Regulation of K₄.3 currents by Ca²⁺/calmodulin-dependent protein kinase II

Gerard P. Sergeant, Susumu Ohya, James A. Reihill, Brian A. Perrino, Gregory C. Amberg, Yuji Imaizumi, Burton Horowitz, Kenton M. Sanders, and Sang Don Koh. Regulation of Kᵥ4.3 currents by Ca²⁺/calmodulin-dependent protein kinase II. Am J Physiol Cell Physiol 288: C304–C313, 2005. First published September 29, 2004; doi:10.1152/ajpcell.00293.2004.—The voltage-dependent K⁺ channel 4.3 (Kᵥ4.3) is one of the major molecular correlates encoding a class of rapidly inactivating K⁺ currents, including the transient outward current in the heart (Iₒ) and A currents (Iₐ) in neuronal and smooth muscle preparations. Recent studies have shown that Iₒ in human atrial myocytes and Iₐ in murine colonic myocytes are modulated by Ca²⁺/calmodulin-dependent protein kinase II (CaMKII); however, the molecular target of CaMKII in these studies has not been elucidated. We performed experiments to investigate whether CaMKII could regulate Kᵥ4.3 currents directly. Inclusion of the autothiophosphorylated form of CaMKII in the patch pipette (10 nM) prolonged Kᵥ4.3 currents such that the time required to reach 50% inactivation from peak more than doubled, with positive shifts in voltage dependence of both activation and inactivation. In contrast, the rate of recovery from inactivation was accelerated under these conditions. CaMKII-inhibitory peptide or KN-93 produced effects opposite to that above; thus the rate of inactivation was increased, and recovery from inactivation decreased. A number of mutagenesis experiments were conducted on the three candidate CaMKII consensus sequence sites on the channel. Mutations at S550A, located at the COOH-terminal region of the channel, resulted in currents that inactivated more rapidly but recovered from inactivation at a slower rate than that of wild-type controls. In addition, these currents were unaffected by dialysis with either autothiophosphorylated CaMKII or the specific inhibitory peptide of CaMKII, suggesting that CaMKII slows the inactivation and accelerates the rate of recovery from inactivation of Kᵥ4.3 currents by a direct effect at S550A, located at the COOH-terminal region of the channel.

THE VOLTAGE-DEPENDENT K⁺ CHANNEL 4.3 (Kᵥ4.3) is one of the major molecular correlates encoding a class of rapidly inactivating K⁺ currents, often referred to as A currents (Iₐ). These currents have been recorded in a number of excitable tissues, including cardiac, neuronal, and smooth muscle preparations (1, 2, 9, 28, 33, 34). The fast-inactivation kinetics displayed by Kᵥ4.3 are an intrinsic feature that facilitates their key role of regulating membrane excitability. For example, in cardiac myocytes, Kᵥ4.3 channels are known to mediate the early repolarization phase of the action potential (9, 16), and in neuronal cells, activation of Kᵥ4.3 channels helps to establish the prolonged interval between action potentials (21, 35). Recent studies have also demonstrated that Kᵥ4.3 is likely to be the major molecular determinant of Iₒ in gastrointestinal smooth muscle cells (1, 2) and that this conductance appears to participate in the maintenance of the resting potential. Kᵥ4.3 channels are known to be influenced by α-adrenergic nerves acting via PKC (30) and also by a number of other factors, including pH, external cations, and a number of pharmacological agents (10, 11, 36, 39); however, relatively little is known about the regulation of these channels. Recent studies have shown that inhibition of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), a multifunctional kinase with widespread expression, including the heart, brain, and smooth muscles, accelerates the inactivation kinetics of transient outward current in the heart (Iₒ) in human atrial myocytes (38) and murine colonic myocytes (17). The target of CaMKII in these studies was likely to be Kᵥ4.3, because Iₒ in cardiac myocytes and Iₐ in smooth muscles are mediated, at least in part, by these channels. CaMKII is also known to regulate a number of ion channels, including Ca²⁺-activated Cl⁻ channels in smooth muscle cells (13, 40) and a range of K⁺ channels such as Drosophila ether-a-γ-goi ( eag) K⁺ channels (14, 41), small- and large-conductance Ca²⁺-activated K⁺ channels (see Refs. 18, 32), and Kv,1.4 channels (31). The purpose of the present study was to test whether CaMKII directly modulates Kᵥ4.3 currents. Voltage-clamp experiments were performed to determine whether autothiophosphorylated CaMKII or inhibition of native CaMKII with KN-93 or a CaMKII-inhibitory peptide would affect Kᵥ4.3 currents. In addition, site-directed mutagenesis at CaMKII consensus sequence sites on the Kᵥ4.3 channel was performed to ascertain potential target sites of action for CaMKII.

