

Calcium–Calmodulin-Dependent Kinase II Modulates Kv4.2 Channel Expression and Upregulates Neuronal A-Type Potassium Currents

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Calcium–calmodulin-dependent kinase II (CaMKII) has a long history of involvement in synaptic plasticity, yet little focus has been given to potassium channels as CaMKII targets despite their importance in repolarizing EPSPs and action potentials and regulating neuronal membrane excitability. We now show that Kv4.2 acts as a substrate for CaMKII *in vitro* and have identified CaMKII phosphorylation sites as Ser438 and Ser459. To test whether CaMKII phosphorylation of Kv4.2 affects channel biophysics, we expressed wild-type or mutant Kv4.2 and the K⁺ channel interacting protein, KChIP3, with or without a constitutively active form of CaMKII in *Xenopus* oocytes and measured the voltage dependence of activation and inactivation in each of these conditions. CaMKII phosphorylation had no effect on channel biophysical properties. However, we found that levels of Kv4.2 protein are increased with CaMKII phosphorylation in transfected COS cells, an effect attributable to direct channel phosphorylation based on site-directed mutagenesis studies. We also obtained corroborating physiological data showing increased surface A-type channel expression as revealed by increases in peak K⁺ current amplitudes with CaMKII phosphorylation. Furthermore, endogenous A-currents in hippocampal pyramidal neurons were increased in amplitude after introduction of constitutively active CaMKII, which results in a decrease in neuronal excitability in response to current injections. Thus CaMKII can directly modulate neuronal excitability by increasing cell-surface expression of A-type K⁺ channels.

Key words: kinase; hippocampus; phosphorylation; potassium channel; synaptic plasticity; memory

Introduction

Calcium–calmodulin-dependent kinase type II (CaMKII) is an abundant neuronal protein that has received much attention for its key role in long-term potentiation (LTP), a cellular substrate for learning. A feature of CaMKII that originally made it an attractive candidate to mediate the synaptic processes underlying learning is that when CaMKII is activated by a Ca²⁺–calmodulin complex, it can be autophosphorylated at Thr286, and this event renders the kinase autonomously active and less sensitive to additional increases in calcium (Hanson et al., 1989). Thus, CaMKII can translate a transient event (influx of Ca²⁺) into a long-lasting change independent of that transient event (autophosphorylation and enzyme activation).

Moreover, CaMKII has been shown to be necessary and sufficient for LTP induction and necessary for various learning para-

digms (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992a,b; Pettit et al., 1994; Lledo et al., 1995; Wang and Kelly, 1995; Giese et al., 1998; Hinds et al., 1998). Studies assessing the downstream targets of CaMKII have justifiably focused on glutamate receptor subunits, because they are principally responsible for excitatory neurotransmission in the CNS and play an important role in LTP expression. There are many other potential substrates for CaMKII phosphorylation in the dendritic membrane, however.

In particular, A-type potassium channels, specifically the Kv4.2 subunit, are expressed at high density on dendrites of hippocampal pyramidal neurons (Sheng et al., 1992; Maletic-Savatic et al., 1995; Hoffman et al., 1997). The Shal family of channels, of which Kv4.2 is a member, comprises four α subunit monomers that tetramerize to form the channel (Choe et al., 1999). The Kv4.2 α subunit can be modulated by interacting partners including Kv β subunits, K⁺ channel interacting proteins (KChIPs), and dipeptidyl peptidase IV-related protein (Nakahira et al., 1996; Perez-Garcia et al., 1999; An et al., 2000; Holmqvist et al., 2002; Nadal et al., 2003). Modulation of Kv4.2 is not limited to protein–protein interactions; Kv4.2 has been shown to be a bona fide substrate for phosphorylation by extracellular signal-regulated kinase (ERK)–mitogen-activated protein kinase (MAPK) and PKA (Adams et al., 2000; Anderson et al., 2000). These post-translational modifications have been shown to change both current density and biophysical properties of A-type

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potassium currents thought to be mediated by Kv4.2 (Hoffman and Johnston, 1998; Yuan et al., 2002). This is significant, because A-type potassium channels are important for regulating the excitability of neurons, and furthermore, dendritic A-type potassium channels have been implicated in both the induction and expression of LTP (Watanabe et al., 2002).

Because CaMKII is so crucial to memory formation and because Kv4.2 seems to be a viable phosphorylation target based on its function and localization, this study sought to determine whether Kv4.2 acts as a substrate for CaMKII, and if so, what the functional consequence of such phosphorylation might be. Here we present evidence that CaMKII phosphorylates the C terminus of Kv4.2 at Ser438 and Ser459. These phosphorylation events on the native channel in the cell result in an increase in total cellular Kv4.2 assessed biochemically. We obtained corroborating physiological data showing that active CaMKII or CaMKII site aspartate point mutants increased surface A-type channel expression as revealed by increases in peak K^+ current amplitudes. The change in peak current amplitude was unaccompanied by changes in the biophysical parameters of Kv4.2-mediated A-current. Finally, we demonstrated that constitutively active CaMKII activity can potentiate native A-currents in hippocampal neurons.

Materials and Methods

Protein expression and purification. The Kv4.2 N-terminal and C-terminal domain proteins were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins using methods modified from Hakes and Dixon (1992) and Anderson et al. (2000). Plasmids containing the N-terminal (amino acid residues 1–133) and C-terminal (amino acid residues 411–630) domain cDNAs were constructed using the GST-fusion vector pGEX-KN (Hakes and Dixon, 1992). A single colony of BL21(DE3)-pLysS cells transformed with the protein plasmid was grown in Luria broth [LB (170 mM NaCl, pH 7.5, 1% tryptone, and 0.5% yeast extract)] containing 20 μ g/ml carbenicillin and then used to seed a 500 ml culture. After growing to an optical density of 0.6–0.8 (A_{600}), the culture was centrifuged for 15 min at $1000 \times g$ (and 4°C) using a Beckman Centrifuge Model J2-21M (Beckman Instruments, Fullerton, CA). The cell pellet was resuspended in 500 ml of LB with carbenicillin. The bacteria was induced by incubation at room temperature (RT) with 200 μ M isopropyl β -D-thiogalactopyranoside for 4 hr and harvested by centrifugation.

The cells were resuspended and incubated in Tris buffer 1 (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mg/ml pepstatin, 10 μ g/ml leupeptin, and 100 μ M PMSF) containing 10 mM β -mercaptoethanol and 100 μ g/ml lysozyme (Sigma, St. Louis, MO) for 15 min at 30°C. After solubilization with 1.5% N-laurylsarcosine, the lysate was incubated with 20 μ g/ml DNase I (Boehringer Mannheim, Indianapolis, IN) and 10 mM MgCl₂. The lysate was then centrifuged for 15 min at $1000 \times g$ (and 4°C) (RT 6000B; Sorvall, Newtown, CT) and adjusted to a 2% Triton X-100 concentration.

The GST-fusion protein was purified using glutathione affinity absorption. Glutathione agarose beads were washed, resuspended in Tris buffer 1, and then incubated with the lysate for 1 hr at 4°C. The beads were washed three times with Tris buffer 1 by repeated centrifugation ($100 \times g$ for 5 min at 4°C; Sorvall). After the final wash, the bead preparation was resuspended in Tris buffer 2 (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM Na₂P₂O₇, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin).

Phosphorylation of Kv4.2 N-terminal and C-terminal GST fusion proteins. Kv4.2 N-terminal or C-terminal GST-fusion proteins were incubated at 37°C in reaction mixtures (25 μ l) containing 70 ng of the catalytic subunit of CaMKII, Tris buffer 2, and ATP mix 1 (100 μ M ATP, 100 mM MgCl₂, and 10 μ Ci of [γ -³²P]ATP). Reactions were stopped by boiling for 5 min with sample buffer (30 mM Tris-HCl, pH 6.8, 200 mM DTT, 40% glycerol, 8% SDS, and 0.04 mg/ml bromophenol blue). The GST-fusion proteins were separated by SDS-PAGE (10%) and visualized

by Coomassie blue staining. Phosphopeptides were identified by autoradiography. As a control, parallel reactions were performed for GST with and without the kinases and ATP mix 1. A time course of kinase phosphorylation was also performed to ensure a maximal extent of phosphorylation.

Phosphopeptide mapping. Phosphorylation reactions were performed as described above with some modifications; a preparative scale (reaction volume of 300–400 μ l) of the reaction was used. The incubation period was adjusted, based on the time course of kinase phosphorylation of the fusion proteins. The phosphorylated GST-fusion protein was separated by SDS-PAGE (10%). After Coomassie blue staining of the gels, the bands corresponding to the N-terminal or C-terminal fusion proteins were excised, and an in-gel digestion with trypsin or Lys-C was performed as described previously, with minor modifications (Frangioni and Neel, 1993; Anderson et al., 2000). After extraction from the gel, the peptides were separated using reverse-phase HPLC with absorption monitoring at 214, 254, and 280 nm. Counts per minute in each HPLC fraction were measured as Cherenkov radiation. Phosphopeptides identified as HPLC fractions containing high radioactivity were applied to Sequelon arylamine membranes (Millipore, Bedford, MA) essentially as described by the manufacturer. After drying, the membrane was rinsed two times sequentially with 10 ml of methanol, with water, and finally with 5 ml of 10% trifluoroacetic acid and 50% acetonitrile in water. The membrane was air-dried, cut into pieces with a scalpel blade, inserted in a BLOTT cartridge, and sequenced in an Applied Biosystems (Foster City, CA) model 477A Protein Sequencer with an in-line 120A PTH-Analyzer using optimized cycles. Instead of butyl chloride, 90% methanol containing phosphoric acid (15 μ l/100 ml) was used to extract the cleaved amino acids. After conversion, 50% of the sample was transferred to the HPLC for PTH-amino acid identification, and the other 50% was collected in the instrument fraction collector for determination of radioactivity by scintillation counting.

