Phosphorylation of S1505 in the Cardiac Na^+ Channel Inactivation Gate Is Required for Modulation by Protein Kinase C

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ABSTRACT Inactivation of both brain and cardiac Na^+ channels is modulated by activation of protein kinase C (PKC) but in different ways. Previous experiments had shown that phosphorylation of serine 1506 in the highly conserved loop connecting homologous domains III and IV ($L_{III/IV}$) of the brain Na^+ channel α subunit is necessary for all effects of PKC. Here we examine the importance of the analogous serine for the different modulation of the rH1 cardiac Na^+ channel. Serine 1505 of rH1 was mutated to alanine to prevent its phosphorylation, and the resulting mutant channel was expressed in 1610 cells. Electrophysiological properties of these mutant channels were indistinguishable from those of wild-type (WT) rH1 channels. Activation of PKC with 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) reduced WT Na^+ current by 49.3 ± 4.2% ($P < 0.01$) but S1505A mutant current was reduced by only 8.5 ± 5.4% ($P = 0.29$) when the holding potential was −94 mV. PKC activation also caused a −17-mV shift in the voltage dependence of steady-state inactivation of the WT channel which was abolished in the mutant. Thus, phosphorylation of serine 1505 is required for both the negative shift in the inactivation curve and the reduction in Na^+ current by PKC. Phosphorylation of S1505/1506 has common and divergent effects in brain and cardiac Na^+ channels. In both brain and cardiac Na^+ channels, phosphorylation of this site by PKC is required for reduction of peak Na^+ current. However, phosphorylation of S1506 in brain Na^+ channels slows and destabilizes inactivation of the open channel. Phosphorylation of S1505 in cardiac, but not S1506 in brain, Na^+ channels causes a negative shift in the inactivation curve, indicating that it stabilizes inactivation from closed states. Since $L_{III/IV}$ containing S1505/S1506 is completely conserved, interaction of the phosphorylated serine with other regions of the channel must differ in the two channel types.

KEY WORDS: heart • ion channels • sodium channel inactivation

INTRODUCTION

Voltage-gated cardiac Na^+ channels carry the Na^+ influx that underlies the upstroke (phase 0) of the action potential in the heart. Thus, cardiac Na^+ channels are critical for the initiation and spread of excitation and synchronous contraction of the heart. cDNA clones encoding the α subunit of cardiac Na^+ channels have been isolated from neonatal rat heart (rH1) (Rogart et al., 1989) and denervated skeletal muscle (SKM2) (Kallen et al., 1990). The Na^+ channel α subunit is predicted to have four homologous domains, each consisting of six transmembrane α helices. The amino acid sequence in the transmembrane segments has the greatest similarity, with the amino acid sequence in the intracellular connecting loops being less well conserved. Cardiac Na^+ channels have physiological and pharmacological properties that distinguish them from brain and muscle Na^+ channels. They are less sensitive to tetrodotoxin, more sensitive to antiarrhythmic drugs, have slower kinetics of activation and inactivation, and both activation and inactivation occur at more hyperpolarized potentials (Satin et al., 1992).

Activation of protein kinase C (PKC) modulates brain type IIA and skeletal muscle Na^+ currents. Activation of PKC decreases the current amplitude and slows the inactivation kinetics of brain type IIA current (Sigel and Baur, 1988; Dascal and Lotan, 1991; Numann et al., 1991; Schreibmayer et al., 1991). The highly conserved intracellular loop connecting homologous domains III and IV ($L_{III/IV}$)[1] is thought to be the inactivation gate (Vassilev et al., 1988, 1989; Stühmer et al., 1989; West et al., 1992; Beck et al., 1994; Hartmann et al., 1994). Preventing phosphorylation by mutation of serine 1506 in $L_{III/IV}$ abolished the effects of PKC (West et al., 1991). PKC activation also decreases Na^+ currents through native (Numann et al., 1994) and cloned (Bendahhou et al., 1995) skeletal muscle Na^+ channels. However, mutation of the analogous serine residue in that channel is reported to have little effect on current reduction by PKC (Bendahhou et al., 1995).

