of the primary subunit [13,22], however, the mechanism of the reduction in Kv4.2 current is unknown.

Activation of the PKC pathway can lead to phosphorylation of Kv4.2 channels and modulation of I_A via activation of the ERK/MAPK pathway [9,11,24]. We propose that PKC may modulate I_A by direct phosphorylation of the Kv4.2 subunit. In this study, we investigate whether the cytoplasmic regions of Kv4.2 N- and C-termini are directly phosphorylated by PKC and the functional effects of the phosphorylation events.

METHODS

DNA

The original rat Kv4.2 and human KChIP3 cDNA were generously provided by Dr. P. J. Pfaffinger. Both constructs were in a cytomegalovirus vector. The C- and N-terminal glutathione *S*-transferase (GST) fusion proteins were expressed in pGEX vectors.

Phosphorylation of the Kv4.2 GST constructs

The amino- and carboxyl-terminal cytoplasmic domains of Kv4.2 were expressed in *E. coli* as (GST) fusion protein constructs, purified and phosphorylated *in vitro* following the methods previously described except for the following modifications [25]. The recombinant proteins were incubated for 20 min at 37°C in reaction mixtures (25 µl) containing 20 ng of the catalytic domain of PKC (Calbiochem), Tris buffer 1 (50 mM Tris-HCl), pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM Na₄P₂O₇, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and ATP mix (100 µM ATP, 100 mM MgCl₂, and 10 µCi [γ -³²P] ATP). Reactions were stopped by boiling for 5 min with sample buffer. The GST-fusion proteins were separated by SDS-PAGE (10%) and visualized by Coomassie blue staining. Phosphopeptides were identified by autoradiography. Parallel reactions were performed with GST alone. A time course of PKC phosphorylation of the Kv4.2 C-terminal peptide was performed.

In the case of phosphorylation of mutant C-terminal constructs, ³²P incorporation into mutant and WT proteins were normalized to total protein for each construct. This was then represented as percentages relative to WT proteins (set at 100%).

Phosphorylation of the Kv4.2 C-terminal wildtype and mutant fusion proteins by ERK (MAP kinase) and PKC were performed as previously described with minor modifications [26]. Briefly, the fusion proteins were incubated for 30 min at 37 °C with 10 μ Ci [γ - 32 P] ATP per reaction in the presence of activated ERK only (as control) and activated ERK plus PKC (Stratagene), HEPES buffer (in mM: 25 HEPES, 0.5 EDTA, 0.5 EGTA, 1 $\text{Na}_4\text{P}_2\text{O}_7$, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin), 10 mM MgCl_2 and 100 μ M ATP. The reactions were stopped and samples were loaded in the SDS-polyacrylamide gel and visualized by Coomassie blue staining. Phosphopeptides were identified by autoradiography. To quantify phosphorylation, autoradiographs were analyzed further by densitometry using NIH Image software. 32 P incorporation was normalized to total protein for all constructs, which was then demonstrated as a percentage of control (ERK phosphorylation of WT construct) which was set at 100%.

Phosphopeptide mapping

The methodology for the phosphopeptide mapping is as previously described except for the following modifications: the reaction volume was increased by a factor of 10, specific activity was increased (20 μ Ci [γ - 32 P]/ 25 μ l reaction volume and 50 μ M ATP), and the incubation period was increased to 60 min based on the time course results. The phosphorylated C-terminal GST-fusion protein was separated by SDS-PAGE (10%). The Coomassie-stained band corresponding to the Kv4.2 C-terminal phospho-protein was excised and used for phosphopeptide mapping as previously described with minor modifications [25,27]. An in-gel digest with trypsin or Lys-C was performed and following extraction from the gel, the peptides were separated using reverse phase HPLC (high-pressure liquid chromatography) with absorption monitoring at 214 nm. Counts/min (CPM) in each HPLC fraction was measured as Cherenkov radiation. Phospho-peptides identified as HPLC fractions containing high radioactivity were sequenced using automated Edmann degradation. Radioactivity was determined with each sequencing cycle by scintillation counting. Notable is that mapping was first attempted using tandem mass spectroscopy as previously described [28]. However, using electrospray ionization mass spectroscopy (API 3000 LC/MS/MS System, PE Sciex, Thornhill, ON, Canada) we were unsuccessful at obtaining peptide sequences of the Kv4.2 C-terminal domain that contained the candidate PKC phosphorylation sites. Thus, we employed the methodology as described in the above paragraph.

Kinetic characterization of the Kv4.2 C-terminal PKC sites

Peptides were synthesized in the Protein Chemistry Core Laboratory (Baylor College of Medicine) which contained the PKC phosphorylation sites within the Kv4.2 C-terminal cytoplasmic domain. The synthetic peptides ANAYMQSKRNGLLC and SRRHKKSFRIPNAC (referred to hereafter as PKC-A and PKC-B peptides, respectively) had a total of 14 amino acid residues corresponding to Kv4.2 channel residues 441-454 (PKC-A) and residues 531-543 (PKC-B) within the Kv4.2 sequence. The phosphorylation site was located in the middle (amino acid number 7) of the peptide, and a cysteine residue for coupling to carrier proteins was located at the carboxyl-terminus (amino acid number 14) of each peptide.

We performed kinetic characterization of the PKC phosphorylation sites using the synthetic peptides in kinase assays as previously described [25]. The catalytic domain of PKC (20 ng) was used and reactions were incubated at 25°C. The assays used to determine K_m and V_{max} were linear with respect to time and linear with added kinase (PKC), and less than 10% of the peptide substrate was converted to product. To obtain the concentration curve for each of the peptides, peptide concentrations ranging from 5-400 μ M were used. As a control parallel reactions were included using the PKC substrate, a synthetic peptide analogue of a fragment of neurogranin [29].