MATERIALS AND METHODS

Expression of Kᵥ4.3 in HEK-293 cells. The full length of rat Kᵥ4.3 was ligated into the mammalian expression vectors, pcDNA3.1 using T4 DNA ligase (New England BioLabs, Beverly, MA). Successful cloning into this vector was confirmed by DNA sequencing. Human embryonic kidney (HEK)-293 cells were transfected with the vector using the calcium phosphate method (4), and cells lines that stably expressed Kᵥ4.3 were obtained by G418 selection (Invitrogen, San Diego, CA). PCR analysis and whole cell patch-clamp recordings confirmed stable expression.

Construction of Kᵥ4.3 mutants (Kᵥ4.3T53A, S516A, and S550A), cell culture, and transfection. The mutants T53A, S516A (C1), and S550A (C2) and the double-mutant S516A and S550A (C1 and C2) of rat Kᵥ4.3 were prepared by performing PCR-based site-directed mutagenesis using the Quick-Change Multi site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The following primers were used for PCR: rKᵥ4.3T53A: 5'−GTGAGTGGC−3'; rKᵥ4.3S516A: 5'−GTGAGTGGC−3'; rKᵥ4.3S550A: 5'−GTGAGTGGC−3'; and rKᵥ4.3S516A and S550A (C1 and C2): 5'−GTGAGTGGC−3'.

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CGTCGGTTCAGCAAGCTGGAGGACACCTGTGGAGG-3’ (nt 156–199; GenBank accession no. U75448); rK4.3.S551A: 5’ GCAAGAT-TACCCATGCAAGGACAGCTGCTGTGCTGCCACTCCGG-CGC-3’ (nt 1,539–1,587); rK4.3.S550A: 5’ CTCCAATCTGGCCG-GCCACCCGGCTTGGCACATGCAAGAGCTCAGCACC-3’ (nt 1,640–1,688). Underlined characters show the mutation sites. PCR was performed in a 25-μl PCR mixture containing 2.5 μl of 10× reaction buffer, 1 μl of dNTP mixture, 10 ng of rK4.3 DNA in pCDNA3.1 (+) (Invitrogen) (4, 28) as a template, 125 ng of each primer, 3 μl of QuickSolution, and 1 μl of QuicKChange Multi enzyme blend. The cycling parameters were as follows: 1 cycle of 1 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. After completion of the PCR reaction, 10 units of Dpn I were added directly to the reaction. The reaction mixture was mixed and incubated at 37°C for 60 min to digest the parental supercoiled, double-stranded DNA. After digestion using Dpn I, the reaction mixture was transformed into Escherichia coli XL1-Blue ultracompetent cells (Stratagene), and the cells were grown overnight on a Lumia Broth (LB; Research Products International, Mt. Prospect, IL) plate containing ampicillin (60 μg/ml). Each sequence of rK4.3 mutants [rK4.3.T53A, rK4.3.S516A (C1), rK4.3.S550A (C2), and rK4.3.S516A and S550A (C1 and C2)] was confirmed by DNA sequencing.

HEK-293 cell lines were used for stable transfection with the calcium phosphate coprecipitation technique, and then G418 (GIBCO-BRL) resistance cells were selected as previously reported (27).

Voltage-clamp experiments. The whole cell configuration of the patch-clamp technique was used in these experiments. Patch pipettes were made from borosilicate glass capillaries pulled with a micropipette puller (P-80/PC; Sutter Instrument, Novato, CA) and polished with a microforge (MF-83; Narishige, Tokyo, Japan). The pipette resistances were 1–3 MΩ. After gigaseals were obtained, currents in response to depolarizing steps were amplified with an Axopatch 1A and/or Axopatch 200B amplifier and digitized with either a 12- or 16-bit analog-to-digital converter (Digidata 1322A and Digidata 1320A, respectively; Axon Instruments, Foster City, CA). Data were stored directly and digitized online using pClamp software (version 8.0; Axon Instruments). Data were sampled at 4 kHz, filtered at 1 kHz with a single exponential function, compared with control cells (n = 6), representing 73 ± 2% and 27 ± 2% (n = 6) of total inactivation (Fig. 1C). Both inactivation time constants decreased as a function of depolarization. Application of the known K4.3 blockers 4-AP and flecainide (15, 34, 42, 43) reduced the amplitude of currents evoked from these cells in a concentration-dependent fashion, with IC50 values of 1.1 ± 0.2 mM (n = 5) and 11.4 ± 2.9 μM (n = 6), respectively (data not shown).