We have reported that activation of PKC inhibited native and heterologously expressed cardiac Na^+ currents in a voltage-dependent manner, with inhibition being greater at depolarized than at hyperpolarized holding potentials (Qu et al., 1994). This inhibition re-

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[1] *Abbreviations used in this paper:* $L_{III/IV}$, loop connecting homologous domains III and IV; WT, wild-type.

sults from a decrease in maximum Na^+ current and a negative shift in the voltage dependence of steady-state inactivation. Because $L_{III/IV}$ is highly conserved, we investigated whether phosphorylation of S1505 of the rat heart channel (analogous to S1506 in the rat brain type IIa Na^+ channel) is required for PKC modulation of cardiac Na^+ current. We report that mutation of serine 1505 to alanine abolished the negative shift in the voltage dependence of steady-state inactivation caused by PKC and reduced the overall reduction in peak Na^+ current from 49.3 ± 4.2% ($P < 0.01$) in wild type (WT) to 8.5 ± 5.4% ($P = 0.29$). Evidently, phosphorylation of this homologous serine residue is required for the differential functional effects of PKC on brain and cardiac Na^+ channels.

MATERIALS AND METHODS

Production of SNa-rH1-S1505A Cell Line

A region containing $L_{III/IV}$ (nucleotides 3185–5120) (Rogart et al., 1989) was cloned into M13mp19 (New England Biolabs Inc., Beverly, MA). Single-stranded uracil-containing DNA was produced, and the antisense oligo 5′-GGTTTCTTGGCGCCCAGC-3′ was used to make the mutant S1505A according to standard procedures for oligonucleotide-directed mutagenesis (Ausubel et al. 1996). Mutation S1505A was confirmed by double-stranded DNA sequencing and subcloned into a full length cardiac Na^+ channel construct in expression vector pCDNA3 (Invitrogen Corp., San Diego, CA) to yield pCDNA3.rH1.S1505A. pCDNA3.rH1.S1505A was transfected into Chinese hamster lung 1610 cells using the transfection reagent *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP, Boehringer Mannheim Biochemicals, Indianapolis, IN). Transfected cells were selected for G418 resistance (400 μg/ml), and 24 clonal lines were expanded for Northern blot analysis. RNA isolated from these cells was separated on a formaldehyde-agarose gel, and two clonal cell lines expressing the 6-kb mRNA expected for the cardiac Na^+ channel were selected for electrophysiological analysis. Of these, the cell line producing the largest currents was studied in detail.

Cell Maintenance and Electrophysiological Recording

Methods for maintenance of the cell lines and electrophysiological recordings are essentially the same as those described previously (Qu et al., 1994). Recordings were made at room temperature (~22°C) using the cell-attached configuration of the patch clamp technique (Hamill et al., 1981). The pipette solution contained (in mM): 150 $NaCH_3SO_3$, 1 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES. The bath solution was designed to "zero" the membrane potential and contained 140 KCH_3SO_3, 10 $NaCH_3SO_3$, 1 mM $CaCl_2$, 1.15 EGTA, 3 $MgCl_2$, and 10 HEPES. The pH of both solutions was adjusted to 7.4 with HCH_3SO_3. Cell-attached patches were allowed to stabilize for 30 min to an hour before recording to allow a gradual negative shift in the voltage dependence of channel kinetics to stabilize (Kimitsuki et al., 1990). Recordings were performed at room temperature (~22°C). 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide at 1 or 10 mM and stored at −20°C until used.

Data Transformation and Analysis

To generate conductance-voltage relationships from peak current versus voltage relationships, conductance was calculated from the peak current (I) elicited at each test pulse voltage (V) as $I/(V - V_r)$ where V_r is the measured reversal potential. Both normalized conductance-voltage and inactivation curves were fit with a Boltzmann equation: $1/\{1 + \exp[(V - V_x)/k]\}$, where V_x is the half activation (V_a) or inactivation (V_h) voltage and k is a slope factor. Least-squares fitting was done with Sigma Plot (Jandel Scientific, San Rafael, CA). Errors are reported as ±SEM.