DNA preparation and site-directed mutagenesis. The original Kv4.2 cDNA was kindly provided by Dr. Paul Pfaffinger (Baylor College of Medicine, Houston, TX) and subsequently subcloned into a pCI:Neo vector (Promega, Madison, WI) containing a cytomegalovirus promoter. Point mutations were made using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers used included 5'-AGGATACGG-GCAGCCAAAGCAGGAAGTGCAAAATGCCTAC-3' for S438A, 5'-AGGATACGGGCAGCCAAAGATGGAAGTGCAAAATGCCTAC-3' for S438D, 5'-AGCAACCAACTACAAGCCTCGGAGGATGAACC-3' for S459A, and 5'-AGCAACCAACTACAAGACTCGGAGGATGAACC-3' for S459D. Mutations were confirmed by restriction enzyme digest and DNA sequencing (Seqwright, Houston, TX). In all cases, the entire Kv4.2 region was sequenced to determine whether any nonspecific mutations were introduced.

COS-7 and Chinese hamster ovary cell transfection and protein harvest. The FuGene 6 transfection reagent was used for COS-7 cell transfections with plasmid DNAs of wild-type or mutant Kv4.2, K^+ channel interacting proteins (KChIP3), and constitutively active CaMKII or green fluorescent protein (GFP) as a control (1:1:0.2 μ g ratio). COS-7 cells were grown on 35 mm plates to a 4×10^5 cell density. Each plate received 3 μ l of FuGene 6 reagent and 2 μ g of total DNA per the FuGene instruction manual. Eighteen to 30 hr after transfection, COS cells were scraped in 1 ml of homogenization buffer (HB) per dish (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA), containing 200 μ M phenylmethylsulfonyl fluoride, 4 mM *para*-nitrophenylphosphate, 1 mM sodium orthovanadate, and a 1:100 dilution of protease inhibitor mixture (P-8340; Sigma) [80 μ M aprotinin, 2.1 mM leupeptin, 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 3.6 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64]. These harvested cells were then centrifuged at $15,000 \times g$ (10,000 rpm in a Hermle Labnet Eppendorf tube centrifuge) at 4°C for 5 min. The supernatant was removed, and each cell pellet was resuspended in 300 μ l of HB followed by sonication [10 pulses at output level 1.5 on a Branson (Danbury, CT) Sonifier 250] to lyse cells. The cell lysate was centrifuged at 60,000 rpm ($100,000 \times g$) at 4°C for 20 min in a Beckman ultracentrifuge to isolate cell membranes. The resultant membrane pellet was suspended in 75 μ l of 10% SDS containing 100 mM DTT, 80 ng/ml microcystin, 200 μ M phenylmethylsulfonyl fluoride, a 1:100 dilution of protease inhibitor mixture (see above), and 25 μ l of 4 \times sample

buffer. Twenty-five microliters of this total 100 μ l were typically loaded onto an SDS-PAGE gel.

Surface biotinylation. We followed the protocol of Ehlers (2000). Briefly, monolayers of COS cells were rinsed with progressively cooler washes of PBS plus 1 mM MgCl₂ and 2.5 mM CaCl₂ (PBS 2+), followed by a 20 min incubation with 1.5 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in PBS 2+ at 4°C. Unreacted biotin was quenched by rinsing three times for 5 min with ice-cold 50 mM glycine in PBS 2+. Membrane proteins were harvested as described above except with a 5% rather than a 10% SDS concentration. This membrane fraction was then incubated with a 1:1 slurry of BSA-blocked neutravidin beads (Pierce) for 2 hr at room temperature. The beads were rinsed three times with HB (above) plus 1% Triton X-100 before proteins were eluted with 1× sample buffer.

Western blotting. Membrane proteins were loaded and run on a 7.5% acrylamide gel to resolve Kv4.2 and a 10% acrylamide gel to resolve ERK-MAPK. Gels were then blotted electrophoretically to Immobilon filter paper using a transfer tank maintained at 4°C, with typical parameters being an overnight transfer at a constant current of 250 mA (transfer buffer: 192 mM glycine, 25 mM Tris, pH 8.3). Immobilon filters were blocked for 1 hr at room temperature in Blotto (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 5% powdered milk, and 0.01% thimerosal). All antibody applications were done in Blotto. Primary antibody concentrations were as follows: 1:500 Kv4.2 polyclonal antibody from Chemicon (Temecula, CA), 1:2 Kv4.2 monoclonal antibody K57/41.1 from the Trimmer Laboratory (Shibata et al., 2003), and 1:3000 total ERK from Cell Signaling Technology (Beverly, MA).

Antibody detection. Immobilon filters were incubated sequentially with primary antibody and a biotin-labeled goat anti-rabbit IgG secondary antiserum (1:20,000) and then developed using enhanced chemiluminescence (Amersham Biosciences). Blots were washed extensively in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 after incubations with primary and secondary antibodies (typically, four washes of 10 min each).

Functional expression in *Xenopus oocytes*. Oocytes were harvested from the ovarian lobe of female *Xenopus laevis* frogs (Schrader et al., 2002). Briefly, the frog was anesthetized by submersion in 0.15% tricaine. The ovarian lobe was then surgically removed and placed in Ca²⁺-free OR2 solution (in mM: 82.5 NaCl, 2.5 KCl, 1 MgCl₂, and 5 HEPES). The frog was allowed to recover and placed back in the tank. The oocytes were digested in 1.6–2.4 mg/ml collagenase (Boehringer Mannheim) for several hours. Digested oocytes were then incubated in ND-96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES, pH 7.4) supplemented with pyruvic acid (2.5 mM) and gentamicinsulfate (50 mg/ml; Invitrogen, San Diego, CA). After ~24 hr, oocytes were injected with 16 ng of each DNA construct using a Nanoject microinjector (Drummond Scientific Co.) into the nucleus of stage V–stage VI oocytes. Currents were recorded after 2–3 d under two-electrode voltage-clamp using an Axoclamp 2A amplifier (Axon Instruments) at room temperature. Microelectrodes were pulled from filamented glass (1.5 × 0.86 mm; A-M Systems) filled with 3 M KCl. The current electrode had resistance of 0.3–0.5 M Ω , whereas the voltage electrode ranged from 0.3 to 1 M Ω . Currents were leak-subtracted on-line using P/4 leak subtraction. Data were digitized at 2 kHz and stored using a Digidata 1200. Current protocols used to obtain data include activation (hyperpolarization to –110 mV and then depolarization to +40 mV for 400–800 msec, repeated in –5 mV step intervals) and inactivation (depolarization to 0 mV and then hyperpolarization to –110 mV for 650 msec, changing this step by +5 mV intervals, then depolarization to 0 mV). The chamber was continuously perfused with ND-96 and with NaOH at a rate of 3–6 ml/min. Data were analyzed using Clampfit, Origin, and Prism programs. Peak currents were obtained, and conductance was determined using a reversal potential of –95 mV. Activation and inactivation curves were fit with a Boltzmann sigmoidal curve with the following equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / [1 + \exp([V_{1/2} - X]/\text{Slope})]$.

Hippocampal primary culture and transfection. Hippocampal cell cultures were prepared as described previously (Wu et al., 2001). Briefly, hippocampal CA1–CA3 regions were dissected from 0- to 3-d-old Sprague Dawley rats, dissociated by trypsin treatment followed by trituration with a siliconized Pasteur pipette, and then plated onto coverslips

coated with matrigel (1:50). Culture media consisted of minimal essential media (MEM; Invitrogen), 0.6% glucose, 0.1 gm/l bovine transferrin (Calbiochem, La Jolla, CA), 0.25 gm/l insulin (Sigma), 0.3 gm/l glutamine, 5–10% fetal calf serum (FCS; Sigma), 2% B-27 supplement (Invitrogen), and 2 μ M cytosine-D-arabino-furanoside (Sigma). Cultures were maintained at 37°C in a 95% air, 5% CO₂-humidified incubator.

The calcium phosphate transfection method (Xia et al., 1996) was used to transfect the neurons. Briefly, 3–7 d *in vitro* cell cultures were washed twice with fresh, serum-free medium (MEM plus 5 gm/l glucose) after saving original media. Thirty microliters of calcium phosphate precipitate–DNA mixtures were added to each coverslip, and cells were put back into the incubator for 30 min. After washing twice with serum-free medium, the cells were returned to the incubator after adding back the original medium.

Electrophysiology. A Zeiss (Oberkochen, Germany) Axioskop, fitted with a 40× water-immersion objective and differential interference contrast, was used to view cultured cells and neurons. Transfected cells and neurons expressing enhanced GFP (EGFP) were identified under a fluorescent microscope. Whole-cell voltage clamp and outside-out patch clamp were done in Chinese hamster ovary (CHO)-K1, COS-7, and cultured hippocampal pyramidal neurons using an Axopatch 200 amplifier. All experiments were performed at RT. For CHO and COS, the bath solution contained (in mM): 134 NaCl, 2 KCl, 5.6 NaOH, 1.8 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.4. The pipette solution contained (in mM): 140 KCl, 1 MgCl₂, 1 EGTA, 0.133 CaCl₂, a ~10 nM free Ca²⁺ concentration, and 10 HEPES, pH 7.4. The K⁺ current reversal potential measured under this condition was approximately –85 mV. The liquid junctional potential between pipette solution and bath solution was measured as –7 mV and was not corrected for all experiments.

For pyramidal neurons, the bath solution contained (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 2 CaCl₂, 1 MgCl₂, and 10 dextrose. The pipette solution contained (in mM): 120 K-gluconate, 20 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 Na₂ATP, 0.3 Tris-GTP, and 14 phosphocreatine, pH 7.25 (with KOH). To isolate K⁺ currents in pyramidal neurons, 1 μ M TTX and 2 mM MnCl₂ were added to the bath to block Na⁺ currents, Ca²⁺ currents, and Ca²⁺-activated K⁺ currents. To isolate the transient component (I_A), a prepulse protocol (a voltage step to –20 mV for 100 msec before the test pulse) was used, and the difference between the currents elicited with and without the prepulse was measured. Pipettes had resistances of between 2 and 5 M Ω . Whole-cell capacitance and series resistances were compensated to >80%, and in addition, series resistances were less than two times the tip resistance. Pulse generation and data acquisition were controlled with custom software written for the Igor Pro analysis environment. Leakage and capacitive currents were digitally subtracted on-line.