Effect of autothiophosphorylated CaMKII on K4.3 currents. The effects of dialysis of HEK-293 cells with autothiophosphorylated CaMKII (10 nM concentration in the patch pipette) were tested on the inactivation properties of K4.3 currents. Representative current traces recorded from a cell dialyzed with autothiophosphorylated CaMKII are shown in Fig. 1. A pipette solution containing 10 mM BAPTA was used for these experiments to minimize endogenous activity of CaMKII. Autothiophosphorylated CaMKII dramatically slowed the rate of K4.3 current inactivation at all test potentials compared with controls and changed the profile of inactivation from a double to a single exponential function. For example, the mean time constant of inactivation in these cells with autothiophosphorylated CaMKII was 102 ± 18 ms and 13 ± 2 ms, respectively (P < 0.05). The voltage dependence of the activation and inactivation was investigated in cells dialyzed with 10 mM BAPTA to reduce intracellular Ca2+ to ~10 nM (17). At this concentration of Ca2+, endogenous CaMKII would be expected to have minimal activity. The voltage dependence of inactivation of the K4.3 current was characterized using the protocol shown in Fig. 2A, inset. Membrane potential was held for 1 s at conditioning potentials ranging from −80 to 0 mV. The conditioning potential was followed by steps to 0 mV. Normalized peak currents elicited by the test steps are plotted in Fig. 2B.
half-inactivation potential, determined from a Boltzmann function fitted to the data, was $-58 \pm 1$ mV ($n = 5$). The voltage dependence of activation of $K_V4.3$ currents was obtained by performing step depolarizations from $-80$ to $+40$ mV in 10-mV intervals (A, inset). A and B are families of currents evoked in the absence and presence of autophosphorylated CaMKII (10 nM) in the patch pipette. In A, the inactivation of $K_V4.3$ currents was fit by two exponentials (inset; thick line denotes actual fitting line at $+40$ mV). After dialysis with autophosphorylated CaMKII, the inactivation of $K_V4.3$ currents was fit by a single exponential. C and D: effects of voltage on the inactivation time constants in the absence and presence of autophosphorylated CaMKII (10 nM) in the patch pipette, respectively. $\tau_{fast}$, fast inactivation time constant; $\tau_{slow}$, slow inactivation time constant.

Previously, our laboratory (17) showed that autophosphorylated CaMKII not only decreased the rate of inactivation of $I_A$ in murine colonic myocytes but also accelerated the rate of recovery from inactivation. Thus we examined the effects of autophosphorylated CaMKII on the time required for $K_V4.3$ channels to recover from inactivation. Double-pulse protocols were designed to characterize recovery from inactivation at $+80$ mV (see Fig. 3A, inset). After a conditioning pulse to 0 mV, the cells were returned to $+80$ mV for recovery.
periods of 0–2 s before a second step to 0 mV. The time course of recovery from inactivation was determined by plotting the normalized peak test current as a function of the recovery interval. Examples of typical responses evoked by this protocol in the absence and presence of autothiophosphorylated CaMKII are shown in Fig. 3, A and C, respectively. The time course of recovery from inactivation was well fit with a single exponential function. Summary plots of recovery from inactivation in control cells and cells dialyzed with autothiophosphorylated CaMKII are shown in Fig. 3, B and D. Under control conditions, the mean time constant for recovery from inactivation was 325 ± 39 ms (n = 11), compared with 146 ± 19 ms (n = 6) in cells dialyzed with autothiophosphorylated CaMKII (10 nM). These data demonstrate that CaMKII significantly increased the rate of recovery of Kv4.3 channels from inactivation (P < 0.05).