RESULTS

Comparison of Na^+ Currents in Stable Cell Lines Expressing Wild-Type or Mutant S1505A Cardiac Na^+ Channel α Subunits

To assess the involvement of serine 1505 in $L_{III/IV}$ of the rat heart Na^+ channel, a cDNA in which serine 1505 had been mutated to alanine (S1505A) was stably expressed in 1610 cells (see MATERIALS AND METHODS). Currents recorded from this cell line expressing S1505A mutant Na^+ channels were compared to currents in an analogous cell line expressing the WT rH1 Na^+ channel α subunit (Qu et al., 1994). The cell-attached macropatch recording configuration (Hamill et al., 1981) was chosen for these experiments designed to study Na^+ channel modulation since it avoids rupture of the cell membrane and, thus, minimally disrupts the intracellular environment. In addition, voltage is well controlled in the small patches of membrane that are used.

Before examining modulation of S1505A mutant channels, we characterized their electrophysiological properties to determine whether these had been altered by the mutation. Fig. 1 *A* shows families of Na^+ currents recorded from patches on cells expressing either WT or S1505A mutant Na^+ channels. The expressed WT and mutant channels had nearly identical macroscopic kinetics of activation and inactivation (Fig. 1 *A*). Current-voltage relationships were also similar for WT and S1505A mutant channels (Fig. 1 *B*). Fits of the Boltzmann equation to conductance-voltage curves derived from such current-voltage relationships had a mean half maximal activation voltage, V_a, of −50.8 ± 10.3 mV with a slope factor, k, of −8.2 ± 1.2 ($n = 8$) for WT Na^+ channels, and a V_a of −52.9 ± 9.4 mV and k of −9.1 ± 1.3 ($n = 6$) for S1505A Na^+ channels (see Fig. 3, control data). Steady-state inactivation curves determined using 98-ms long prepulses to a range of potentials were also similar for WT and S1505A mutant Na^+ channels (Fig. 1 *C*). Fits of inactivation curves with a Boltzmann relationship gave a mean voltage for half maximal inactivation (V_h) of −82.3 ± 8.4 mV with a k of 7.3 ± 0.8 mV ($n = 8$) for WT Na^+ channels, and a V_h of −84.8 ± 4.3 mV with a k of 7.2 ± 1.7 mV ($n = 6$) for S1505A mutant Na^+ channels. None of the values for WT and S1505A channels differed significantly, indicating that this mutation did not affect the basic electrophysiological properties of the rat cardiac Na^+ channel. These results also suggest that there is little basal phosphorylation of serine 1505 in 1610 cells.

