Activation of Protein Kinase A Modulates Trafficking of the Human Cardiac Sodium Channel in Xenopus Oocytes

Jingsong Zhou, Jianxun Yi, NingNing Hu, Alfred L. George, Jr, Katherine T. Murray

Abstract—Voltage-gated Na\(^+\) channels are critical determinants of electrophysiological properties in the heart. Stimulation of \(\beta\)-adrenergic receptors, which activate cAMP-dependent protein kinase (protein kinase A [PKA]), can alter impulse conduction in normal tissue and promote development of cardiac arrhythmias in pathological states. Recent studies demonstrate that PKA activation increases cardiac Na\(^+\) currents, although the mechanism of this effect is unknown. To explore the molecular basis of Na\(^+\) channel modulation by \(\beta\)-adrenergic receptors, we have examined the effects of PKA activation on the recombinant human cardiac Na\(^+\) channel, hH1. Both in the absence and the presence of h\(\beta_1\) subunit coexpression, activation of PKA caused a slow increase in Na\(^+\) current that did not saturate despite kinase stimulation for 1 hour. In addition, there was a small shift in the voltage dependence of channel activation and inactivation to more negative voltages. Chloroquine and monensin, compounds that disrupt plasma membrane recycling, reduced hH1 current, suggesting rapid turnover of channels at the cell surface. Preincubation with these agents also prevented the PKA-mediated rise in Na\(^+\) current, indicating that this effect likely resulted from an increased number of Na\(^+\) channels in the plasma membrane. Experiments using chimeric constructs of hH1 and the skeletal muscle Na\(^+\) channel, hSKM1, identified the I-II interdomain loop of hH1 as the region responsible for the PKA effect. These results demonstrate that activation of PKA modulates both trafficking and function of the hH1 channel, with changes in Na\(^+\) current that could either speed or slow conduction, depending on the physiological circumstances. (Circ Res. 2000;87:33-38.)

Key Words: sodium channels ▪ protein kinases ▪ heart

Voltage-gated Na\(^+\) channels play a pivotal role in the normal conduction of electrical impulses in the heart. In addition, dysfunction of Na\(^+\) channels can cause life-threatening cardiac arrhythmias by slowing impulse conduction\(^1\) or prolonging cardiac repolarization.\(^2\) Thus, the factors that regulate Na\(^+\) channel function are of great interest from both a pathophysiological and a therapeutic standpoint. Neurohumoral stimulation of \(\beta\)-adrenergic receptors, which activate cAMP-dependent protein kinase (protein kinase A [PKA]), can modulate cardiac electrophysiology and enhance conduction in normal ventricular myocardium.\(^3,4\) On the other hand, adrenergic stimulation is a potent stimulus for arrhythmogenic events in both the congenital long-QT syndrome\(^5\) and cardiomyopathic states.\(^5\) It is likely that modulation of ion channel function plays a role in this arrhythmogenesis.

The effects of \(\beta\)-adrenergic receptor stimulation on cardiac Na\(^+\) channel function have been controversial because of conflicting results of previous studies. More recent studies using rat ventricular myocytes have demonstrated a consistent increase in \(I_{\text{Na}}\) with PKA stimulation.\(^6,7\) with similar results obtained using recombinant cardiac Na\(^+\) channels. After expression in Xenopus oocytes, activation of PKA increased Na\(^+\) current derived from both the rat (rH1) and human (hH1) channels.\(^8,9\) In addition, in experiments to investigate “slip-mode” conductance, or Ca\(^{2+}\) permeation of cardiac Na\(^+\) channels, hH1 current was enhanced by activation of PKA when the channel was expressed in Chinese hamster ovary (CHO) cells.\(^10\) The mechanism of this PKA-mediated increase in cardiac Na\(^+\) current is currently unknown. However, it is recognized that protein kinases can regulate the activity of multiple receptors and transporters by modulating intracellular trafficking and the number of functional proteins at the cell surface.\(^11\) Recently, Lu et al\(^6\) proposed that PKA stimulation increased \(I_{\text{Na}}\) in rat ventricular myocytes by increasing the number of Na\(^+\) channels in the plasma membrane on the basis of single-channel recordings.

To investigate the molecular mechanisms of kinase modulation of the human cardiac Na\(^+\) channel, we have examined the effects of potent activation of PKA on hH1 currents. Our results demonstrate evidence for both direct effects to modulate channel function that are consistent with previous studies, as well as a previously unrecognized effect of PKA activation to alter trafficking of the channel.

Materials and Methods

Materials

Reagent-grade chemicals, 8-chlorophenylthio cAMP (8-cpt-cAMP), 3-isobutyl-1-methylxanthine (IBMX), forskolin, monensin, and chloro-
serotonin were obtained from Sigma. Enzymes and buffers were from Boehringer Mannheim and Promega. Both IBMX and forskolin were dissolved in DMSO to generate 400 and 20 mmol/L stock solutions, respectively (total bath concentration of DMSO was 0.3%).