Generation of antisera

The PKC-A and PKC-B synthetic peptides containing the phosphorylated Ser447 and Ser537 PKC sites, respectively were coupled to Keyhole Limpet Hemocyanin and injected into rabbits according to standard protocol at Cocalico Biological Labs (Reamstown, PA). The antisera were screened by western blotting using the phosphorylated and unphosphorylated ovalbumin-coupled synthetic peptides and GST-fusion proteins. The antisera were affinity purified against the phosphorylated PKC-A and PKC-B synthetic peptides using Hi-trap columns (Amersham Pharmacia Biotech) [25].

Site-directed mutagenesis

The Kv4.2 point mutations were made using the site-directed mutagenesis kit (Stratagene, La Jolla, CA) as previously described [28,30]. The following primers were used for mutant constructs: S447A: FORWARD 5'-GCAAATGCCTACATGCAGG**C**CAAGCGGAATGGGTTAC-3', REVERSE 5'-GTAACCCATTCCGCTTGG**C**CTGCATGTAGGCATTTGC-3'; S447D: FORWARD 5'-GTGCAAATGCCTACATGCAGG**A**CAAGCGGAATGGGTTACTGAGC-3', REVERSE 5'-GCTCAGTAACCCATTCCGCTTGT**C**CTGCATGTAGGCATTTGCAC-3'; S537A: FORWARD 5'-CGGAGACACAAGAA**G**GCTTTCCGAATC-3', REVERSE 5'-GATTCGGAAAG**C**CTT**C**TTGTGTCTCCG-3'; S537D: FORWARD 5'-GCTCACGGAGACACAAAAA**G**ATTTCCGAATCCCAAATGCC-3', REVERSE 5'-GGCATTTGGGATTCGGAAA**T**CTTTTTTGTGTCTCCGTGAGC-3' (bold underline denotes a difference compared to the original sequence). All mutations were confirmed by DNA sequence analysis through the entire coding region.

Functional expression in *Xenopus* oocytes

Oocytes were harvested as described previously [30]. After ~24 h, oocytes were injected with 3–10 ng of DNA Kv4.2 [wild type (WT) or mutants] + KChIP3 in a 1-to-1 ratio using a Nanoject microinjector (Drummond Scientific), into the nucleus of stage V–VI oocytes. Currents were recorded after 2 days under two-electrode voltage clamp with an Axoclamp 2A amplifier (Axon Instruments) at room temperature. Microelectrodes were pulled from filamented glass (1.5 mm × 0.86 mm; A-M Systems) filled with 3 M KCl. The current electrode had a resistance of 0.30–0.50 MOhms, whereas the voltage electrode ranged from 0.3 to 1.0 MOhms.

Currents were leak subtracted online with P/4 leak subtraction. Data were digitized at 2 kHz and stored on a computer equipped with Digidata 1200 software. Current protocols used to obtain data included: 1) activation: hyperpolarization to –110 mV and then depolarization to +40 mV for 400–800 ms, repeated in –5- or –10-mV step intervals; 2) inactivation: depolarization to 0 mV and then hyperpolarization to –110 mV for 650 ms, changing this step by +5-mV intervals, then depolarization to 0 mV.

The chamber was continuously perfused at a rate of 3–6 ml/min with ND-96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES pH 7.4 with NaOH). Oocytes expressing mutant DNA and WT (control) DNA were always recorded on the same day and were recorded in at least three batches of oocytes. The data from oocytes expressing WT DNA from different days were not different; therefore, all WT data were combined. Data were analyzed with Clampfit, Origin, and Prism programs. Peak currents were obtained and conductance (G) was determined with a reversal potential (V_{rev}) of –95 mV according to the equation $G = I_p / (V_c - V_{rev})$, where I_p is the peak of the current at a given voltage command (V_c). Activation and inactivation curves were fit with a Boltzmann sigmoidal curve with the equation $G/G_{max} = 1 / [1 + \exp((X - V_{1/2}) / \text{slope})]$, where X is equal to the test potential (V_m) and G_{max} is maximum conductance. The mean ± SE voltage at which half the currents are activated ($V_{1/2}$) was determined from the

Boltzmann fit and compared among the mutants and WT with a One-Way ANOVA and post hoc test.

Studies in the COS-7 expression system

COS7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin (all from Invitrogen) in a humidified incubator at 37°C under 5% CO₂. Cells were maintained in poly-L-lysine-coated 6-well plates. Cells were transfected with mammalian expression vectors for Kv4.2 with FuGene6 (Roche) transfection reagents using the manufacturers' protocols. Cells expressing Kv4.2 wildtype and Ser to Alanine (Ala) and Ser to Aspartate (Asp) mutants were stained 48 hours post-transfection using an immunofluorescence protocol [31]. Briefly, after fixing cells with 4% paraformaldehyde for 30 min, and incubation in 0.3% Triton X-100 in PBS for 20 min at room temperature, the cells were blocked by 10% fetal bovine serum in PBS for 60 min at room temperature, and then incubated for 60 min at room temperature or overnight at 4°C with primary antibodies: polyclonal Kv4.2 antibody from Alomone Labs (Jerusalem, Israel) generated against residues 454-469 (SNQLQSSEDEPAFVSK) on the C-terminal or a monoclonal Kv4.2 antibody against an ectodomain [31]. The Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) secondary antibody was used for detection of primary antibody binding. Microscopic analysis was performed with a Nikon TE200 inverted fluorescence microscope equipped with a digital camera (Coolmax Fx, RS Photometrics, Tucson, AZ).