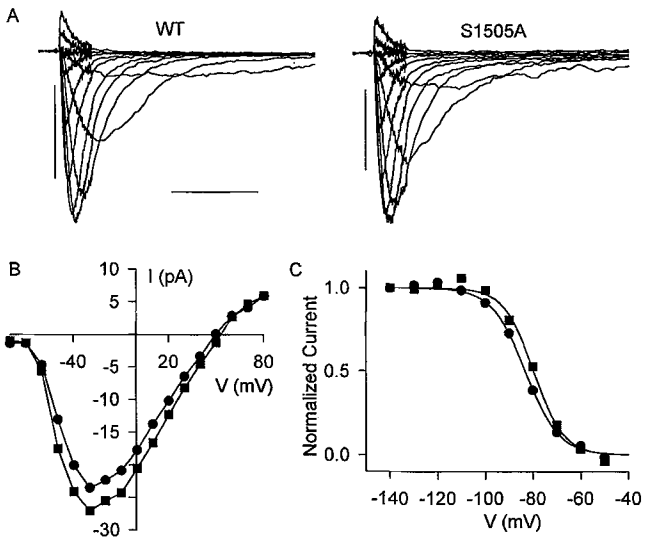

FIGURE 1. Comparison of currents due to WT and S1505A Na^+ channels stably expressed in 1610 cells. (*A*) Current traces recorded in 1610 cells transfected with WT (*left*) or S1505A mutant (*right*) Na^+ channels. Currents recorded during depolarizations to −74, −54, −44, −34, −24, −14, +6, +26, +46, +66, and +86 mV from a holding potential of −114 mV are shown. Calibration bars, 24 pA, 5 ms. (*B*) Current-voltage relationships measured from cells expressing WT (●) and mutant S1505A (■) Na^+ channels (same cells shown in *A*). (*C*) Voltage dependence of inactivation measured from the same WT (●) and mutant S1505A (■) cells with superimposed fits of a Boltzmann equation to the data (*lines*). Inactivation curves were obtained using 98-ms prepulses to various potentials followed by a test pulse to −24 mV. For these two cells, V_h = −83.2 mV and k = 6.3 mV for WT, and V_h = −79.7 mV and k = 7.1 mV for S1505A.

Modulation of WT and S1505A Na^+ Currents by Activation of PKC

Previous experiments demonstrated that activation of PKC with 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) strongly inhibited cardiac Na^+ currents in the cells expressing the WT channels (Qu et al., 1994). The degree of inhibition depended on the holding potential with greater inhibition being observed at more depolarized potentials (Qu et al., 1994). In the present series of experiments, the mean reductions in WT cardiac Na^+ current by application of 10 μM OAG were 49.3 ± 4.2% ($P <$ 0.01) at a holding potential of −94 mV, 36.5 ± 3.1% ($P <$ 0.01) at −114 mV, and 28.6 ± 1.4% ($P <$ 0.01) at −134 mV (n = 8) (Fig. 2, *WT*). Such effects of OAG are due entirely to PKC activation since they are essentially completely blocked by a specific peptide inhibitor of PKC (Qu et al., 1994). When cells expressing S1505A channels were studied contemporaneously using identical recording conditions, 10 μM OAG decreased Na^+ current by 8.5 ± 5.4% (P = 0.29) at a holding potential of −94 mV, by 9.3 ± 3.9% (P = 0.10) at −114 mV, and by 4.6 ± 3.5% (P = 0.20) at −134 mV (n = 6) (Fig. 2, *S1505A*). For S1505A, none of the reductions reached statistical significance by t test nor did the reductions of Na^+ current observed at different holding potentials differ significantly from each other. Thus, the potential-dependent effects of OAG were abolished and overall reduction of current by OAG was nearly completely prevented in cells expressing S1505A mutant channels.

To test whether the reduced responsiveness to OAG was due to diminished effectiveness of a particular OAG concentration in the cells expressing mutant channels, 100 μM OAG was also tested on S1505A mutant Na^+ currents. This OAG concentration produced an average reduction of 3.3 ± 5.8% (P = 0.70) at −94 mV, 8.1 ± 4.2% (P = 0.13) at −114 mV, and 6.8 ± 5.1% (P = 0.10) at −134 mV (n = 5). These reductions were not greater than those observed with 10 μM OAG. Thus, serine 1505 is required for response of the cardiac Na^+ channel to activation of PKC, consistent with the effect of mutation of the analogous serine in the rat brain type IIA Na^+ channel (West et al., 1991).

In addition to reducing the current, activation of PKC by application of OAG also hyperpolarized the voltage dependence of inactivation (Qu et al., 1994). This hyperpolarizing shift is partially responsible for the holding-potential dependence of the reduction in current by OAG. Whereas 10 μM OAG caused a 15-mV hyperpolarizing shift in the voltage dependence of inactivation of rH1 without changing the voltage dependence of activation, it did not alter either the voltage dependence of activation or inactivation significantly in mutant S1505A (Fig. 3). This is consistent with the finding that the small reduction of S1505A current by OAG does not depend on the holding potential (Fig. 2). Thus, the reduction in current is substantially reduced, and the negative shift in the voltage dependence of inactivation is eliminated in mutant S1505A.