**Na**⁺ Channel Expression**

DNA constructs of hH1, hβ1, hSKM1, and the hH1-hSKM1 chimeric channels (in a modified pSP64T vector) were linearized with XbaI or EcoRI and cRNA transcribed using the SP6 RNA polymerase (SP6 Cap-Scribe, Boehringer Mannheim). Defolliculated oocytes were obtained from *Xenopus laevis* after anesthesia with intraperitoneal tricaine (3%) and prepared as previously described. The protocol was approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Oocytes were injected with ≤20 nL of RNA diluted with RNase-treated water to achieve Na⁺ currents of ≤7.5 µA for experimentation (under control conditions, Na⁺ current at −20 mV was −2.9±0.7 and 3.6±0.7 µA for hH1 and hH1+hβ1, respectively). Where indicated, an excess of undiluted hβ1 RNA (in a ratio of 5:1 with hH1 RNA) was combined with α-subunit RNA in ratios that achieved maximal effect (as assessed during electrophysiological recordings after coexpression with hSKM1). In all experimental groups, data were obtained 24 to 48 hours after RNA injection.

**Electrophysiological Recording and Data Analysis**

Na⁺ current recordings were performed using the two-electrode voltage-clamp technique as previously described. The holding potential was −120 mV, and the cycle time for all pulse protocols was 1 second or slower to allow full recovery from inactivation between pulses, unless otherwise specified. Cell membrane electrical capacitance was measured as detailed previously.

In a representative group of cells (ie, those expressing hH1+hβ1), capacitance averaged 342±44 nF (n=10). Experiments were conducted at room temperature (22±2°C).

Data analysis was performed using custom programs designed to read and analyze pClamp data files. The reversal potential was estimated using a linear fit to the ascending limb of the current-voltage relationship to construct activation curves. Activation and inactivation curves were fitted with a Boltzmann equation. The time course of recovery from Na⁺ current inactivation was fitted with an exponential function using a nonlinear least-squares algorithm. Comparison of the voltage-dependent and kinetic properties of Na⁺ currents after PKA stimulation to control values was performed using a paired t test (P≤0.05 was considered significant). Results are presented as mean±SEM.

**Results**

**Effect of PKA Activation on hH1 and hH1 Currents**

To investigate the effects of PKA activation, Na⁺ currents derived from expression of the hH1 α subunit alone were studied initially. To stimulate PKA in *Xenopus* oocytes, cells were perfused with a combination of kinase activators (8-cpt-cAMP 200 µmol/L, IBMX 1 mmol/L, and forskolin 10 µmol/L), which has been shown to activate the cystic fibrosis transmembrane conductance regulator (CFTR) in a rapid, potent manner. Figure 1 demonstrates that stimulation of PKA caused a significant increase in hH1 current amplitude (+67±14% at −20 mV in 55 minutes; n=8). The time course of this effect was slow, as shown in Figure 1B, with a continual increase in current for nearly 1 hour without saturation. Activation of PKA did not alter cell membrane electrical capacitance (0±0% in 55 minutes), indicating that measurable changes in cell surface area were not involved in the effect observed. In a separate group of cells, Na⁺ currents were unchanged after bath superfusion of DMSO for a similar time period (+3±4% in 55 minutes; n=6), eliminating the possibility of a nonspecific effect due to either experimental time or vehicle. In contrast to hH1, PKA stimulation had no effect on Na⁺ currents derived from expression hSKM1 (−1±2% at −20 mV in 55 minutes; n=6), as demonstrated in Figure 1B.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of PKA activation on hH1 and hSKM1 currents. A, hH1 currents are shown under control conditions (left) and after bath superfusion of PKA activators (right; voltage was stepped from a holding potential of −120 mV, to −100 mV to +30 mV at 10-mV intervals). B, Time course of effect of PKA activators on peak Na⁺ current (at −10 mV) is illustrated for hH1 (○) and hSKM1 (●). Averaged data are expressed normalized to predrug value. Time 0 (arrow) indicates start of drug infusion. C, Using the voltage-clamp protocol described in panel A, peak hH1 currents were plotted as a function of test potential under control conditions (○) and after PKA stimulation (●). D and E, Similar data are shown for hH1+hβ1 and hSKM1+hβ1 currents.
Coexpression of hH1 and hβ1 Subunits

Additional experiments were performed to determine whether coexpression of hβ1 with hH1 modified the effects of PKA activation. As shown in Figure 1D, Na⁺ currents derived from expression of hH1+hβ1 increased after bath superfusion of kinase activators (+26±5% at −20 mV in 55 minutes; n=10), although the magnitude of the increase was smaller than for hH1 alone (+67±14%). Na⁺ current increased slowly with a time course similar to that for hH1 alone (data not shown). For hSKM1, Na⁺ currents remained unchanged with stimulation of PKA despite coexpression of hβ1 (n=5), as illustrated in Figure 1E.