Biotinylation of Surface Proteins

Kv4.2 WT+KChIP3 or Kv4.2 AA (Ser447Ala/Ser537Ala) mutant + KChIP3 - transfected COS7 cells were washed with ice-cold PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS/Ca²⁺ plus Mg²⁺, pH 8.0) then treated with sulfo-NHS-LC-biotin (0.5 mg/ml, Pierce) at 4 °C for 40 min as described previously [28]. Cells were lysed in radioimmune precipitation assay buffer (RIPA -150 mM NaCl, 0.5% sodium deoxycholate, 20 mM Na₂HPO₄, 1% Triton X-100, 0.1% SDS, pH 7.4), supplemented with 0.01 mM PMSF, 0.005 µg/ml leupeptin, and 0.005 µg/ml pepstatin for 1 h at 4 °C. Lysates were centrifuged at 20,000 × g for 30 min at 4 °C, and the protein concentration in the supernatants was determined using the BCA assay kit (Pierce). UltraLink Immobilized NeutrAvidin beads (50 µl, Pierce) were added to each group sample, and the mixture was incubated for 1 h at room temperature or 4 °C overnight. The beads were washed four times with cold RIPA buffer and eluted by boiling with 50 µl of Laemmli loading buffer (Bio-Rad) for 5 min at 95 °C. The eluates (25 µl) were resolved by SDS-PAGE gel and immunoblotted with the Kv4.2 antibody. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody (1:10,000). Immunoreactivity values of surface Kv4.2 channels were normalized to levels of actin immunoreactivity in total cell extracts to preclude errors that accompany sample loading and transfer.

Hippocampal slice studies

The N- and C-terminal phospho-selective antibodies were used to evaluate modulation of PKC phosphorylation of Kv4.2 in rat hippocampal slices. Transverse hippocampal slices were prepared using the methodology of Roberson et al. [32]. Slices were incubated in artificial cerebrospinal fluid (aCSF) containing in mM: NaCl 125; KCl 2.5; NaH₂PO₄ 1.25; NaHCO₃ 25; CaCl₂ 2; MgCl₂ 1; glucose 25) at 32°C for approximately 1 hour. Slices were then incubated in vehicle (DMSO) or 10 µM PDA for 10 minutes. Slices were rapidly frozen on dry ice. The slices were subsequently sonicated, normalized according to protein assay, and then centrifuged (Beckman, 100,000g, 20 min, 4°C) and the pellet was resuspended in the 10% SDS solution with 200mM DTT and protease/phosphatase inhibitors as previously described [25]. After resuspension of the pellet sample buffer was added. The hippocampal membrane proteins

were then used for western blotting. 4-5 slices were used for each condition and pooled for sonication.

Data analysis

Immunoreactivity was measured using densitometry (public domain, Scion Image). Densitometry data were analyzed with a Student's t-test, Mann Whitney test for nonparametric data analysis or with ANOVA and post-hoc test. The GraphPad Prism software package was used for statistical analysis. Error bars represent standard error of the mean.

Materials

The catalytic subunit of PKC (rat brain) was obtained from Calbiochem. Glutathione agarose bead and [γ - ^{32}P] ATP were obtained from Amersham Pharmacia Biotech. The FuGene 6 Transfection Reagent was obtained from Boehringer Mannheim. PDA (phorbol diacetate) was obtained from Sigma.

Synthetic peptides

The PKC-A and PKC-B peptides were synthesized in the Protein Core Chemistry Laboratory at Baylor College of Medicine, Houston TX.

RESULTS

The Kv4.2 amino acid sequence contains PKC consensus sites

Voltage-gated K⁺ channel primary subunits are characterized by 6 transmembrane domains with prominent intracellular N- and C-termini [33]. A schematic diagram of the putative Kv4.2 topology (Figure 1A) and the Kv4.2 amino acid sequence (Figure 1B) are shown. Analysis of the amino acid sequence of Kv4.2 with NetPhosK (<http://www.cbs.dtu.dk/services/NetPhosK/>) [34] predicted multiple serine and threonine residues (greater than 50% probability) as candidate PKC phosphorylation sites. Candidate sites for PKC phosphorylation are shown in bold and italic. Four consensus sites for PKC phosphorylation exist in the N-terminal and six consensus sites exist in the C-terminal domains of Kv4.2. The putative consensus PKC sequence is **Ser/Thr-X-Arg/Lys**, where X symbolizes an uncharged residue [35]. The presence of PKC consensus sites within the N- and C-termini suggested that PKC may phosphorylate Kv4.2 at both intracellular termini.

The C-terminal of Kv4.2 is a substrate for PKC

To determine if the Kv4.2 N- and C-termini are directly phosphorylated by PKC, Kv4.2 GST-fusion proteins of the putative intracellular N- and C-termini were expressed in bacteria and purified. The resulting purified proteins were incubated with PKC and 10 μCi [γ - ^{32}P] ATP *in vitro* (see methods). Reaction products were separated on an SDS-PAGE gel. Figure 2A shows the bands corresponding to the N- and C-termini GST fusion proteins on the Coomassie Blue stained gel (left panel — Coomassie). The autoradiogram (left panel — Autorad) revealed ^{32}P incorporation into the C-terminal only, suggesting that the C-terminal but not the N-terminal of Kv4.2 was phosphorylated by PKC. In addition, purified GST protein was used as a control and incubated with ^{32}P and PKC. There was no ^{32}P incorporation into the GST protein, indicating that GST was not phosphorylated by PKC (data not shown). In order to determine the optimal time for the saturation of PKC phosphorylation of the Kv4.2 C-terminal substrate, a time course of PKC phosphorylation was done (Figure 2A; right panel). Saturation of phosphorylation is desirable for mapping the phosphorylation sites in order to be certain that sites are not missed during mapping. Phosphorylation of the Kv4.2 C-terminal reached saturation levels in 40 minutes. Therefore, incubations of 60 minutes ensured complete phosphorylation of the protein.

Phospho-site mapping of the PKC sites within Kv4.2

To determine the individual PKC phosphorylation sites in the C-terminal of Kv4.2, phosphopeptide mapping and protein sequencing was performed. For these studies Kv4.2 C-terminal fusion protein was incubated with PKC *in vitro* as described above except that the reaction volume was increased by a factor of 10. The reaction products were separated on an SDS-PAGE gel. The phosphorylated protein bands were excised, eluted and enzymatically digested. Following digestion, the peptides were separated using reverse phase high pressure liquid chromatography (HPLC) with absorption monitoring at 214, 254, and 280 nm (Figure 2B; left panel — 214 nm shown). Counts per min (cpm) in each HPLC fraction were measured as Cerenkov radiation [25]. The phosphopeptide map for the C-terminal construct demonstrated two peaks in radioactivity, in HPLC fraction 17-19 and 67-75 (Figure 2B; right panel). HPLC fractions 17-19 (black arrow on HPLC trace) and 67-75 (gray arrows on HPLC trace) were sequenced using Edman degradation. Two phosphorylation sites were identified in the C-terminal construct at residues Ser447 and Ser537 (Figure 2C; left and right panels, respectively).