Effect of inhibition of CaMKII on Kv4.3 inactivation kinetics. Because dialysis with autothiophosphorylated CaMKII slowed the rate of inactivation, we tested whether inhibition of endogenous CaMKII with a CaMKII-inhibitory peptide could produce opposite effects. Cells were dialyzed with pipette solution containing 0.1 mM EGTA and the CaMKII inhibitory peptide (nt 281–301; 100 μM). Repetitive pulses to +40 mV throughout the dialysis period elicited large, inactivating outward currents as described earlier. Figure 4A shows representative current traces immediately after rupture of the patch and after dialysis of the CaMKII inhibitor for ~3 min. Inclusion of the inhibitory peptide in the pipette solution significantly increased the rate of inactivation of Kv4.3 currents. For example, τI decreased from 136 ± 19 to 101 ± 9 ms (n = 9, P < 0.05). τr was similarly decreased, from 30 ± 3 to 23 ± 3 ms (n = 9, P < 0.05). Figure 4B summarizes data from nine experiments.

KN-93, a membrane-permeable methoxybenzenesulfonamide inhibitor of CaMKII with little or no effect on other protein kinases such as PKA and PKC (37), has been used to determine the role of CaMKII in a number of cell types (12, 13, 23). Therefore, we tested whether application of KN-93 could produce effects similar to those observed after dialysis with the CaMKII inhibitory peptide. Cells were dialyzed with pipette solution containing 10 mM BAPTA for this series of experiments. Figure 4C shows currents evoked from a cell expressing Kv4.3 upon a voltage step to +40 mV before and after 3-min exposure to 0.3 μM KN-93. The effect of KN-93 was maximal within this time period. The mean time for KN-93 to exert a maximal effect in nine cells was 152 ± 14 s. The CaMKII inhibitor significantly decreased the time for recovery from inactivation (P < 0.05). To confirm that the effects of KN-93 were specific, we also tested the effects of KN-92, an analog of KN-93 without inhibitory effects on CaMKII. KN-92 had no significant effect on Kv4.3 current amplitude (i.e., 3.4 ± 0.3 nA under control conditions and 3.3 ± 0.3 nA in the presence of KN-92). The mean time to reach 50% of peak amplitude was also not significantly affected by KN-92: 16.9 ± 2.8 ms under control conditions and 18.7 ± 3.4 ms in the presence of KN-92 (n = 7; P = 0.4).
Effect of mutations on CaMKII binding sites on the kinetics of Kv4.3 currents. There are three consensus sequences for phosphorylation by CaMKII in the sequence of Kv4.3 channels. We performed site-directed mutagenesis [i.e., T53A on the NH2 terminus, S516A (C1) and S550A (C2) on the COOH terminus] to test whether the effects shown in Figs. 1–4 attributed to CaMKII were due to direct effects of CaMKII on the pore-forming α-subunit of Kv4.3 channels. Cells stably expressing either wild-type Kv4.3 channels or Kv4.3 with T53A, S516A (C1), or S550A (C2) mutations were stepped from −80 mV to test potentials ranging from −80 to +40 mV (500-ms steps; Fig. 5). Cells were dialyzed with pipette solution containing 0.1 mM EGTA. Figure 5F shows summary data of the mean “half-decay time” of currents (e.g., measured as the time to 50% inactivation of the maximal current amplitude at 0 mV). This measurement was chosen instead of comparisons of rate constants because of the variability in the inactivation kinetics of wild-type Kv4.3 currents in pipette solutions containing 0.1 mM EGTA. The mean half-decay time decreased significantly in all mutants except C1 alone (P < 0.05). For example, the mean time taken to reach half decay was 68 ± 5 ms (n = 24) in control cells, compared with 50 ± 6 ms (n = 21) in T53A mutants, 32 ± 8 ms (n = 29) in cells with double mutations at C1 and C2, 62 ± 8 ms (n = 22) in C1 mutants, and 34 ± 2 ms (n = 19) in C2 mutants. These data show that although the rate of inactivation increased in the T53A mutants, the most dramatic effects on Kv4.3 inactivation occurred in C2 mutations or in double mutations at C1 and C2. Both groups of cells with these mutations expressed currents with similar changes in the rate of inactivation. Mutations at C1 alone, however, resulted in currents that were not quantitatively different from controls. These data suggest that the primary site on the Kv4.3 channel for phosphorylation by CaMKII is C2. Therefore, we tested whether the rate of recovery from inactivation and the responses to the application of autothiophosphorylated CaMKII or inhibition of CaMKII were affected by mutations at this site.