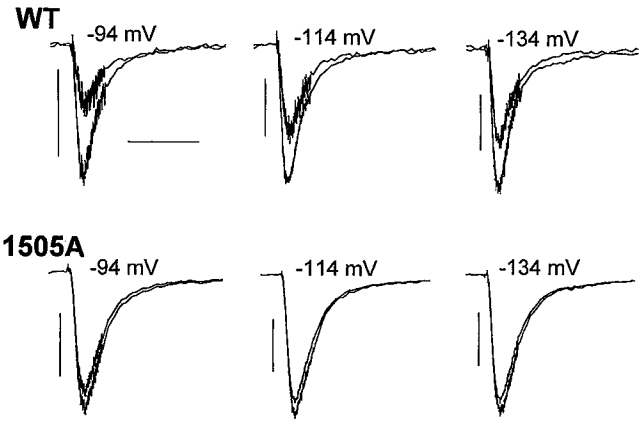

FIGURE 2. Effect of the PKC-activator OAG on WT and S1505A Na^+ currents. Currents were recorded in response to test depolarizations to −24 mV from the indicated holding potentials. Calibration bars: 12 pA, 4 ms for WT, and 24 pA, 4 ms for S1505A.

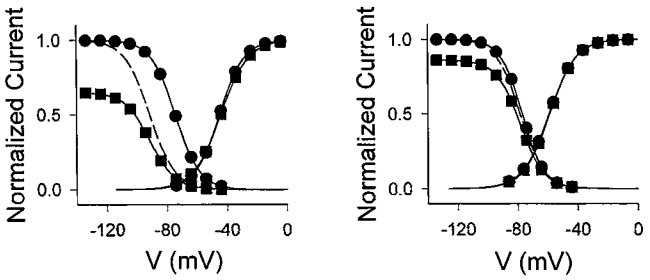

FIGURE 3. Effects of OAG on the mean voltage dependence of activation and inactivation of WT and mutant S1505A Na^+ channels. Mean steady-state Na^+ current activation and inactivation curves determined from cells expressing WT (*A*) and S1505A mutant (*B*) Na^+ channels in control solution (●) and, in the same experiments, after exposure to 10 μM OAG (■) using pulses to the potentials indicated by the symbols. The dashed lines are the data obtained in OAG after normalization. Conductance-voltage relationships and inactivation curves from individual experiments were fit with the Boltzmann equation. The data shown were derived from the mean values of V_x and k from those fits. The mean parameters obtained by fitting the control data are given in the text. The mean data determined in 10 μM OAG were, for activation of WT, $V_a = -52.0 \pm 9.5$ mV, $k = -9.2 \pm 1.2$ mV ($n = 6$), for activation of S1505A, $V_a = -52.5 \pm 6.5$ mV, $k = -8.9 \pm 1.5$ mV ($n = 6$); for inactivation of WT, $V_h = -97.2 \pm 11.5$ mV, $k = 8.7 \pm 2.8$ mV ($n = 6$), for inactivation of S1505A, $V_h = -87.1 \pm 4.7$ mV, $k = 7.9 \pm 0.8$ mV ($n = 6$).

DISCUSSION

Phosphorylation of S1505 is Required for PKC Effects on Rat Heart Na^+ Current

We have shown previously that the biophysical properties of WT rH1 Na^+ channels expressed in 1610 cells are virtually identical to those of native cardiac Na^+ channels in neonatal rat cardiac cells (Qu et al., 1994). Activation of PKC reduces current through both the stably expressed and the native cardiac Na^+ channels (Qu et al., 1994) and is associated with a negative shift in the voltage dependence of channel inactivation, resulting in stronger inhibition of the current at more depolarized holding potentials. This voltage dependence of PKC action is central to its physiological effect since much of the reduction in current observed at normal resting potentials results from the partial inactivation and stronger inhibition observed at these potentials. Serine 1506 in $L_{III/IV}$ is essential for modulation by PKC in rat brain rIIA Na^+ channels (West et al., 1991). Our results here show that mutation of the analogous serine (S1505A) in the rH1 rat heart Na^+ channel abolished the negative shift of the inactivation curve and inhibited decreases in current at all holding potentials. The remaining decrease in current through S1505A channels in response to PKC activation was not significantly larger than the response of WT channels when a peptide inhibitor of PKC was included in the recording pipette (Qu et al., 1994). Thus, the residual decrease in current may indicate that additional sites of PKC phosphorylation are involved in the response to PKC or that OAG has a small direct inhibitory effect on Na^+ current under our experimental conditions. Biochemical experiments detect multiple sites in the loop connecting homologous domains I and II of the rH1 cardiac Na^+ channel which are also phosphorylated by PKC (B.J. Murphy and W.A. Catterall, unpublished results), consistent with the idea that phosphorylation of additional sites is important in the action of PKC.