Kinetic characterization of the PKC sites was performed using synthetic peptides (see methods) containing the phosphorylation site and 6 flanking residues on either side. The concentration curves for the PKC Ser537 site revealed that the Kv4.2 Ser537 site was a good substrate for PKC with a K_m of 6.7 μ M and a V_{max} of 771 nmol/min/mg of protein (averages of two experiments performed in duplicate under steady-state conditions, data not shown). The kinetic characterization for the Ser447 site was not obtained since there was no 32 P incorporation into the synthetic peptide containing this site. A possible explanation for the lack of 32 P incorporation into this synthetic peptide is that there are tertiary structural determinants necessary for phosphorylation at this site that are disrupted in the small (14 amino acids) peptide used for the kinetic experiments. The full C-terminal GST fusion protein construct was not used for the kinetic experiments as it remained on the beads and thus protein concentrations could not accurately be measured, which would be a critical aspect for determining the kinetic values. An additional possible explanation for the lack of phosphorylation in the peptide is that phosphorylation of the Ser537 site may be necessary for phosphorylation at the Ser447 site. Our data from phosphorylation of alanine site mutants suggest that this, however, is not the case. The Ser447 site is still phosphorylated in the Ser537A site mutant protein (Figure 3).

Confirmation of Phosphorylation Sites

These mapped sites were confirmed using site-directed mutagenesis of the C-terminal GST fusion protein. Ser537 and Ser447 of the Kv4.2-GST fusion protein were mutated to an alanine to block phosphorylation. PKC incubation *in vitro* with the mutant C-terminal construct in which both Ser537 and Ser447 were mutated to an alanine (Ser447A, Ser537A, AA) showed no 32 P incorporation (Figure 3A), suggesting that these sites were indeed the only sites within the C-terminal that were phosphorylated by PKC (Figure 3B). Furthermore, mutation of the individual sites (Ser447A alone or Ser537A alone) showed that both sites were phosphorylated by PKC and phosphorylation of each site was sufficient to mimic phosphorylation levels of the WT construct. Interestingly, each site was phosphorylated equally well in the absence of phosphorylation at the other site. These data provide additional support that the C-terminal is indeed phosphorylated at both Ser447 and Ser537 *in vitro*.

Generation of phospho-site specific Kv4.2 antibodies

We generated site-selective phospho-specific antibodies to determine if PKC phosphorylates the native Kv4.2 channel in the hippocampus at the sites that were identified. Two synthetic phosphorylated peptides were produced, corresponding to amino acids $_{441}$ ANAYMQSKRNGLLC $_{454}$ and $_{531}$ SRRHKKSFRIPNAC $_{543}$ with phosphate groups attached to the appropriate serine residues (Ser447 and Ser537, respectively). Antisera against

the two peptides were generated in separate rabbits. After the terminal bleed, the antisera were affinity-purified using columns with the appropriate peptide. Antibodies were first screened for immuno-reactivity to the synthetic peptides (phosphorylated and unphosphorylated) that were coupled to ovalbumin for the western blotting. Both the PKC-A antibody (Ser447; Figure 4A — left) and the PKC-B antibody (Ser537; Figure 4B — left) were specific for the phosphorylated peptide, as there was no recognition of the unphosphorylated peptide. In addition, each antibody was specific for the peptide for which it was made, and there was no cross-reactivity with the peptide generated for the other site (data not shown). The antibody was tested against the C-terminal fusion protein. While the PKC-A antibody was not phospho-specific for the phosphorylated fusion protein (Figure 4A — right), the PKC-B antibody was indeed specific for the C-terminal fusion protein only after phosphorylation by PKC, indicating that the PKC-B antibody was phospho-specific (Figure 4B — right).

The PKC-B antibody was further tested in the hippocampus to determine if it recognized the full-length phosphorylated Kv4.2 protein and to determine if Kv4.2 is phosphorylated at Ser537 in the hippocampus. Rat hippocampal slices were treated with PDA (10 μ M) to stimulate PKC activation or vehicle (control) and then the membranes were extracted for Western blotting. The PKC-B antibody recognized a band at 69 kDa, suggesting basal phosphorylation of Kv4.2 at that site (Figure 4C; Control). In addition, the immunoreactivity of the PKC-B antibody increased in response to PDA application ($+96 \pm 31\%$ over vehicle DMSO, $p < 0.05$, $n = 4-6$, Figure 4C lower), suggesting increased phosphorylation at Ser537 within Kv4.2 sequence in response to PKC activation.