Effect of C2 mutations on rate of recovery from inactivation. We further tested whether mutations of CaMKII consensus sites on the COOH-terminal region of Kv4.3 channels would affect the rate of recovery from inactivation using the same double-pulse protocol illustrated in Fig. 3. Current responses from control cells and cells with mutations at C2 and at C1 and C2 are shown in Fig. 6, A–C, respectively. Figure 6D shows normalized τ values for the rate of recovery from inactivation between the different cell types. The rate of recovery from inactivation was significantly slowed in cells with the COOH-terminal mutations. τ values decreased by 41 ± 9.5% (n = 15) in C2 mutants and by 46 ± 8.1% (n = 14) in cells with C1 and C2 mutations.
Effects of autophosphorylated CaMKII on inactivation kinetics of Kv4.3 channels with COOH-terminal mutations. Dialysis of autophosphorylated CaMKII prolonged the inactivation of wild-type Kv4.3 channels (Fig. 1). We tested the effects of autophosphorylated CaMKII on channels with C2 mutations and those with C1 and C2 mutations. As demonstrated previously, the mean half-decay time for wild-type Kv4.3 channels was significantly slower in cells dialyzed with autophosphorylated CaMKII \((P < 0.05)\). Inactivation of currents in cells expressing either C2 or C1 and C2 mutations was not significantly affected by autophosphorylated CaMKII \((P > 0.05)\) for each; Fig. 7).

Effects of CaMKII inhibitors on channels with COOH-terminal mutations. As shown in Fig. 4A, inhibition of CaMKII with the specific CaMKII-inhibitory peptide (nt 281–301) increased the rate of inactivation of Kv4.3 currents. In control cells for this series of experiments, the mean half-decay time of Kv4.3 currents was significantly decreased (by 59 \(\pm\) 9\%) by the CaMKII-inhibitory peptide nt sequence 281–301 \((P < 0.05)\). The mean time to reach this level of inhibition was 7.4 \(\pm\) 1.7 min of dialysis (Fig. 8A). Dialysis with the CaMKII inhibitory peptide had no effect on currents in cells expressing Kv4.3 channels with C2 \((n = 6)\) or C2 and C1 \((n = 4)\) mutations after 17.3 \(\pm\) 2.5 and 14.4 \(\pm\) 3.5 min, respectively.

![Image](https://example.com/figure5.png)

**Fig. 5.** Effect of mutagenesis on specific CaMKII consensus sequences on Kv4.3 currents. **A–E:** representative current traces evoked by stepping from a holding potential of \(-80\) mV to test potentials from \(-80\) to \(+40\) mV for durations of 500 ms from cells stably expressing either wild-type Kv4.3 (A) or with mutations at T53A on the NH2-terminal (T-53; B) or at sites S516A (C1) and S550A (C2) on the COOH terminus (C–E). Cells were dialyzed with pipette solution containing 0.1 mM EGTA. **F:** summary data depicting the mean “half-decay time” \((\tau_{1/2})\) measured as the time to reach decay of 50% of maximal current amplitude from peak amplitude after a step to 0 mV.

![Image](https://example.com/figure6.png)

**Fig. 6.** Effect of C2 mutagenesis on the rate of recovery from inactivation. The same double-pulse protocol illustrated in Fig. 3 was used to investigate effects of mutations of CaMKII consensus sites on the COOH-terminal region of the Kv4.3 channel on the rate of recovery from inactivation. Typical current responses to this protocol from controls and cells with double mutations at C1 and C2 and C2 only are shown in **A–C**, respectively. **D:** normalized \(\tau\) values for the rate of recovery from inactivation between the different cell types.
Fig. 7. Effect of C2 mutagenesis on Kv4.3 channel inactivation kinetics in response to application of autothiophosphorylated CaMKII. A and B: representative current responses from cells transfected with Kv4.3 to depolarizing pulses to 0 mV in the absence and presence of autothiophosphorylated CaMKII, respectively. The mean half decay time was significantly slower in cells dialyzed with autothiophosphorylated CaMKII (C; \( P < 0.05 \)). Results from similar experiments on the C1 and C2 mutants are shown in D–F. Dialysis of autothiophosphorylated CaMKII did not significantly affect the rate of inactivation in these mutants (\( P > 0.05 \)). Likewise in the C2 mutants, autothiophosphorylated was without significant effect (\( P > 0.05 \), G–I, respectively).

(Figs. 8, B and C). Figure 8D shows plots of the half-decay time (as described above) as a function of time from rupture of patches.