Na⁺ Current Gating and Kinetics

To characterize the mechanism of the PKA effect, we examined whether kinase activation altered the voltage dependence or kinetics of channel gating. For both hH1 and hH1+hβ1 (Figure 2), PKA stimulation resulted in a small but significant shift in the activation curve to more negative potentials (midpoint or V1/2 was −32±2/−36±2 and −33±2/−37±2 mV before/after PKA for hH1 and hH1+hβ1, respectively). For hSKM1+hβ1, this effect was not apparent (V1/2 was −28±2/−29±1 mV before/after PKA). Similarly, activation of PKA was associated with a significant shift in the voltage dependence of channel availability or inactivation for both hH1 and hH1+hβ1 (Figure 2; V1/2 was −77±1/−82±2 and −64±1/−70±2 mV before/after PKA, respectively), as well as for hSKM1+hβ1 (V1/2 was −60±1/−62±1 mV before/after PKA). During control experiments with DMSO, the availability curve for hH1 was stable over time (V1/2 was −70±3/−71±3 mV before/after DMSO; n=4), indicating that the effects observed for the different experimental groups indeed resulted from PKA activation. Using a 2-pulse protocol (initial 100-ms pulses to 0 mV), the time course of recovery from fast inactivation (at −120 mV) was examined. Activation of PKA resulted in slowing of the recovery process (τ=4.7±0.1/5.8±0.3 ms before/after PKA). Although PKA stimulation caused alterations in the voltage dependence of channel gating and recovery of fast inactivation, these effects could not account

for the increase in Na⁺ current derived from hH1+hβ1, and hH1 alone that was observed.

Effects of Chloroquine and Monensin

The slow, nonsaturable increase in Na⁺ current with PKA activation suggested a mechanism other than direct modulation of channel subunit function by phosphorylation. Typically, such direct modulation occurs rapidly and in a saturable fashion, as we have recently shown for relief of β subunit–mediated K⁺ current inactivation of the Kv1.5+Kv1.3 ion channel complex when the β subunit is phosphorylated by PKA.14 Therefore, we hypothesized that activation of PKA altered trafficking of hH1, resulting in an increase in the number of channels at the cell surface membrane.

The monovalent carboxylic ionophore monensin disrupts transport of membrane vesicles from the Golgi complex to the plasma membrane.16 In addition, it increases lysosomal pH to interrupt recycling of cell surface proteins such as receptors for LDL, transferrin, and insulin.17–19 A similar lysosomal effect is produced by chloroquine.20–22 To determine whether activation of PKA disrupts trafficking of the hH1 channel, cells were preincubated in either chloroquine (100 μmol/L) or monensin (100 μmol/L) before exposure to kinase activators. Figure 3 demonstrates that in cells coexpressing hH1 and hβ1, bath superfusion of chloroquine led to a marked reduction in Na⁺ current (−70±4% in 25 minutes;
Figure 4. Correlation of initial Na\textsuperscript{+} current amplitude and response to PKA. Percent change in Na\textsuperscript{+} current (hH1+h\(\beta_1\)) with PKA stimulation was plotted as a function of Na\textsuperscript{+} current amplitude under control conditions.

n=4) that occurred rapidly (\(\tau\) for Na\textsuperscript{+} current decline was 4.6±0.5 minutes). This decline in Na\textsuperscript{+} current resembled the loss of cell surface expression seen for membrane proteins such as P-glycoprotein and LDL receptors after exposure to either chloroquine and monensin, an effect attributed to interruption of insertion of membrane vesicles into the plasma membrane in the face of continued internalization.\textsuperscript{17,20} After preincubation with chloroquine, subsequent addition of PKA activators to the bath had no effect on Na\textsuperscript{+} current amplitude (+4±5% in 55 minutes; n=3). A similar effect was seen with monensin, which caused Na\textsuperscript{+} current to decline (−16±4% in 25 minutes; n=4) with a rapid time course (\(\tau\)=3.9±1.0 minutes) and prevented the effects of PKA to increase Na\textsuperscript{+} current (+1±8% in 50 minutes; n=3). Na\textsuperscript{+} current derived from expression of hH1 alone also declined in response to chloroquine and monensin (−67±5% and −23±5% in 25 minutes, respectively; n=7 and 6). Thus, 2 different compounds that disrupt recycling of plasma membrane by distinct mechanisms could prevent the effect of PKA to increase Na\textsuperscript{+} current, indicating that this phenomenon likely resulted from an increase in the number of channels in the plasma membrane.