Functional characterization of PKC sites within Kv4.2

PKC activation causes a reduction in I_A in the dendrites of hippocampal pyramidal neurons [11] and dorsal horn neurons [9], and Kv4.2 is the major pore-forming subunit of I_A in these neurons [5,7]. Furthermore, activation of PKC with phorbol esters decreases the amplitude of Kv4.2 and Kv4.3 currents in *Xenopus* oocytes [23]. This could be due to direct PKC phosphorylation of the channel at the sites we have mapped. In order to evaluate the functional effects of the PKC phosphorylation sites within Kv4.2, we generated alanine (A - to block phosphorylation) and aspartate (D - to mimic phosphorylation) mutants of the phosphorylation sites. We made single site mutants (Ser447Ala or Asp or Ser537Ala or Asp) and mutants where both sites were mutated to alanines (AA mutant) or aspartates (DD mutant). These mutant channel constructs and the WT construct were transfected into the COS7 expression system to compare expression and localization of the channel protein. Immunohistochemistry of COS7 cells expressing the various mutant channel constructs revealed that mutation of Ser447 to Ala or Asp or Ser537 to Ala or Asp or both sites to an Ala (Ser447A/Ser537A, AA mutant) or Asp (Ser447D/Ser537D, DD mutant) had no significant effect on channel protein expression (data not shown). However, in order to more specifically study changes in surface localization, we evaluated surface expression of the WT and double AA mutant channels using surface biotinylation (see methods). We observed that there was a significant increase in the surface expression of the AA mutant compared to the WT ($+61 \pm 25\%$ over WT, $p = 0.028$, Mann-Whitney test, $n=4$; Figure 5A). These data suggest that basal phosphorylation of the channel at the Ser447 and Ser537 sites reduces channel surface expression. This reduction in channel surface expression provides a possible mechanism for the reduction in current in response to activation of PKC previously observed [23].

Next, we evaluated the effects of mutation of both of these sites (AA or DD mutant channel) on the kinetics of the Kv4.2 current. Recordings were conducted in oocytes expressing the WT and mutant (AA and DD) channels with KChIP3. There was no significant difference in the kinetics of the WT channel control for AA and DD mutants, therefore, the WT data were combined. Figure 5B shows typical current responses to voltage depolarizations from the WT

channel and AA and DD mutant channels. Figure 5C is the summary I-V curve for the WT and mutant channels. Consistent with the increase in surface expression of the AA mutant channel, the average peak current was significantly greater in the AA mutant channel recordings compared to WT and DD mutant channels ($p < 0.001$; Repeated Measures One Way Anova with post-hoc Tukey's). Furthermore, no significant differences in the half-activation (see table 1 — $F(2,39) = 0.31$; $p = 0.74$) or half-inactivation voltage (see table 1 — $F(2,27) = 0.28$; $p = 0.76$) were observed between WT and mutant (AA or DD) channels. Together, these data suggest that surface expression of the AA mutant is enhanced, but the kinetics of the channel is not modulated in the mutant channels.

PKC phosphorylation of Kv4.2 increases ERK phosphorylation efficacy *in vitro*

Interestingly, the Ser537 PKC phosphorylation site lies within a putative ERK docking domain in the Kv4.2 C-terminal sequence (Figure 6A), which consists of a series of basic amino acid residues [36] corresponding to amino acid residues 532-540 in the Kv4.2 sequence. Because the Ser537 site is localized within this domain, we hypothesized that PKC phosphorylation of the Kv4.2 channel could modulate ERK phosphorylation of the channel. To test the possibility we evaluated the interaction of PKC and ERK phosphorylation of the Kv4.2 C-terminal fusion protein construct *in vitro*. The C-terminal GST fusion protein was incubated with activated PKC for 15 minutes, and subsequently incubated with activated ERK to determine the effect of prior PKC phosphorylation of the C-terminal on ERK phosphorylation. Prior phosphorylation of the C-terminal GST fusion protein by PKC caused a significant increase in phosphorylation of the Kv4.2 C-terminal by ERK ($157 \pm 47\%$ of PKC/ERK phosphorylation vs $100 \pm 45\%$ of ERK phosphorylation alone, $p < 0.05$, Figure 6A). This effect was blocked in the Ser537A mutant ($157 \pm 47\%$ vs. $72 \pm 28\%$, $p < 0.05$, Figure 6A) and the AA mutant C-terminal construct ($157 \pm 47\%$ vs. $42 \pm 12\%$, $p < 0.01$, Figure 6A). These data suggest that PKC phosphorylation of the C-terminus of Kv4.2 facilitates ERK phosphorylation of this protein and that phosphorylation of the Ser537 site is necessary for this effect.

We have previously shown that ERK phosphorylates the Kv4.2 C-terminal at 3 amino acid residues, Thr602, Thr607 and Ser616 [26] and that there are functional effects of phosphorylation of these sites [24]. In addition, phospho-specific antibodies for the individual ERK phosphorylation sites were generated. These antibodies were used to determine whether PKC activation affects ERK phosphorylation at one, two or all three of these sites within the Kv4.2 C-terminal GST fusion protein. We found that there was a significant increase in ERK phosphorylation at the Thr602 and Thr607 sites, but not at Ser616 in response to prior phosphorylation of the GST fusion protein by PKC (Figure 6B). Densitometry showed that phospho-specific immunoreactivity at the Thr602 [$+84 \pm 46\%$ over WT control (PKC-, ERK +), $p < 0.05$, $n=3$] and the Thr607 [$+68 \pm 24\%$ over WT control (PKC-, ERK+), $p < 0.05$, $n=3$] sites significantly increased, while phospho-specific immunoreactivity against the Ser616 site was unaffected (Figure 6B). These data suggest that PKC phosphorylation of the Kv4.2 C-terminus enhances phosphorylation at two of the ERK phosphorylation sites, Thr602 and Thr607 in the Kv4.2 C-terminal.

DISCUSSION

In this study we showed that the C-terminal of Kv4.2 is phosphorylated by PKC at two sites, Ser447 and Ser537. Using a phospho-selective antibody against the Ser537 PKC site within Kv4.2 we determined an increase in Kv4.2 phosphorylation at this site following PKC pathway activation in hippocampal slices, indicating that PKC couples to Kv4.2 phosphorylation in a system where it is natively expressed. Additional functional data were evident in the COS7 expression system using phospho-site mutants. These studies suggested that PKC phosphorylation of Kv4.2 regulates surface expression of the channel. These studies showed

that when the PKC sites within Kv4.2 were mutated to alanines to block phosphorylation there was a significant increase in surface expression of Kv4.2 compared to wildtype channels. In addition, PKC phosphorylation of Kv4.2 enhanced ERK phosphorylation of the channel *in vitro*, suggesting the possibility that Kv4.2 is a locus for PKC and ERK cross-talk.