DISCUSSION

In the present study, we have shown that CaMKII modulates the gating properties and kinetics of Kv4.3 channels. This conclusion is supported by the following three observations. 1) Dialysis of cells with autothiophosphorylated CaMKII decreased the rate of inactivation and increased the rate of recovery from inactivation. 2) Dialysis of cells with the CaMKII inhibitory peptide (nt 281–301) increased the rate of inactivation of Kv4.3 currents. 3) Treatment of cells with the membrane permeable CaMKII inhibitor KN-93 increased the

Fig. 8. Effect of C2 mutagenesis on Kv4.3 channel inactivation kinetics in response to inhibition of CaMKII. A: dialysis with the CaMKII inhibitory peptide 281–301 accelerates the rate of inactivation of Kv4.3 currents evoked by depolarizing steps to 0 mV (arrow indicates the traces every 30 s after rupture of membrane). Typical current responses to dialysis with CaMKII inhibitory peptide 281–301 in cells transfected with Kv4.3 containing double mutations at C1 and C2 or at C2 alone are shown in B and C, respectively. D: plots of half-decay time (\( \tau_{50\%} \)), measured as the time taken to reach decay of 50% of maximal current amplitude from peak amplitude after a step to 0 mV.
rate of inactivation of Kv4.3 currents and slowed the recovery from inactivation. Similar effects of KN-93 have also been observed on native I_o and A-type currents (17, 38) and are distinctly opposite the effects of dialysis of cells with autophosphorylated CaMKII.

Regulation of Kv4.3 currents by CaMKII appears to be mediated by direct phosphorylation of the pore-forming α-subunit of the channels. This conclusion is based on the observations that site-directed mutation at Ser^350 (i.e., S550A), a CaMKII consensus site near the COOH terminus, blocked effects due to dialysis with autophosphorylated CaMKII and rendered the channels unresponsive to inhibition of CaMKII. Mutations at this site (C2) hastened inactivation kinetics of mutated channels, suggesting that basal phosphorylation by CaMKII regulates the kinetics of wild-type Kv4.3 channels.

Kv4.3 channels contribute significantly to I_o in rat, canine, and human cardiac myocytes (9). The principal function of I_o is to set the level of the plateau phase of the cardiac action potential (AP) by initiating the early repolarization (phase 1 or the “notch” of the AP). Activation of cardiac I_o is crucial because it affects the activity of other voltage-dependent currents. The rapid inactivation kinetics displayed by I_o tend to limit the influence of this current to the early repolarization phase of the cardiac AP. Our finding that CaMKII decreased the rate of inactivation of Kv4.3 and increased current availability after steady-state inactivation suggests that this mechanism might tend to shorten the cardiac AP and could be important in regulating excitation-contraction coupling. Consistent with this hypothesis is the finding that inhibition of I_o resulted in prolongation of cardiac AP (19) and prolonged QT intervals (5). Kv4.3 channels are also molecular components of I_A expressed in neurons and smooth muscle cells (2, 28, 33, 34). The outward currents generated by activation of these channels oppose membrane depolarization and tend to stabilize membrane potential or reduce excitability. The physiological effects of Kv4.3 are therefore likely to depend on the kinetics and/or voltage dependence of activation and inactivation. Our data suggest that a rise in cytosolic Ca^{2+} would activate CaMKII, causing slowing of inactivation of Kv4.3 current and a shift of the window current range to more positive potentials. The shift in window current range may matter very little in cells with very negative resting potentials that depolarize through the entire range of voltage-dependent activation and inactivation during AP (i.e., cardiac muscle and neurons); however, smooth muscle cells that appear to use Kv4.3 currents to regulate membrane potential would be expected to have altered contributions to resting potential from A-type currents as a function of CaMKII activity.