Whole-cell current-clamp recordings were made using an Axoclamp 2A (Axon Instruments) amplifier in “bridge” mode at room temperature and were analog-filtered at 3 kHz. Series resistances were in the range of 10–15 M Ω . Heteropodatoxin-3 (HpTx-3) (NPS Pharmaceuticals, Salt Lake City, UT) was dissolved in water and added to the external solution with 0.1% BSA.

Data analysis. Data were analyzed by using a self-written analysis program in Igor. Steady-state activation and steady-state inactivation curves were constructed and fitted with the normalized Boltzmann equation. Time course of the recovery from inactivation was fitted with single exponentials. The current inactivation kinetic was also determined by single exponential fitting.

Significance ($p < 0.05$) among multiple groups was determined by ANOVA. A paired or two-sample t test was used when comparisons were restricted to two means. Error bars represent SEM.

Results

CaMKII phosphorylates the C terminus of Kv4.2 *in vitro*

To determine whether Kv4.2 is phosphorylated by CaMKII, we expressed the cytoplasmic N and C termini of Kv4.2 as GST fusion proteins, purified them using affinity chromatography, and exposed these proteins to ³²P-labeled ATP, CaMKII, and the appropriate cofactors *in vitro*. The proteins were separated via SDS-

PAGE, and autoradiography revealed ^{32}P labeling in the C terminus but not in the N terminus of Kv4.2 (Fig. 1A). GST alone was not phosphorylated by CaMKII (data not shown). To determine the phosphorylation sites within the C terminus, we first separated CaMKII-phosphorylated C-terminal fusion protein via SDS-PAGE and stained bands with Coomassie blue. The band representing the C terminus was excised, eluted, and digested with Lys-C. We then performed phosphopeptide mapping and amino acid sequencing of the phosphorylated Kv4.2 C-terminal fusion protein to directly determine sites of CaMKII phosphorylation.

Two CaMKII phosphorylation sites were identified in the C-terminal cytoplasmic domain. The phosphopeptide map for the C-terminal construct demonstrated three peaks in radioactivity in HPLC fractions (Fig. 1C) corresponding to four HPLC absorbance peaks (Fig. 1B). These HPLC fractions were directly sequenced using automated Edman degradation, and the amount of radioactivity released with each sequencing cycle was measured (Fig. 1D,E). This revealed two phosphopeptides: one corresponding to Kv4.2 C-terminal residues 438–444, with a phosphorylation site at Ser438 (Fig. 1D), and another corresponding to Kv4.2 C-terminal residues 449–468, with a phosphorylation site at Ser459 (Fig. 1E). The Kv4.2 sequence from amino acids 432–445 is RIRAAKSGSANAYM, and the sequence from amino acids 453–466 is LSNQLQSSEDEPAFV. Thus the Ser438 phosphorylation site is more of a prototypical CaMKII phosphorylation site, containing an immediately preceding lysine and several other preceding positive charges. These data thus implicate CaMKII in Kv4 channel regulation.

CaMKII phosphorylation of Kv4.2 leads to increased total cellular Kv4.2 protein

Because the above data supported the hypothesis that CaMKII regulates Kv4.2 through direct phosphorylation, we subsequently wanted to determine the functional effect of such phosphorylation. To do so, we used two approaches. One approach entailed coexpression of Kv4.2 with either of two truncated, constitutively active forms of CaMKII to test for effects of CaMKII on Kv4.2. One version of CaMKII was truncated at amino acid 316 (hereafter referred to as CaMKII $\Delta 316$) (Lledo et al., 1995), and the other is a GFP-labeled, truncated version of CaMKII (hereafter referred to as tCaMKII) (Hayashi et al., 2000). The second approach was to use site-directed mutagenesis to test the specificity of the two CaMKII phosphorylation sites we identified.

We transfected wild-type Kv4.2 plus an accessory subunit KChIP3 and tCaMKII or GFP into COS-7 cells and harvested these cells 24 hr after transfection. KChIP3 was included because it promotes channel synthesis and renders the channel properties

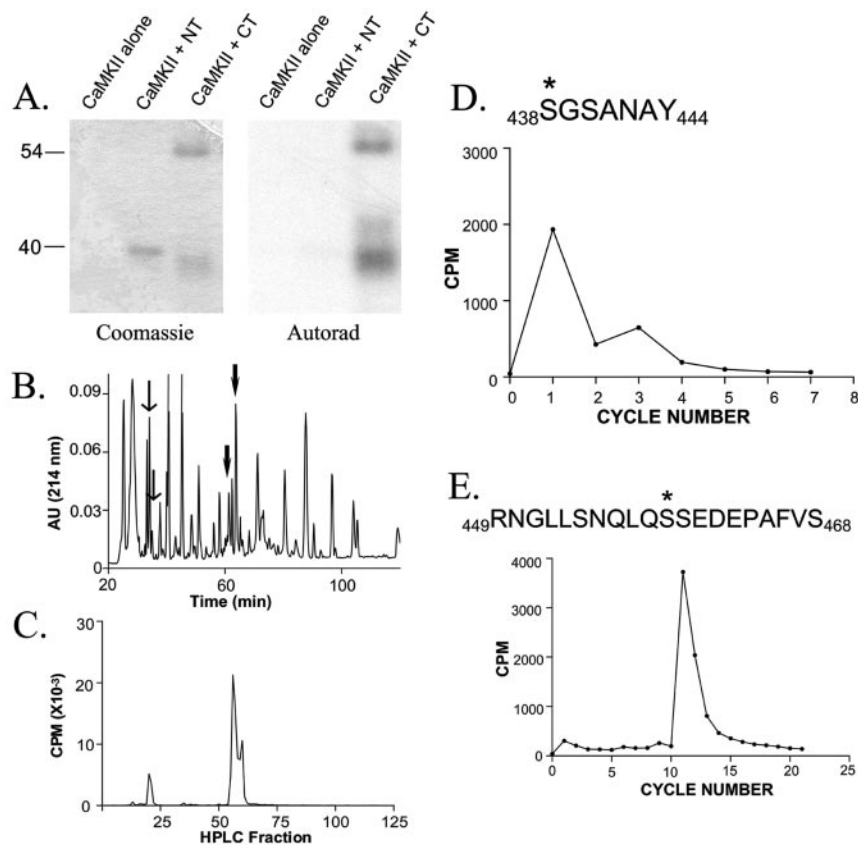


Figure 1. *In vitro* phosphorylation of Kv4.2 and identification of CaMKII-phosphorylated residues. *A*, Purified recombinant GST fusion proteins expressing the N-terminal (NT) or C-terminal (CT) cytoplasmic domains of Kv4.2 were reacted with CaMKII and the appropriate cofactors *in vitro*. Proteins were separated via SDS-PAGE. Left, Coomassie staining of proteins. Right, Autoradiogram demonstrating ^{32}P incorporation into the C terminus only. *B*, Phosphopeptide mapping was performed after CaMKII phosphorylation of the Kv4.2 GST C-terminal fusion protein. The arrows indicate the absorbance peaks at 214 nm corresponding to the HPLC fractions with peaks in radioactivity. *C*, The radioactivity plot illustrates peaks of radioactivity in two different HPLC fractions (one from fractions 20–21 and one from fractions 56–57). ^{32}P counts per minute (CPM) measured as Cherenkov radiation are represented on the y-axis. *D*, *E*, The sequences of the phosphopeptides in HPLC fractions 20–21 (*D*) and fractions 56–57 (*E*) that were determined by automated amino acid sequencing correspond to amino acids 438–444 (*D*) and amino acids 449–468 (*E*) within the C terminus of Kv4.2. The radioactivity released with each sequencing cycle was measured. There is a peak in sequence cycle 1 for the peptide containing amino acids 438–444, indicating that Ser438 is the phosphorylated amino acid in that peptide (*D*). There is a peak in sequence cycle 12 for the peptide containing amino acids 449–465, indicating that Ser459 is the phosphorylated amino acid in that peptide (*E*). Phosphorylated residues are marked with an asterisk.

more native-like (An et al., 2000; Schrader et al., 2002). We then prepared membrane fractions from these cells, separated proteins via SDS-PAGE, and probed for total Kv4.2 protein via Western blotting. When Kv4.2 was cotransfected with tCaMKII, there appeared to be an approximately fourfold increase in total Kv4.2 protein immunoreactivity ($374 \pm 70\%$ of control; $n = 13$; $p < 0.05$) (Fig. 2A–C). To verify that the changes in immunoreactivity seen with constitutive CaMKII expression were not an artifact of increased availability of the particular epitope recognized by the Kv4.2 antibody we used (Chemicon), we demonstrated that the immunoreactivity to Kv4.2 in the membrane fraction also increased when a monoclonal antibody recognizing an extracellular epitope of Kv4.2 was applied (Fig. 2D). These data demonstrate an effect of CaMKII on Kv4.2, specifically a pronounced increase in protein expression levels.