Mimicking or blocking phosphorylation of Kv4.2 at the PKC sites (Ser447 and Ser537) did not modulate the kinetics of the Kv4.2-mediated current, as demonstrated by electrophysiological recordings of the DD and AA Kv4.2 channel mutants. Specifically, there were no differences in the half-activation and -inactivation voltages between the WT and double mutant channels (AA and DD). However, as noted above we demonstrated that surface expression of the AA mutant increased as indicated by an enhanced peak current in oocytes expressing the AA mutant channel. This suggests that basal phosphorylation of the channel by PKC (or ERK/MAPK) reduces trafficking or maintenance of the channel in the membrane.

We found that prior phosphorylation of the Kv4.2 C-terminal by PKC caused an increase in ERK phosphorylation at two of the three ERK/MAPK sites, Thr602, Thr607, but not Ser616 [26]. This is interesting in the context of our previously reported results which showed that phosphorylation at 602 and 607 sites had an overall inhibitory effect on the current, which mimicked the effect of ERK/MAPK activation seen in the dendrites of CA1 pyramidal neurons [11], whereas phosphorylation at Ser616 caused an opposite effect [24]. The Thr607Asp (T607D) mutation mimicked the effect of ERK seen in neurons, which includes a right shift in the activation curve and an overall decrease in current. This suggests that at least the Thr607 site is the relevant site phosphorylated in response to PKC activation in neurons [11]. Moreover, the Thr607 site effect is dominant when all 3 sites are phosphorylated. Phosphorylation of the C-terminal by PKC prior to ERK may augment phosphorylation at the Thr607 site, increasing the balance of phosphorylation at that site over the Ser616 site to ensure a decrease in current. This does not preclude phosphorylation at Ser616 in response to different physiological stimulation.

Previous data suggest that filamin is a scaffold protein that links Kv4.2 and the actin cytoskeleton. Furthermore, mutation of amino acids 601-604 region in the Kv4.2 C-terminal to alanines completely abolished the interaction of Kv4.2 and filamin in a yeast two-hybrid assay [37]. This suggests that this region is important for the interaction of Kv4.2 with filamin and possibly for Kv4.2 localization and stabilization in the membrane. Phosphorylation of the ERK amino acid residues in this region on the Kv4.2 C-terminal (Thr602 and Thr607) may alter the interaction of filamin and Kv4.2 such that Kv4.2 becomes unstable at the surface membrane. Enhanced phosphorylation of these sites mediated by phosphorylation by PKC may play a role in 'tagging' Kv4.2 subunits for internalization. Further studies are necessary to determine the exact role of the interaction of PKC and ERK phosphorylation sites and the interaction with filamin.

Kv4.2 is the primary subunit that contributes to I_A in the dendrites of CA1 pyramidal cells, and the threshold for induction of synaptic plasticity in the CA1 pyramidal cell dendrites is lower in the Kv4.2 KO animals as compared to control [5]. This suggests that a decrease in I_A can augment induction of synaptic plasticity. I_A is regulated by activation of PKC, and the effect is blocked by the ERK/MAPK inhibitor U0126 [11]. This suggests the possibility that direct phosphorylation of the Kv4.2 subunit by both PKC and ERK/MAPK may modulate the current. Indeed, our phospho-site specific antibody demonstrates that Kv4.2 is phosphorylated at Ser537 in the hippocampus in response to PKC activation.

We have previously shown that the Kv4.2 current is modulated by direct phosphorylation of Kv4.2 by ERK/MAPK [24]. These data provide further support for interplay in the regulation of the A-type currents by various kinases and provide another possibility of Kv4.2 as a site of

signal integration in neurons and cardiac myocytes. The modulation of ERK phosphorylation of the channel by PKC phosphorylation may be a mechanism of fine-tuning the current in response to a physiological stimulus. Moreover, a greater decrease in current mediated by both direct phosphorylation of the channel by PKC and ERK/MAPK may be a mechanism to dynamically regulate current amplitude and induction of synaptic plasticity.

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Abbreviations used

PKC, protein kinase C; ERK, extracellular signal-regulated kinases; MAPK, Mitogen-activated protein kinase; GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography.

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B

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1  MAAGVAAWLPFARAAA IGWMPVASG PMPAPPRQERKRTQDALI VLVNVS T
51 RFQTWQDTLERYPDTLLGS SERDFFYHPETQQYFFDRDPD IFRHILNFYR
101 TGKLHYPRHECISAYDEELAFFGLI PEI IGDCCYEEYKDRRRENAERLQD
151 DADTDNTGESALP TMTARQRVWRAFENPHT STMALVFYYVTGFFIAVSVI
201 ANVVE TVPCGSSPGHI KEL PCGERYAVAFFCLDTACVMIFTVEYLLRLAA
251 APSRYRFVRSVMSIIDVVA ILPHYI GLVMTDNEDVSGAFVTLRVFRVFR I
301 FKFSRHSQGLRILGYTLKSCASELGFLFSLTMAIII FATVMFYAEKGSS
351 ASKFTSI PAAFWYTIVTMT TLGYGDMVPKTI AGKIFG SICSLSGVLVIAL
401 PVPVIVSNFSRIYHQNQ RADKRR AQKKARLARIRAAKSGS ANAYMQ SKRN
451 GLLSNQLQSS EDEPAFVSKSGS SFETQHHL LHCLEKTTNHEFVDEQVFE
501 ESCMEVATVNRPS SHSPSLSSQQGV TSTCC SRRHKK SFRIPNANVSG SHR
551 GSVQELSTIQ IRCVERTPL SNSRSS SLNAKMEE CVKLNCEQPYVTTAI ISI
601 PTPPVT TPEGDDRPE SPEYSGGNI VRVSAL

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Figure 1. Candidate PKC consensus sites within the Kv4.2 channel subunit

(A) Schematic diagram of the Kv4.2 subunit with a putative tertiary structure of six transmembrane regions, two intracellular loops and cytoplasmic N-terminal and C-terminal domains. The asterisks denote ten candidate PKC phosphorylation sites in the intracellular domains predicted by NetPhosK program (<http://www.cbs.dtu.dk/services/NetPhosK/>). (B) The full-length Kv4.2 subunit consists of 630 amino acid residues. The amino acid sequence of the Kv4.2 channel subunit is shown with the six transmembrane spanning domains underlined. We inspected the primary amino acid sequence of the intracellular domains of the Kv4.2 subunit for known PKC consensus sequences as these regions may be accessible to

intracellular PKC. Four candidate PKC sites on the N-terminal and six candidate sites on the C-terminal are shown in bold and italic.