Effects of PKC Phosphorylation in the Inactivation Gate Differ in Different Na^+ Channels

The effects of phosphorylation of S1505 on inactivation are consistent with its location in $L_{III/IV}$ which is virtually completely conserved between different Na^+ channels and is proposed to form the Na^+ channel inactivation gate (West et al., 1992). Mutation of this serine to an alanine in either the brain Na^+ channel (West et al., 1991) or the cardiac Na^+ channel studied here has no effect on the channel properties before stimulation of PKC. However, this mutation prevents significant effects of PKC activation. In both Na^+ channel types, phosphorylation of this serine by PKC alters inactivation, but different aspects of inactivation are affected. In the brain channel, PKC slows the inactivation of open channels which is prevented by mutation of the analogous serine residue, but there is no change in steady-state inactivation. In the cardiac channel studied here, phosphorylation of S1505 causes a strong shift of steady-state channel inactivation in the negative direction which is completely blocked by mutation S1505A. $L_{III/IV}$ is involved in both processes since a variety of mutations of residues in this loop in both brain and cardiac channels affect both inactivation of open channels and the voltage dependence of steady-state inactivation of closed channels (Moorman et al., 1990; West, et al., 1992; Hartmann et al., 1994). However, the effects of adding a phosphate group seem fundamentally different in the two channels. In the rIIA brain channel phosphorylation slows and destabilizes open-state inactivation without affecting steady-state inactivation (Numann et al., 1991). This contrasts with the rH1 cardiac channel where phosphorylation of a serine in the same position stabilizes closed-state inactivation at negative membrane potentials as indicated by the negative shift in the inactivation curve. Since $L_{III/IV}$ containing the phosphorylated serine is virtually identical in the two channels, these results suggest that the residues with which this loop interacts as the channel inactivates differ between the two channels. Perhaps a positive charge in another region of the cardiac but not the brain channel interacts with the phosphate group on S1505 as the inactivation gate closes and stabilizes the inactivated state.

The effects of PKC on μ1 skeletal muscle Na^+ channels expressed in HEK293 cells (Bendahhou et al., 1995) are

similar to the effects we observe on the rH1 Na^+ channel expressed in 1610 cells or in native heart cells (Qu et al., 1994), but μ1 Na^+ channels expressed in a native skeletal muscle satellite cell line or in native skeletal muscle myoballs are modulated more similarly to brain Na^+ channels (Numann et al., 1994). Surprisingly, when the analogous serine was mutated in the μ1 skeletal muscle channel (S1321) and the resulting construct was expressed in HEK293 cells, PKC effects on reduction of peak Na^+ currents and voltage dependence of steady-state inactivation were unchanged (Bendahhou et al., 1995). Thus, despite the similar overall biophysical effects of PKC on cardiac Na^+ channels and on μ1 skeletal muscle Na^+ channels expressed in HEK293 cells, the role of the serine at this position in the inactivation gate may be quite different. Since the effect of PKC on the skeletal muscle Na^+ channel seems sensitive to the cellular background in which it is expressed, it is unclear whether these same effects will be observed when WT and mutant μ1 Na^+ channels are studied in other expression systems. Differences in modulation of μ1 Na^+ channels in different cell types may reflect preferential phosphorylation of different sites by the predominant PKC isozymes expressed in those cells.

In summary, our experiments show that S1505 in the inactivation gate of the cardiac sodium channel is critically involved in PKC modulation of the cardiac channel as it is for the brain channel. However, the different effects of phosphorylation of the same serine in the two α subunits suggests that the exact orientation of the phosphate group as well as the residues with which it interacts may also be important in modulation of the Na^+ current.

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