To ascertain, in part, whether this effect was mediated by the Kv4.2 phosphorylation sites we had identified, we used site-directed mutagenesis to convert Ser438 and Ser459 to alanines to prevent phosphorylation and also converted the sites to aspartates to mimic constitutive phosphorylation. When the S438D–

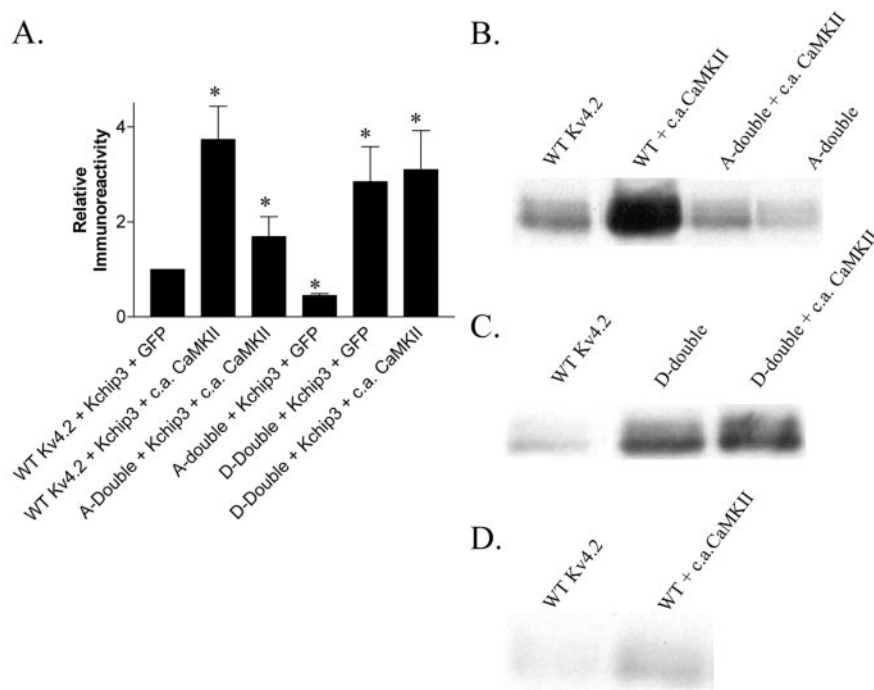


Figure 2. Changes in immunoreactivity against Kv4.2 by coexpression of constitutively active CaMKII or mutating the identified CaMKII phosphorylation sites. *A*, Expression of either the tCaMKII or the $\Delta 316$ constitutively active form of CaMKII (collectively labeled c.a. CaMKII) instead of GFP, along with Kv4.2 and KCHIP3, increased the immunoreactivity against Kv4.2 in the membrane proteins harvested from transfected COS cells ($374 \pm 70\%$ of control; $n = 13$; $p < 0.05$). CaMKII failed to have as large an effect on Kv4.2 where both Ser438 and Ser459 have been mutated to alanines (A-double) ($169 \pm 84\%$ of control; $n = 4$; $p < 0.05$). The A-double-mutant in the absence of constitutively active CaMKII was decreased compared with control ($45 \pm 4\%$ of control; $n = 4$; $p < 0.05$). Mutagenesis of both Ser438 and Ser459 to aspartates to mimic constitutive phosphorylation (D-double) recapitulated the CaMKII effect ($287 \pm 74\%$ of control; $n = 16$; $p < 0.05$). Addition of constitutively active CaMKII to the D-double-mutant failed to add substantially to the effect of the D-double-mutant in the absence of constitutively active CaMKII ($309 \pm 82\%$ of control; $n = 9$; $p < 0.05$). *B*, Representative Western blot showing the effect of CaMKII activity on Kv4.2 immunoreactivity as well as the effect of mutating the two CaMKII phosphorylation sites to alanines (A-double). *C*, A separate representative Western blot showing the effect of introducing aspartates into the two identified CaMKII phosphorylation sites. *D*, CaMKII phosphorylation does not simply change the availability of the antibody epitope, because the same phenomenon is observed with a separate monoclonal Kv4.2 antibody. WT, Wild type.

S459D double-mutant was transfected into COS cells with KCHIP3, the total level of Kv4.2 immunoreactivity mimicked the increased immunoreactivity on Western blots seen with the cotransfection of tCaMKII ($287 \pm 74\%$ of control; $n = 16$; $p < 0.05$) (Fig. 2*A,C*). This phenotype was unchanged after cotransfection with tCaMKII ($309 \pm 82\%$ of control; $n = 9$; $p < 0.05$). Conversely, when the double-mutant S438A–S459A was introduced into COS cells, there was a significant reduction in immunoreactivity for Kv4.2 ($45 \pm 4\%$ of control; $n = 4$; $p < 0.05$) (Fig. 2*A,B*). When tCaMKII was cotransfected with the double-mutant S438A–S459A, the total immunoreactivity of Kv4.2 increased, but not nearly to the extent that the wild-type channel was modified ($169 \pm 84\%$ of control; $n = 4$; $p < 0.05$). This suggests that there may be multiple mechanisms by which CaMKII affects Kv4.2; however, the primary mechanism appears to be via direct phosphorylation of the C terminus of Kv4.2 at the identified phosphorylation sites. Overall, these results suggested to us that CaMKII may be regulating either the net rate of creation or degradation of the Kv4.2 channel; however, we favor the idea of degradation, because the effects of the aspartate point mutants must of necessity be post-translational.

Because the immunoreactivity of the S438A–S459A double-mutant was low and sometimes not even detectable, we decided to ensure that we could accumulate this mutant channel by in-

hibiting its degradation. Therefore, we applied the proteasome inhibitor MG132 at $20 \mu\text{M}$ for 12 hr. We found that inhibiting the proteasome in such a manner led to recovery of protein in the S438A–S459A Kv4.2 mutant and increased overall channel protein immunoreactivity in all variants of Kv4.2 (Fig. 3*A*). These observations suggest that Kv4.2 is at least in part degraded by the proteasome and that CaMKII may somehow be regulating the net half-life of the protein in the cell. This might occur by trafficking the channel to a stabilized pool or decreasing the rate of protein breakdown.

It has become increasingly clear that ubiquitination of proteins is an important regulatory step in protein degradation and cycling proteins into and out of cell membranes, particularly for proteins associated with the postsynaptic density (Ehlers, 2003). Because we had already shown that Kv4.2 was capable of being regulated by the proteasome and that CaMKII phosphorylation causes cellular accumulation of Kv4.2, we took particular notice of a lysine residue at amino acid 437, immediately preceding one of the CaMKII phosphorylation sites at Ser438. We hypothesized that phosphorylation of Ser438 might be blocking access of a ubiquitin ligase to Lys437, thereby leading to a stalling of the degradation process. To test this hypothesis, we used site-directed mutagenesis to introduce an alanine at the Lys437 position of Kv4.2. We suspected that the total Kv4.2-like immunoreactivity of the K437A mutant of Kv4.2 might be increased in the same way the S438D–S459D

mutant total immunoreactivity is increased in COS cells. However, the K437A mutant displayed significantly decreased total immunoreactivity compared with wild-type channel (Fig. 3*B*). This result suggests that Lys437 is not involved in Kv4.2 degradation, and we interpret the decrease in immunoreactivity to represent an inability of CaMKII to properly recognize the mutant Kv4.2, thereby resulting in decreased phosphorylation of the channel. This model is consistent with the known role of upstream positively charged residues as being involved in the CaMKII substrate recognition consensus sequence.

In our Western blotting experiments, we made the serendipitous observation that Kv4.2 is often processed into a doublet of immunoreactivity (Fig. 4*B*). Thus, we were intrigued by the observation that inhibiting CaMKII activity pharmacologically with $10 \mu\text{M}$ KN-93 could alter the immunoreactivity of Kv4.2 from a doublet to a singlet in transfected COS cells (Fig. 4*A*) along with decreasing total Kv4.2 protein as expected. The effect on the CaMKII doublet was first observed when KN-93 was present in the media 18 hr before harvesting the cells. Previous observations have suggested that the upper band of immunoreactivity represents protein specifically trafficked to the cell surface, whereas the lower band represents a pool of channel that can exist either intracellularly or at the surface (Shibata et al., 2003). We confirmed these previous observations by applying the Golgi traffick-

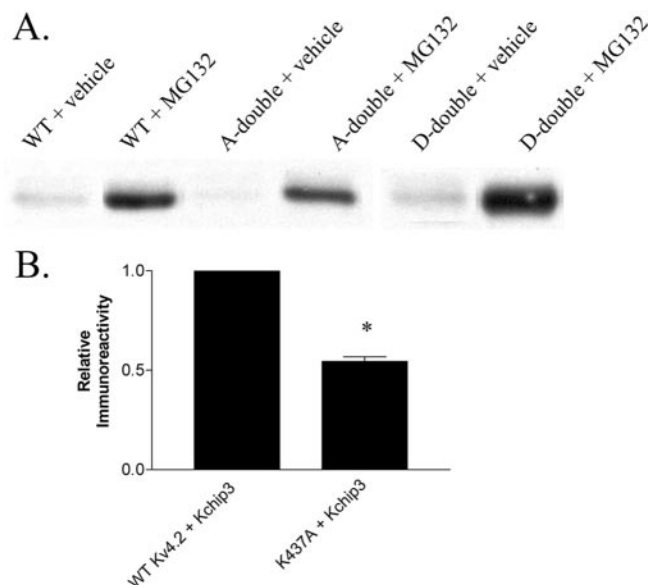


Figure 3. Inhibition of the proteasome causes accumulation of Kv4.2. *A*, To ascertain the presence of a band in the S438A–S459A double-mutant of Kv4.2, we applied a 21 μ M concentration of the proteasome inhibitor MG132 to transfected COS cells for 12 hr. Obvious accumulation of protein in the wild-type (WT) channel, the S438A–S459A double-mutant (A-double), and the S438D–S459D double-mutant (D-double) was seen with MG132 after harvesting the cells. *B*, To test the hypothesis that Lys437, immediately preceding CaMKII phosphorylation site Ser438, is involved in the ubiquitin-mediated degradation of Kv4.2, we substituted Lys437 for an alanine. This mutant of Kv4.2 displayed decreased immunoreactivity when cotransfected with KChip3 in COS cells compared with the wild-type channel plus KChip3 ($54 \pm 4\%$ of control; $n = 3$; $p < 0.05$). Our interpretation of this result is that Lys437 does not play a role in ubiquitin-mediated degradation of Kv4.2 but may be necessary as part of the consensus sequence for CaMKII recognition of Ser438.

ing inhibitor Brefeldin A to COS cells transfected with wild-type Kv4.2 and KChip3 with or without tCaMKII at the time of transfection. The immunoreactivity of Kv4.2 from cells expressing either channel alone or channel plus tCaMKII was consistently restricted to the lower molecular weight band after Brefeldin A treatment (Fig. 4*B*), suggesting to us the hypothesis that CaMKII may be specifically increasing the surface pool of Kv4.2 concurrently with increasing total cellular protein.