We used KN-93 and CaMKII autoinhibitory peptide to inhibit the activity of endogenous CaMKII in HEK-293 cells. Because endogenous CaMKII activity depends on intracellular Ca^{2+} concentration, we tested the effects of CaMKII on the inactivation time constant in two different Ca^{2+} concentrations. CaMKII autoinhibitory peptide mainly inhibits Ca^{2+}-dependent CaMKII (7). Therefore, we used 0.1 mM EGTA internally to keep relatively high concentration of intracellular Ca^{2+} for CaMKII autoinhibitory peptide experiments. Cells dialyzed with 0.1 mM EGTA (~100 nM intracellular Ca^{2+} concentration) had 136 ms (τ_i) and 30 ms (τ_d) of inactivation time constants at +40 mV. The treatment with the CaMKII autoinhibitory peptide decreased the inactivation time constants. The inactivation time constants after CaMKII autoinhibitory peptide treatments were similar in cells dialyzed with 10 mM BAPTA (~10 nM intracellular Ca^{2+} concentration) which also decreased the time inactivation constants to 92 ms (τ_i) and 20 ms (τ_d). These data suggest that intracellular Ca^{2+} concentration can affect the time constants of Kv4.3 currents through CaMKII. Furthermore, even though cells were dialyzed with 10 mM BAPTA, Ca^{2+}-independent CaMKII is present in the cells (B. Perrino, unpublished observation). Therefore, we tested KN-93 effects on BAPTA-dialyzed cells to examine the effect of KN-93 on Ca^{2+}-independent CaMKII. The CaMKII autoinhibitory peptide inhibited Ca^{2+}/CaM-dependent activity because it mimicked the autoinhibitory domain of CaMKII. Autophosphorylation of CaMKII at Thr^286 blocked the autoinhibitory domain, as well as the autoinhibitory peptide containing the autoinhibitory domain amino acid sequence, from interacting with the catalytic domain (7). KN-93 can inhibit the Ca^{2+}-dependent (Thr^286 autophosphorylated) form of CaMKII in vivo because its inhibition is competitive with respect to CaM, causing Ca^{2+}-independent CaMKII activity to decrease as endogenous phosphatases dephosphorylate Thr^286 (37). Even though intracellular Ca^{2+} levels in BAPTA would be ~10 nM, the preexisting Ca^{2+}-independent CaMKII would be active and also would be inhibited by KN-93. Indeed, KN-93 significantly decreased τ_i and τ_d in cells dialyzed with 10 mM BAPTA. However, the specificity of KN-93 and KN-62 recently was questioned with regard to whether blockers of CaMKII produce nonspecific inhibitory effects on K_v currents in portal vein myocytes (20). This nonspecificity, however, does not affect our interpretation of the effects of KN-93 in the present study, because the HEK-293 cells (used to express Kv4.3) lack contaminating K_v currents. In addition, unlike a previous study (20), KN-93 (0.3 μM) had no discernible effects on current amplitude in our experiments. However, we should also note that higher concentrations of KN-93 (>3 μM) than we typically use in our experiments reduced K_v4.3 current amplitude (data not shown). Furthermore, KN-92, an inactive form of KN-93, was without effect on I_o on Kv4.3 currents in this study. Taken together, our data suggest that KN-93 had a specific inhibitory effect on CaMKII in our experiments.

Kv4 channels are modulated by a family of Ca^{2+} sensor proteins termed K_v channel-interacting proteins (KChIP) (3). When Kv4 channels were coexpressed with KChIP in a mammalian cell line, the amplitude of I_i was enhanced more than eightfold. KChIP increased trafficking of channels from the endoplasmic reticulum to the membrane (3). KChIP are known to be encoded by at least four genes, KChIP1–4 (3, 25), and are members of the recoverin/NCS family of Ca^{2+}-binding proteins, providing a potential pathway for regulation of Kv4 channels by Ca^{2+}. KChIP interact with Kv4 channels via the NH2 terminus, resulting in increased current density, decreased rates of inactivation, and accelerated recovery from inactivation. Recent studies have shown that the effects of KChIP on current density and inactivation kinetics occur independently of Ca^{2+}, whereas the effects of KChIP on the rate of recovery from inactivation are Ca^{2+} dependent (29). The effects of KChIP on Kv4 inactivation are similar to those produced by CaMKII in the present study. This suggests the possibility that CaMKII regulation may be mediated by mechanisms affecting...
KChIP expression or interactions with Kv4.3 channels. Our studies do not support this hypothesis, however, because we observed effects of CaMKII in the absence of KChIP. We also found that direct mutation of Kv4.3 (at Ser550) eliminated the effects of CaMKII, suggesting that the channel subunit is the direct target for CaMKII. Our data suggest that direct regulation of native I_{ca} by CaMKII should occur in cardiac myocytes, but further experiments are required to test this hypothesis on native currents.

In summary, the data presented in this study suggest that CaMKII regulates Kv4.3 currents kinetics by direct phosphorylation of α-subunits at Ser550. These data may provide novel insights into the cellular regulation of membrane excitability in cardiac, neuronal, and many smooth muscle cells.

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