Figure 2. PKC phosphorylates the Kv4.2 C-terminal but not the N-terminal cytoplasmic domains *in vitro*

GST fusion protein constructs of the Kv4.2 N-terminal and C-terminal regions were generated and incubated with the PKC catalytic subunit and [γ -³²P] ATP *in vitro*. Reaction products were separated using SDS-PAGE and visualized by Coomassie Blue staining (Coomassie). ³²P incorporation was evaluated with autoradiography (Autorad). For phospho-peptide mapping the GST C-terminal construct was phosphorylated *in vitro* as described above and an in-gel digest of the excised band corresponding to the GST C-terminal construct was performed. This was followed by HPLC and amino acid sequencing of the radiolabelled HPLC fractions. (A) Coomassie Blue-stained gels show bands at 54 kDa (GST Kv4.2 CT) and at 40 kDa (GST

Kv4.2 NT) representing the C-terminal and N-terminal constructs, respectively (Coomassie). The autoradiogram shows ^{32}P incorporation into the GST Kv4.2 C-terminal construct only (Autorad) after 30 minutes incubation, suggesting that only the Kv4.2 C-terminal domain is a PKC substrate. Note that the PKC alone lane (all reaction components except without peptide substrates) showed no ^{32}P incorporation. A time course of phosphorylation of the GST Kv4.2 C-terminal fusion proteins by PKC was performed (right panel). The average normalized optical density of the autoradiograph is shown. By 40 minutes, the phosphorylation of C-terminal domains reached saturation levels. Data are expressed as mean $\pm$ S.E. Three independent experiments were performed. **(B)** The HPLC plot is shown (left panel) with the x-axis representing the time (minutes) of fraction collection and the y-axis representing the absorbance units at 214 nm. The radioactivity plot is shown (right panel) with the x-axis representing HPLC fraction number and the y-axis representing counts per minute for beta emission. The radioactivity plot illustrates peaks in radioactivity that correspond to HPLC fractions 17-19 (black arrow in HPLC trace) and 67-68, 69-70, 71-72, 73-75 (open arrows). **(C)** HPLC fractions 17-19 and 67-75 were sequenced using Edman degradation and the radioactivity released with each sequencing cycle was measured. The sequence of the phosphopeptide in HPLC fractions 17-19 corresponded to amino acids 438-452 within the C-terminal (left panel). There was a peak in radioactivity in sequence cycle number 10 indicating that Ser447 was the phosphorylated amino acid. The phosphorylated amino acid, Ser447, is labeled with a circled P in the peptide sequence. The sequence of the phosphopeptide in fractions 67-75 corresponded to amino acids 536-560 within Kv4.2 (right panel). There was a peak in sequence cycle number 2 indicating that Ser537 was the phosphorylated amino acid. The phosphorylated amino acid residue, Ser537, is labeled with a circled P in the peptide sequence.

Figure 3. Mutation of Ser447 and Ser537 to alanine blocks PKC phosphorylation within the K4.2 C-terminal domain *in vitro*

The Kv4.2 C-terminal cytoplasmic domain contains two predicted PKC consensus sites (Ser447 and Ser537). We used serine to alanine mutants (Ser447Ala, Ser537Ala and double mutation, Ser447Ala/Ser537Ala (AA)) within the GST Kv4.2 C-terminal fusion protein to evaluate PKC phosphorylation *in vitro*. (A) The top panel shows a Coomassie Blue-stained gel (Coomassie) with bands at 54 kDa representing the Kv4.2 C-terminal WT and mutant constructs. The lower panel represents an autoradiogram (Autorad) of the Coomassie Blue-stained gel, which shows ^{32}P incorporation into the WT, Ser447A and Ser537A mutants but not the AA mutant constructs. (B) The bar graph shows the densitometry of ^{32}P incorporation

into the WT and mutant GST fusion protein as a percentage of the WT. The single site mutants did not affect phosphorylation while mutation of both sites to alanine (AA) blocked PKC phosphorylation significantly compared to WT control (*, $p < 0.05$). Data are expressed as mean $\pm$ S.E. One-way ANOVA with post-hoc analysis was used for comparison, $n = 3$.

Figure 4. Generation and characterization of the Kv4.2 phospho-specific antibodies for the Ser447 and Ser537 site

To measure PKC phosphorylation of the Kv4.2 subunits, phospho-selective antisera were generated for phospho-Ser447 and phospho-Ser537 and the affinity-purified antibody was screened by Western blotting of the unphosphorylated and phosphorylated synthetic peptides and the GST C-terminal Kv4.2 construct. (A) The peptide sequence (PKC-A peptide, residues 441-454) containing the phosphorylated Ser447 site labeled with a circled P is shown. The 447 site antibody showed specific immunoreactivity for the phosphorylated peptide (left), and that immunoreactivity was blocked by antibody preincubation with the antigen (Ab + Ag, middle). The antibody also recognized the phosphorylated and unphosphorylated GST fusion protein,