We therefore tested the hypothesis that CaMKII phosphorylation might regulate the extent of cell-surface expression of Kv4.2 through several independent means. First, we biotinylated the surface proteins of COS cells expressing Kv4.2 and KChip3 and either tCaMKII or GFP and then pulled down these biotinylated proteins with neutravidin beads. Cells expressing tCaMKII displayed increased Kv4.2 in this surface pool compared with those cells expressing GFP plus Kv4.2 (Fig. 4*C*). Analysis of the nonsurface pool of Kv4.2 also showed an increase in immunoreactivity (data not shown), and this finding is consistent with the hypothesis that CaMKII activity increases both the cell-surface pool and the total cellular content of Kv4.2. In an additional test to determine whether CaMKII increases surface Kv4.2 expression, we took advantage of a C-terminal-directed, PKA-phosphorylated Kv4.2 antibody that selectively recognizes Kv4.2 expressed at the cell surface (Anderson et al., 2000; Shibata et al., 2003). Importantly, this phosphoselective antibody does not nonspecifically cross-react with CaMKII-phosphorylated isoforms of Kv4.2 (Anderson et al., 2000). In brief, the antibody is a phospho-site-specific antibody that recognizes Kv4.2 when it is phosphorylated by PKA in its C-terminal domain (Anderson et al., 2000). Shibata et al. (2003), using this antiserum, found that it

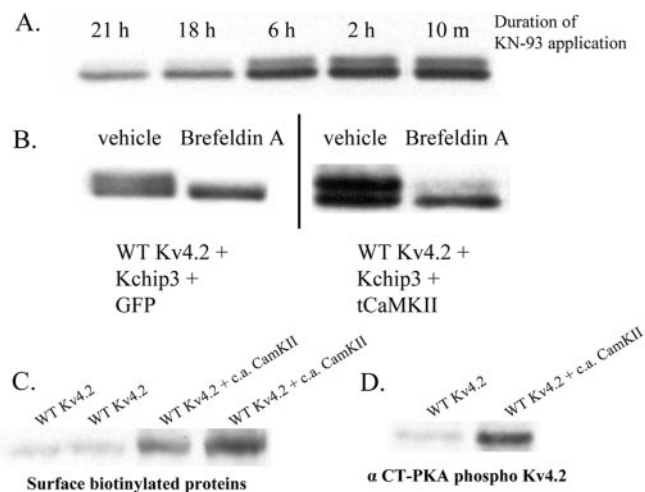


Figure 4. Treatment with KN-93 or Brefeldin A can change the immunoreactivity pattern of Kv4.2 from a doublet to a singlet. *A*, Immunoreactivity to Kv4.2 on a Western blot typically appears as a doublet, and CaMKII seemed to have a specific effect on increasing the immunoreactivity of the upper molecular weight species. We applied the CaMKII inhibitor KN-93 at 10 μ M for various periods of time to COS cells expressing wild-type (WT) Kv4.2 and KChip3. There is an obvious decrease in the immunoreactivity of the upper band of Kv4.2, beginning at 18 hr of KN-93 application. *B*, There has been previous evidence that this upper molecular weight band represents a pool of Kv4.2 trafficked to the surface and that the lower molecular weight band represents an intracellular pool of Kv4.2. To test this more directly, we applied the Golgi trafficking inhibitor Brefeldin A at 10 μ g/ μ l for 12 hr to COS cells expressing Kv4.2, KChip3, and GFP; Kv4.2, KChip3, and tCaMKII; or the S438D–S459D double-mutant of Kv4.2, KChip3, and GFP. We found that such treatment with Brefeldin A changed the immunoreactivity of Kv4.2 from a doublet to a single, lower molecular weight species. This supports the idea that the upper molecular weight band of Kv4.2 represents a pool of channel trafficked to the surface. *C*, Isolation of the surface pool of Kv4.2 in COS cells transfected with Kv4.2, KChip3, and either tCaMKII or GFP by biotinylation and avidin pulldown reveals that CaMKII phosphorylation can increase the amount of Kv4.2 at the cell surface. Biotinylation of Kv4.2 causes a change in the electrophoretic mobility of the protein such that it runs at 200 kDa. *D*, CaMKII phosphorylation increases the immunoreactivity of a PKA-phosphorylated, phosphospecific antibody that has been shown previously to selectively label the cell surface.

selectively recognizes Kv4.2 when it is at the cell surface. The total immunoreactivity detected with this antibody is greater in COS cells expressing Kv4.2, KChip3, and tCaMKII compared with those expressing Kv4.2, KChip3, and GFP as a control (Fig. 4*D*). This result is consistent with the hypothesis that the Kv4.2 protein increase that we see is in part attributable to a change in cell-surface Kv4.2. In addition, the results with this antibody indicate that the increase in Kv4.2 immunoreactivity is not simply an idiosyncratic effect restricted to the immunoreactivity of a single antiserum. Together, the simplest explanation of our surface biotinylation experiments, surface-selective antibody Western blots, and Brefeldin A experiments is that CaMKII phosphorylation of Kv4.2 is causing an increase in the surface expression of Kv4.2. In our next series of studies, we added a physiological correlate to this biochemical evidence by recording surface membrane currents of Kv4.2 expressed in a heterologous cell expression system.

Constitutively active CaMKII potentiates Kv4.2 currents in transfected COS cells

To address the question of increased surface expression, we decided to measure whole-cell current density in expression systems. We transfected CaMKII Δ 316 along with wild-type Kv4.2, KChip3, and GFP into cultured COS cells. Twenty-four to 48 hr after transfection, we performed whole-cell patch recordings on transfected COS cells identified by the GFP marker. The kinetics of expressed I_A shared much similarity with what was reported

Table 1. Current kinetics of various expressed Kv4.2-encoded channels

| | Activation time constant (msec) | Inactivation time constant (msec) |
|--|---------------------------------|-----------------------------------|
| Wild type ($n = 10$) | 3.2 ± 0.3 | 58.2 ± 3.1 |
| Wild type plus CaMKII $\Delta 316$ ($n = 7$) | 3.8 ± 0.4 | 61.3 ± 5.5 |
| S438D–S459D ($n = 8$) | 4.0 ± 0.7 | 60.8 ± 4.5 |
| Wild type ($n = \sim 8$ –11) | 3.0 ± 0.2 | 53.2 ± 2.6 |
| S438A–S459A ($n = 6$) | 3.4 ± 0.3 | 58.2 ± 5.1 |
| S438A–S459A plus CaMKII $\Delta 316$ ($n = 9$) | 3.1 ± 0.2 | 52.0 ± 3.4 |

Above measurements were from currents elicited by a voltage step to 0 mV ($V_h = -80$ mV). Multigroup comparisons within two groups showed no significant difference (ANOVA; $p > 0.05$).

previously for Kv4.2 plus KCHIP3 (An et al., 2000; Holmqvist et al., 2002), and we did not find a dramatic difference in the activation or inactivation time constant among various experimental groups (Table 1).

However, the average current density evoked from a step to +20 mV from a -80 mV holding potential was 191.8 ± 23.8 pA/pF ($n = 13$) for the wild-type channel and 422.9 ± 52.4 pA/pF ($n = 10$) for those cells in which CaMKII $\Delta 316$ was cotransfected, representing a $>200\%$ increase (Fig. 5A). Again, we were interested in confirming that this effect was being mediated in part by direct phosphorylation at the identified sites on the C terminus of Kv4.2, and we therefore used the phospho-site mutants in this assay system. The average current density in COS cells expressing the S438D–S459D double-mutant, when stepped to +20 mV, was 526.7 ± 70.3 pA/pF ($n = 10$), thus recapitulating the effect of constitutively active CaMKII on native Kv4.2 currents. Conversely, the average current density mediated by the S438A–S459A double-mutant in CHO cells was 180.8 ± 47 pA/pF ($n = 8$) when stepped to +20 mV, representing a substantial decrease compared with the wild-type channel expressed in CHO cells (wild-type current density at +20 mV, 371.1 ± 26.4 pA/pF; $n = 12$). CaMKII activity, however, could not potentiate the current density mediated by this double A-mutant channel, resulting in a similar current density (217.1 ± 23.1 pA/pF; $n = 9$). These results indicate a necessity of phosphorylation at these sites for CaMKII to increase surface expression of Kv4.2 (Fig. 5B). Because we did not measure single-channel conductance of Kv4.2 or the mean open time, we cannot rule out a CaMKII effect on these parameters; however, the most parsimonious explanation for our findings is that CaMKII phosphorylation of Kv4.2 increases not only total cellular amounts of Kv4.2 but also a functionally relevant pool of Kv4.2 protein at the cell-surface plasma membrane, contributing to greater average whole-cell currents. This increase, however, was not attributable to changes in the voltage dependence of Kv4.2-mediated A-type channels, as shown below.