suggesting that the antibody was not phospho-specific for the entire protein (right). **(B)** The peptide sequence (PKC-B peptide, residues 531-543) containing the phosphorylated Ser537 site labeled with a circled P used for antibody generation is shown. The Ser537 site antibody also showed specific immunoreactivity for the phosphorylated peptide (left) that was blocked by the antigen (middle). This antibody specifically recognized only the phosphorylated GST fusion protein (right) suggesting it was a phospho-specific antibody. **(C)** The phospho-specific antibody against the Ser537 site was further tested in the hippocampus to determine if it recognized the full-length phosphorylated protein and to determine if indeed Kv4.2 is phosphorylated at Ser537 in the hippocampus. For these studies acute hippocampal slices were prepared and incubated with vehicle (control) or phorbol diacetate (PDA; 10 μ M) for 10 min *in vitro*. Reactions were stopped by placing slices on dry ice and hippocampal membranes were prepared. Hippocampal membrane preparations from vehicle and PDA-treated slices were probed with the Ser537 phospho-specific antibody (upper panel). A bar graph of the densitometry data (lower panel) show that there is a significant increase in immunoreactivity with the phospho-Ser537 antibody in response to PDA application to hippocampal slices ($n = 4 - 6$, * $p < 0.05$). Data are expressed as mean $\pm$ S.E. Student's unpaired t test was used for analysis.

Figure 5. Blockade of phosphorylation at the PKC sites increases the surface expression of Kv4.2 channels in COS-7 cells

Double PKC site mutant constructs of the full-length Kv4.2 subunit were generated with alanine mutations at Ser447 and Ser537 (AA) or aspartate mutations at these sites (DD) and compared to wildtype full-length Kv4.2 constructs (WT) expressed in COS-7 cells (AA and WT coexpressed with KChIP3) or oocytes (AA, DD, and WT coexpressed with KChIP3). (A) To evaluate whether phosphorylation at the PKC sites affects the surface expression of the Kv4.2 channel subunits, surface biotinylation was performed in COS-7 cells expressing the WT or AA Kv4.2 constructs. Total lysate (upper blot) and biotinylated surface (lower blot) proteins of the co-transfected COS-7 cells probed with Kv4.2 antibody revealed a 69 kDa bands

representing Kv4.2 WT and AA channel proteins that were unchanged in total lysate. However, there was a significant increase in surface expression of the AA mutant construct compared to WT (densitometry of the surface WT and AA shown in the lower panel; *, $p < 0.05$, $n = 4$). Surface expression was normalized to Kv4.2 expression levels in total lysate. Data are expressed as mean $\pm$ S.E., unpaired Student's t test was used for analysis. Untransfected COS-7 cells are shown as a negative control and as expected the Kv4.2 antibody did not recognize any protein bands in untransfected cells (upper panels). **(B)** An example recording of current from WT, AA and DD mutant channels coexpressed with KChIP3 in oocytes is shown. The currents recorded in response to depolarizations from -60 to +40 mV in 20 mV increments are shown. **(C)** The current-voltage plot of peak current (mean $\pm$ SE) recorded from oocytes expressing WT + KChIP3 ($n = 13$; ■), AA + KChIP3 ($n = 8$; ▲) and DD + KChIP3 ($n = 5$; ○) is shown. Consistent with the increase in surface expression, the mean peak current recorded from AA + KChIP3 was significantly greater than WT or DD + KChIP3 ($p < 0.001$, One Way Repeated Measures Anova with post-hoc Tukey's).

Figure 6. PKC phosphorylation of the Kv4.2 C-terminal augments ERK phosphorylation of the Kv4.2 C-terminal *in vitro*

(A) Inspection of Kv4.2 C-terminal amino acid sequence reveals that a possible ERK docking domain (bold italic residues underlined, in Kv4.2 residues 532-540) exists on the C-terminal and the Ser537 site is within that sequence. ERK phosphorylation sites (Thr602, Thr607 and Ser616) are shown in bold and italic in the sequence (upper panel). To evaluate potential interaction of the PKC sites with ERK phosphorylation of Kv4.2, the GST fusion protein constructs of the Kv4.2 C-terminal domain representing wildtype (WT), alanine mutation at Ser537 (S537A), and alanine mutation at Ser447 and Ser537 (AA) were incubated first with the catalytic domain of PKC followed by incubation with activated ERK *in vitro*. Prior PKC

phosphorylation of the WT Kv4.2 C-terminal fusion protein significantly augmented ERK phosphorylation of this construct *in vitro* (*, $p < 0.05$, $n = 3$; lower panel). This effect was blocked by the Ser537A (*, $p < 0.05$, $n = 3$) and the AA (**, $p < 0.01$, $n = 3$) mutant fusion proteins. Data are expressed as mean $\pm$ S.E. One-way ANOVA with post-hoc analysis was used for comparison. **(B)** The WT GST C-terminal Kv4.2 construct was incubated without (PKC -) and with (PKC +) PKC, followed by incubation with ERK (ERK +). Western blotting was performed with the ERK single phospho-site specific antibodies against Thr602, Thr607, and Ser616 within the Kv4.2 C-terminal domain (upper panel). Densitometry of Western blotting (lower panel) demonstrated that phosphorylation of the C-terminal GST fusion protein by PKC increased immunoreactivity at the ERK Thr602 phosphorylation site (*, $p < 0.05$) and at the Thr607 site (*, $p < 0.05$), while the Ser616 site was unaffected ($p > 0.05$) as measured by the ERK phospho-site specific Kv4.2 antibodies. For these studies the effect the wildtype PKC +, ERK + condition was normalized to that seen for the wildtype PKC -, ERK + (wildtype control) condition. Data are expressed as mean $\pm$ S.E., unpaired Student's *t* test was used for analysis, $n=3$.

Table 1

Half-activation and -inactivation voltage of WT and mutant channels

	WT + KChIP3	AA + KChIP3	DD + KChIP3
V $\frac{1}{2}$ Activation	-21 $\pm$ 1.8 mV, n = 26	-22.2 $\pm$ 1.5 mV, n = 9	-19 $\pm$ 2.3 mV, n = 7
V $\frac{1}{2}$ Inactivation	-63.5 $\pm$ 2.9 mV, n = 12	-64.9 $\pm$ 1.2 mV, n = 8	-61.6 $\pm$ 1.5 mV, n = 10