Kv4.2 channel biophysics are unchanged by CaMKII phosphorylation

Because phosphorylation events are often multifunctional, we wondered whether CaMKII phosphorylation altered the channel biophysical properties of Kv4.2 in addition to increasing the average peak current amplitude. To assess this, we examined the voltage-dependent properties of Kv4.2 subunits under various conditions in COS cells, CHO cells, and *Xenopus* oocytes. We found that the voltage dependence of activation and the voltage dependence of inactivation were unchanged by introduction of either alanine or aspartate into the 438 and 459 sites or by coexpression with constitutively active CaMKII (Fig. 6). All data are summarized in Table 2. These data suggest that CaMKII does not regulate the voltage dependence of activation or inactivation of Kv4.2. These data also serve as an important control, demonstrat-

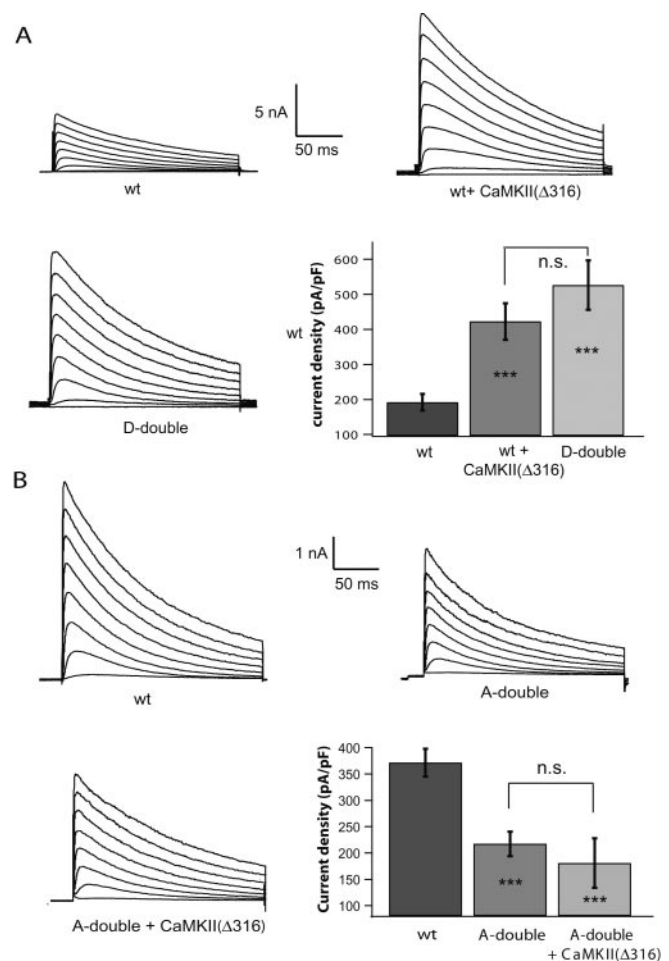


Figure 5. CaMKII activity can increase the peak current amplitude of Kv4.2 currents expressed in a heterologous system. *A*, We recorded the whole-cell current responses to a family of voltage steps in COS cells expressing wild-type (wt) Kv4.2, KCHIP3, and either GFP (wild type) or constitutively active CaMKII ($\Delta 316$). The peak current amplitude of cells expressing constitutively active CaMKII was significantly greater than control (191.8 ± 23.8 pA/pF, $n = 13$, for the wild-type channel; 422.9 ± 52.4 pA/pF, $n = 10$, for a voltage step to +20 mV from a -80 mV holding potential) for cells cotransfected with constitutively active CaMKII. Similarly, the S438D–S459D double-mutant (D-double) plus KCHIP3 and GFP displayed significantly increased peak current amplitudes compared with the wild-type channel (526.7 ± 70.3 pA/pF, $n = 10$, for the same voltage step). *** $p < 0.005$; ANOVA. Asterisks within bars indicate a significant change compared with control. n.s., Not significant. *B*, Expression of the S438A–S459A double-mutant (A-double) plus KCHIP3 and GFP in CHO cells resulted in significantly decreased peak current amplitudes in response to a family of voltage steps compared with the wild-type channel plus KCHIP3 and GFP (wt) (371.1 ± 26.4 pA/pF, $n = 12$, for wt; 180.8 ± 47 pA/pF, $n = 8$, for S438A–S459A). This reduction in peak current amplitude could not be rescued by addition of constitutively active CaMKII, demonstrating the specificity of the sites (217.1 ± 23.1 pA/pF; $n = 9$ for S438A–S459A plus KCHIP3 and constitutively active CaMKII). *** $p < 0.005$; ANOVA. Asterisks within bars indicate a significant change compared with control. n.s., Not significant.

ing that the mutant channels are functionally, and thus most likely structurally, indistinguishable from native Kv4.2 in their basic properties. In other words, the decrease in Kv4.2 current expression with the serine-to-alanine mutants is not attributable to rendering the channel incapable of conducting potassium ions or detecting changes in membrane potential.

CaMKII potentiates A-type currents in hippocampal pyramidal neurons

There is evidence to suggest that Kv4.2 underlies the A-type K^+ current in the somata and dendrites of hippocampal pyramidal

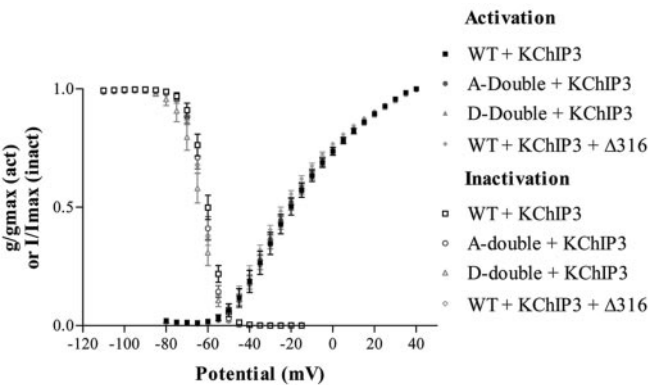


Figure 6. CaMKII does not affect the biophysical properties of the Kv4.2 channel. To assess whether CaMKII could affect the biophysical properties of the Kv4.2 channel, we expressed Kv4.2, KChIP3, and GFP in *Xenopus* oocytes and used a two-electrode voltage clamp to perform activation and inactivation protocols. Neither constitutively active CaMKII ($\Delta 316$) expression nor substitution of the CaMKII phosphorylation sites with alanines (A-double-mutant) or aspartates (D-double-mutant) affected the voltage dependence of activation or inactivation. WT, Wild type.

neurons (Serodio and Rudy, 1998; Song et al., 1998; Tkatch et al., 2000). Because we observed a CaMKII-mediated increase in Kv4.2 currents expressed in a heterologous expression system, we decided to test whether CaMKII could also regulate native A-type current in neurons. To this end, we transfected tCaMKII and GFP into dissociated cultured hippocampal neurons. We used CA1–CA3-enriched primary cultures, including CA1 pyramidal neurons, CA3 pyramidal neurons, and various types of interneurons. To avoid variations in native I_A density and to reduce the complexity of the molecular identity of native I_A among different cell types, we chose to restrict our studies to responses from presumed CA1 pyramidal neurons. Our criteria for a CA1 pyramidal neuron included the presence of a major apical dendrite, some basal dendrites, and a relatively small soma that had whole-cell capacitance of <20 pF (Fig. 7A). Twenty-four to 48 hr after transfection, we recorded whole-cell K^+ currents from expressing neurons, and the transient A-current component was isolated by a prepulse voltage protocol (Fig. 7B). Most recordings were obtained from GFP control and tCaMKII–GFP fusion construct-expressing neurons with similar shapes and sizes. In addition to Kv4 subunits, Kv1.4 subunits were also expressed in hippocampal pyramidal neurons (Veh et al., 1995; Grosse et al., 2000); thus they may also underlie the native I_A of CA1 pyramidal neurons. It was reported that CaMKII activity also regulates Kv1.4-mediated

currents in an expression system (Roeper et al., 1997). One of the aspects of regulation was that the channel inactivation was dramatically slowed by CaMKII activity, resulting in a larger time constant (τ) of current decay. Under our experimental condition, when GFP neurons were held at -80 mV and stepped to 0 mV, the averaged τ was 24.5 ± 1.7 msec. To avoid the contribution from Kv1.4 subunits in the native I_A amplitude upregulated by CaMKII, we focused our studies on these tCaMKII-expressing neurons with a current decay τ that was not larger than 35 msec. The mean τ of these tCaMKII neurons was 29.2 ± 2.2 msec and is not statistically different from that of GFP control ($p > 0.1$). The peak A-current amplitude obtained from a step to 0 mV from a holding potential of -80 mV in tCaMKII-expressing neurons was significantly greater than the current density obtained in control neurons (624.1 ± 79.2 pA, $n = 11$ for control neurons vs 1162.3 ± 67.2 pA, $n = 14$ in tCaMKII expressing neurons) (Fig. 7C). Although it was observed that the somata of tCaMKII neurons tended to be somewhat round in shape compared with that of GFP control, the difference in whole-cell capacitance (an indicator of cell size) was not statistically significant ($p > 0.5$) between tCaMKII neurons and GFP control. As a result, the current density of tCaMKII neurons (78.8 ± 4.9 pA/pF) was significantly larger than control (38.7 ± 3 pA/pF; $p < 0.001$) (Fig. 7D). These results demonstrate that CaMKII has the ability to upregulate the amplitude of somatodendritic A-type currents in hippocampal neurons. Thus, the effect of CaMKII on Kv4.2-encoded A-currents is likely not limited to heterologous systems, but also occurs in native neurons expressing endogenous A-type channels.

Constitutive CaMKII expression alters hippocampal neuronal firing properties

Because the A-type K^+ current (I_A) is important for regulating neuronal excitability and the repetitive firing of neurons, we hypothesized that the CaMKII-mediated increase in neuronal A-currents might affect the firing properties of hippocampal pyramidal neurons. We compared the responses of control hippocampal pyramidal neurons and neurons that had been transfected with constitutively active CaMKII. As described above, recordings were obtained from GFP- and tCaMKII-expressing neurons with similar shapes and sizes. First, the resting membrane potential (V_m) of tCaMKII neurons was more depolarized than that of GFP control cells (59.7 ± 1.5 vs -68.3 ± 3.1 mV; $n = 7$; $p < 0.05$) (Fig. 8C). Second, tCaMKII caused a small but statistically significant increase in threshold current (21.7 ± 3 pA; $n = 7$) compared with GFP-expressing neurons (13.5 ± 1.3 pA;

Table 2. Voltage-dependent properties of various Kv4.2-encoded K^+ currents

| | Activation | | Inactivation | |
|--|-----------------|----------------|-----------------|---------------|
| | $V_{1/2}$ (mV) | Rate | $V_{1/2}$ (mV) | Rate |
| COS | | | | |
| Wild type ($n = 5$) | -6.1 ± 3.5 | 22.5 ± 0.8 | -70.3 ± 2.3 | 8.2 ± 1.1 |
| Wild type plus CaMKII $\Delta 316$ ($n = 4$) | -13.7 ± 2.7 | 21.8 ± 1.4 | -68.3 ± 1.7 | 5.3 ± 0.5 |
| S438D–S459D ($n = 4$) | -8.4 ± 2.6 | 21.2 ± 1.7 | -68.7 ± 2.1 | 5.8 ± 1.5 |
| CHO | | | | |
| Wild type ($n = 4$) | -1.4 ± 2.9 | 21.9 ± 0.7 | -60 ± 1.8 | 4.1 ± 0.3 |
| S438A–S459A ($n = 4$) | -7.8 ± 2 | 20.5 ± 1.3 | -63.5 ± 0.7 | 7.1 ± 0.2 |
| S438A–S459A plus CaMKII $\Delta 316$ ($n = 5$) | -4 ± 3 | 21.7 ± 0.3 | -59.5 ± 1.7 | 4.6 ± 0.4 |
| Oocytes | | | | |
| Wild-type ($n = 4$) | -18.0 ± 2 | 15.3 ± 0.4 | -60.3 ± 0.9 | 3.5 ± 0.1 |
| S438A–S459A ($n = 10$) | -18.6 ± 0.8 | 15.2 ± 0.3 | -61.6 ± 0.9 | 3.5 ± 0.1 |
| S438D–S459D ($n = 9$) | -17.4 ± 0.9 | 15.4 ± 0.5 | -64.1 ± 1.4 | 3.5 ± 0.1 |
| Wild type plus CaMKII $\Delta 316$ ($n = 16$) | -21.2 ± 1 | 15.2 ± 0.1 | -67.2 ± 0.6 | 3.6 ± 0.1 |

Multigroup comparisons for each parameter within three groups showed no significant difference (ANOVA; $p > 0.05$).

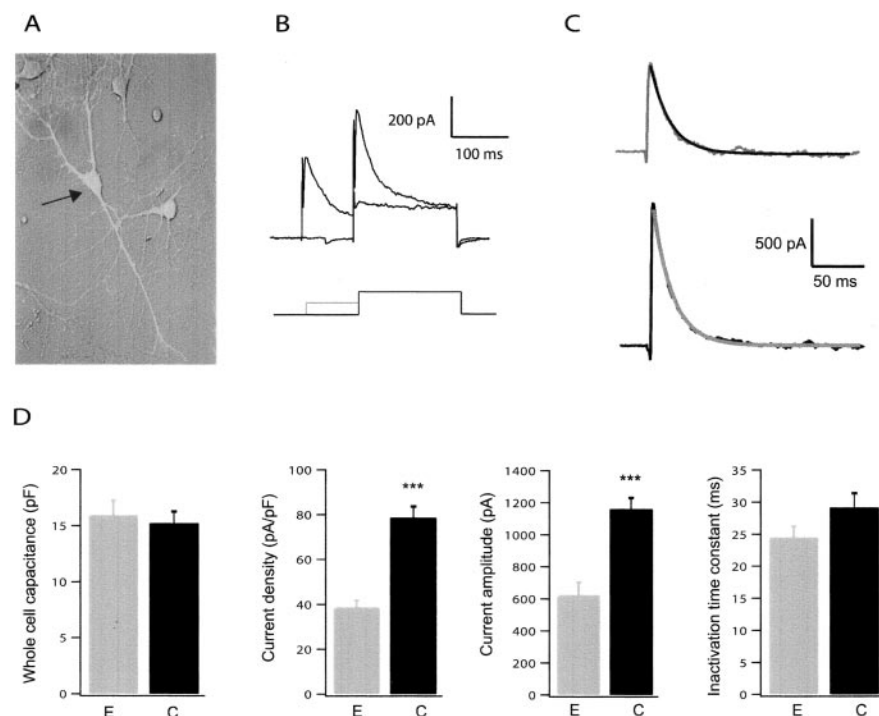


Figure 7. Active CaMKII increased A-type K^+ current in cultured hippocampal neurons. *A*, Fluorescence image of an EGFP-transfected CA1 pyramidal neuron (arrow). *B*, Schematic diagram of the prepulse voltage protocol used to isolate native A currents. The prepulse was to -40 or -20 mV. *C*, Representative A currents obtained from a control pyramidal neuron expressing EGFP (top) and a similar-sized pyramidal neuron expressing tCaMKII (bottom). Neurons were held at -80 mV and stepped to 0 mV. Decay of the A currents was fit with a single exponential. CaMKII activity increased the transient A-type K^+ current amplitude without a change in the inactivation time constant. *D*, Summary data for whole-cell capacitance, peak current at 0 mV, current density, and inactivation time constant for EGFP (E) and tCaMKII (C) transfected neurons. (***) $p < 0.001$.

$n = 7$; $p < 0.05$) when V_m was held at ~ -70 mV. Third, in response to a 1.5–2 times threshold current for 700 msec, the control neurons fired rhythmically without a decrease or with only a slight decrease in amplitude of subsequent action potentials (Fig. 8A). tCaMKII-expressing neurons, however, exhibited a disrupted firing pattern. To quantify the change in firing pattern, we measured the delay to the first action potential in response to a 700 msec current injection. CaMKII-expressing neurons showed a significantly prolonged onset to fire the first AP (403.6 ± 31.3 pA; $n = 7$) compared with GFP controls (230.1 ± 19.3 pA; $p < 0.001$) (Fig. 8A,C). To test the contribution of Kv4 subunits to the prolonged onset observed in tCaMKII neurons, we used HpTx-3, a spider toxin shown to be specific for Kv4 subunits (Sanguinetti et al., 1997; Ramakers and Storm, 2002). As shown in Figure 8, when the injected currents were kept the same, the prolonged onset for APs in tCaMKII neurons was reversely shortened by application of 50–100 nM HpTx-3 from 405 ± 19.2 to 117.4 ± 11.9 msec ($n = 4$; $p < 0.01$) (Fig. 8B,C). These data are consistent with our hypothesis of CaMKII increasing neuronal cell-surface Kv4 family potassium channels. Although we cannot rule out the possibility of CaMKII affecting other conductances (for example, those that contribute to the resting membrane potential), it is worth noting that neurons selectively overexpressing only Kv4.2 subunits, resulting in a subsequent increase in I_A density, display firing properties similar to what we observed in tCaMKII-transfected neurons (Shibata et al., 2000). This observation supports the hypothesis that the changes in firing properties seen with introduction of constitutively active CaMKII are mediated via increases in A-type K^+ currents.

Discussion

Our findings support the idea that CaMKII regulates a novel target, the voltage-gated potassium channel Kv4.2, and that this interaction has the functional consequence of increasing channel expression and decreasing neuronal excitability. We began with a biochemical approach, identifying the cytoplasmic C terminus of Kv4.2 as the major site of phosphorylation and, using phosphopeptide mapping and direct amino acid sequencing, found Ser438 and Ser459 as specific phosphorylation sites. We demonstrated that CaMKII phosphorylation of Kv4.2 results in increased total cellular Kv4.2 protein, but it was unclear to us whether this also resulted in an increase in functional channels at the cell surface or whether the channel was merely trapped in an intracellular membrane such as the endoplasmic reticulum, Golgi body, or endosomes. Using a surface biotinylation approach and by recording whole-cell A-currents from COS cells transfected with Kv4.2 and constitutively active CaMKII, we demonstrated that a significantly increased fraction of Kv4.2 reaches the cell surface when CaMKII is present, resulting in increased peak Kv4.2 currents. This increase in peak current amplitude was unaccompanied by changes in the voltage dependence of activation and inactivation of Kv4.2. We also demonstrated

that CaMKII not only potentiates Kv4.2 currents in a heterologous expression system but also increases the peak amplitudes of endogenous A-type currents in hippocampal neurons. Finally, we found that introduction of constitutively active CaMKII into hippocampal neurons causes a change in neuronal firing patterns such that the neuron has a smaller probability of firing an action potential in response to a given current input, and that the onset of action potentials is significantly delayed.

Our finding that constitutively active CaMKII leads to decreased neuronal excitability is consistent with several other studies suggesting a role for CaMKII in altering neuronal excitability. Neurons from mutant *Drosophila* expressing a CaMKII inhibitory peptide display increased spike frequency, increased action potential duration, and decreased stability of spike frequency coding, hallmarks of increased neuronal excitability (Yao and Wu, 2001). Interestingly, these neurons also display a significant decrease in A-type potassium current density. Mice with a null mutation for α -CaMKII have increased neuronal excitability and display both spontaneous seizures and a decreased threshold for evoked seizures that involve the hippocampus (Butler et al., 1995). Similarly, CaMKII inhibition in cultured rat hippocampal neurons through an antisense oligonucleotide resulted in increased spike frequency and epileptiform activity (Churn et al., 2000). Prolonged application of the CaMKII inhibitor KN-62 to cultured embryonic mouse hippocampal neurons caused a significant decrease in A-type currents with a concomitant decrease in whole-cell capacitance, and it was suggested that this resulted from decreased CaMKII-mediated fusion of channel-bearing

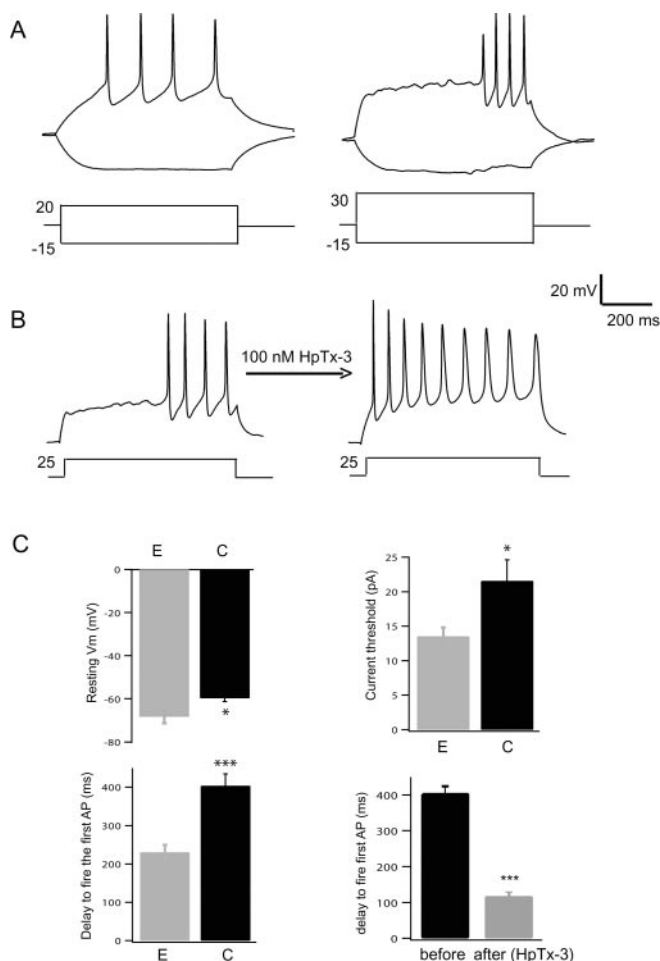


Figure 8. Active CaMKII changed the basic firing properties of hippocampal neurons. *A*, Representative traces of the action potential firing pattern of an EGFP-expressing (left) and a tCaMKII-expressing (right) pyramidal neuron in response to current injections. The amplitude of the injected current was 1.5–2 times threshold. Constitutively active CaMKII (tCaMKII) increased the time to fire the first AP under similar conditions. *B*, The prolonged onset to fire the first AP observed in the tCaMKII-expressing neuron (left) could be reversed by application of 50–100 nM Hptx-3 (right). *C*, Summary data for resting membrane potential, current threshold to fire APs, and delay to fire the first AP before and after Hptx-3 are shown for EGFP-expressing (E) and CaMKII-expressing (C) neurons (* $p < 0.05$; *** $p < 0.001$).

vesicles with the plasmalemma (Wu et al., 1998). Clearly, from these studies and our own, CaMKII activity is required for maintaining normal cellular excitability in many systems.

Our results are distinct from accounts of CaMKII regulation of A-type currents in non-neuronal cell types as well as data suggesting a role for CaMKII in regulating the biophysical properties of other molecular mediators of A-type currents. For example, in murine colonic myocytes, inhibition of CaMKII with KN-93 or KN-62 causes an increased rate of A-type channel inactivation and slows the recovery from inactivation with no change in peak current amplitude (Koh et al., 1999). In human atrial myocytes, KN-93 also accelerates the inactivation of the transient outward potassium current (I_{to}), and in this case the amplitude of the maintained component is also decreased (Tessier et al., 1999). Thus, CaMKII seems to have similar effects on Kv1.4, which also mediates an A-type current. In this vein, increasing CaMKII activity in human embryonic kidney cells expressing Kv1.4 caused a slowing of inactivation and accelerated recovery from inactivation of Kv1.4 currents (Roepke et al., 1997). Overall, the trend for CaMKII modulation of A-type currents outside the brain or en-

coded by non-Kv4 molecular species has been one of altering channel biophysics, whereas CaMKII modulation of neuronal Kv4.2 increases the current amplitude. However, in all cases, CaMKII activity ultimately increases the availability of the underlying A-type channel.

Our studies do not identify the precise molecular mechanisms by which CaMKII increases Kv4.2 surface expression levels. One possibility is that CaMKII is increasing surface expression via mass action, by increasing protein half-life and increasing total cellular Kv4.2 protein. This potential mechanism is in contrast to CaMKII specifically trafficking Kv4.2 to the cell surface via phosphorylation-dependent mechanisms. Which of these is the most likely mechanism is particularly hard to ascertain, because these two possibilities are not mutually exclusive. In addition, surface membrane insertion is known to be able to stabilize proteins and increase their half-life. Thus, increased surface trafficking could result in a secondary increase in total protein in the cell. Alternatively, as mentioned above, simply increasing half-life can increase total protein and drive protein into the surface membrane. At present, each of these remains a viable alternative based on our observations.

Our findings are surprising, in that CaMKII phosphorylation of Kv4.2 and the resulting alteration in A-type current has the opposite effect of several other major kinases implicated in learning. For example, it has already been demonstrated that PKA and PKC shift the activation curve for A-type channels ~ 15 mV in the depolarizing direction in the distal dendrites of CA1 pyramidal neurons, a net inhibition of channel function (Hoffman and Johnston, 1998). Application of inhibitors of mitogen- and extracellular signal-regulated kinase (MEK), an upstream activator of ERK, to hippocampal pyramidal neurons results in an increased availability of A-type K^+ channels, indicating that ERK inhibits A-type channel function (Watanabe et al., 2002). Similarly, stimulation of β -adrenergic receptors in hippocampal slices via isoproterenol application boosts backpropagating action potential amplitude in a PKA- and ERK-MAPK-dependent manner (Yuan et al., 2002), a finding consistent with ERK inhibition of A-type channels. Furthermore, PKC suppresses Kv4.2 currents in *Xenopus* oocytes (Nakamura et al., 1997), and glial-derived neurotrophic factor application to dopaminergic neurons, acting through the ERK-MAPK pathway, suppresses A-type channels, leading to increased neuronal excitability (Yang et al., 2001). Thus PKA, PKC, and ERK-MAPK phosphorylation of Kv4.2 appear to result in suppression of the channel, leading to increased excitability, whereas CaMKII phosphorylation enhances channel activity through increasing surface expression. This dichotomy was the opposite of our expectation entering into these studies.

Given the multifaceted role of CaMKII in synaptic plasticity, one may wonder how a neuron might use a CaMKII-mediated upregulation of A-type current in this context. Because CaMKII has been shown to be involved in synaptic potentiation, one possibility is that CaMKII-mediated increases in Kv4.2 channel density constitute a negative feedback response, affecting the ability of synapses to undergo subsequent potentiation. Thus, CaMKII regulation of Kv4.2 insertion might be one specific mechanism contributing to a sliding threshold for LTP/long-term depression induction.

A related idea is that CaMKII-mediated upregulation of Kv4.2 could be involved in maintaining the homeostasis of neuronal firing properties (Marder and Prinz, 2002). In support of the homeostasis hypothesis, it has been demonstrated in the crab stomatogastric ganglion that several hours of depolarizing cur-

rent pulses lead to increased peak current amplitudes of A-type potassium current (Golowasch et al., 1999). These increases were reversible after cessation of activity and were also inhibited by blocking calcium entry into cells. Our results offer a mechanism by which such homeostasis may occur: CaMKII activity can act as a readout of average Ca^{2+} influx and directly phosphorylate the C terminus of Kv4.2. In turn, this results in an increase in average A-currents that dampens the response of the neuron to the increased activity, until equilibrium is reached.

Finally, it has been demonstrated both electrophysiologically (Hoffman et al., 1997; Yuan et al., 2002) and anatomically (Varga et al., 2000) that there exists a gradient of A-type current and the underlying Kv4.2 α subunit, respectively, along hippocampal dendrites with increasing concentration away from the soma. However, the mechanisms establishing and maintaining this gradient are poorly understood. CaMKII-mediated upregulation of Kv4.2 offers a plausible mechanism for maintaining this gradient, especially in that there exists a gradient of synaptic activity along a dendrite, which could lead to a gradient of average Ca^{2+} influx and CaMKII activity.

Numerous long-term changes in synaptic responses and membrane excitability have been implicated in LTP. The nature of and mechanisms underlying the synaptic conductance change have been extensively investigated. In particular, increased synaptic activity involving CaMKII drives glutamate receptors into synapses to potentiate synaptic responses (Hayashi et al., 2000) and also leads to increased current flux through the channel (Barria et al., 1997; Derkach et al., 1999; Poncer et al., 2002).

However, there are often changes in excitability and EPSP–spike coupling associated with neuronal plasticity, the mechanisms of which remain unknown. From the results of this study, we propose that one mechanism by which CaMKII activity may influence neuronal excitability is via regulation of potassium channels. Specifically, we propose that CaMKII phosphorylation of Kv4.2 subunits that mediate dendritic $I_{K(A)}$ promotes surface insertion of the channel in cells. These studies are particularly exciting in that they are the first to suggest a precise molecular mechanism underlying activity-dependent long-term modification of dendritic function per se. The implication of these studies is more general, in that K^+ channels can be applied as an additional site beyond synaptic AMPA receptors for the regulation of ion channels by protein kinases during LTP. Together with the potentiation of synaptic conductances provided by AMPA receptor insertion, CaMKII-mediated insertion of Kv4.2 channels may provide a new dimension of plasticity that shapes the integrative properties of neurons and extends the computational capacity of neuronal networks.

In conclusion, our data have identified Kv4.2 as a novel target for CaMKII regulation with effects on channel surface expression and peak current amplitudes that are specific to the identified sites of phosphorylation on the C terminus of Kv4.2. CaMKII-mediated increases in peak A-current amplitude have strong effects on dampening neuronal excitability in hippocampal neurons. Our findings provide a mechanism by which changes in CaMKII activity can alter neuronal excitability and also offer a plausible mechanism underlying homeostatic neuronal plasticity. These results offer a framework with which to address future questions about not only the mechanisms behind CaMKII-mediated increased surface expression of Kv4.2 channels but also the consequences of calcium-triggered changes in channel expression in a time-specific and location-specific manner.

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