To control for nonspecific effects of the test compounds, we preincubated cells coexpressing Kv1.5 and Kv\textbeta1.3 with chloroquine followed by exposure to PKA activators. In contrast to the results for hH1, Figure 3B demonstrates that chloroquine had little effect on K\textsuperscript{+} current in these cells (−8±4% in 25 minutes; n=5), suggesting that the K\textsuperscript{+} channel subunits under study were less susceptible to the recycling process than hH1. Moreover, chloroquine did not prevent the rapid increase in K\textsuperscript{+} current amplitude with PKA stimulation due to \(\beta\)-subunit phosphorylation and relaxation of \(\beta\)-mediated inactivation (+33±6% in 17 minutes; n=5). These results indicate that chloroquine did not disrupt the ability of the cells to activate PKA to account for its effects on the hH1 current response.

The increase in hH1 current with PKA activation could result from either reduced internalization or increased insertion of channel subunits into the cell membrane. If internalization is reduced, the relative Na\textsuperscript{+} current increase should be a function of the number of channels present in the membrane under control conditions. In Figure 4, the percent change in \(I_{\text{Na}}\) is plotted as a function of the initial or control value. No correlation exists between these 2 parameters. These data, along with the known effects of both chloroquine and monensin on other plasma membrane proteins, support the concept that PKA stimulation increases hH1 current by promoting insertion of channels into the surface membrane.

Role of the I-II Interdomain Loop of hH1 in the Effects of PKA

The amino acid sequence of hH1 contains a number of putative sites for phosphorylation by PKA. The number of sites present varies depending on the consensus sequence used, but they are primarily concentrated in the I-II and II-III interdomain regions of the channel. Given that hSKM1 current is not affected by PKA stimulation, hH1-hSKM1 chimeric channels were used to localize the region in hH1 responsible for the PKA effect. The chimeric constructs included hH1 containing either the I-II (hH1-A) or II-III (hH1-B) interdomain region of hSKM1, and hSKM1 with either the I-II (hSKM1-A) or II-III (hSKM1-B) interdomain loops of hH1.\textsuperscript{12} As shown in Figure 5A, replacing the I-II interdomain region of hH1 with the analogous region of hSKM1 (hH1-A) caused loss of the PKA response to increase Na\textsuperscript{+} current, whereas the opposite chimera (hSKM1-A) demonstrated gain of function, with a rise in Na\textsuperscript{+} current after PKA activation. Chimeric channels involving the II-III interdomain loop (hH1-B, hSKM1-B) retained the wild-type response (Figure 5B). These results localize the I-II interdomain loop of hH1 as the region in the channel responsible for the PKA response.
Discussion

Our results demonstrate that PKA activation modifies Na\(^+\) current derived from expression of the human cardiac Na\(^+\) channel by at least the following 2 mechanisms: modulation of protein function to alter channel gating and perturbation of channel trafficking to cause an increase in Na\(^+\) current.

With respect to channel gating, our findings are largely similar to those of previous investigations of cardiac Na\(^+\) currents in native myocytes and the recombinant hH1 channel. In studies of \(I_{Na}\) in cardiac myocytes, the most reproducible finding has been a shift in the voltage dependence of channel availability to more negative potentials,\(^{23-26}\) which we also observed. Moreover, when the hH1 channel was expressed in HEK cells, similar findings were obtained.\(^{27}\) In another study using Xenopus oocytes, a shift in steady-state inactivation of hH1 did not occur.\(^{9}\) However, stimulation of PKA was accomplished by intracellular injection of individual kinase activators and therefore may have been less robust than in the present study. For channel activation, other investigators have also reported a negative shift with PKA using both myocytes\(^{26}\) and recombinant cardiac Na\(^+\) channels,\(^{8,27}\) although this effect has been more variable.

Prior studies indicate that the effects of PKA activation on Na\(^+\) current amplitude have been controversial. Early studies with mammalian ventricular myocytes demonstrated a reduction in cardiac \(I_{Na}\) in response to PKA.\(^{23,24,28-29}\) However, relatively positive holding potentials were used. In subsequent investigations in which cells were held at more negative voltages (that would minimize the effects of the negative shift in inactivation), Na\(^+\) currents were increased by kinase stimulation.\(^{6,7,25,26,30}\) For hH1, Frohnwieser et al\(^{9}\) also reported that PKA activation caused a slow increase in Na\(^+\) current, although the mechanism of this effect was not investigated. When hH1 was expressed in HEK cells (holding potential of \(-100\) mV), Na\(^+\) current declined in response to isoproterenol.\(^{27}\) However, this effect occurred in the background of a significant negative shift in the voltage dependence of inactivation, as well as mild rundown in \(I_{Na}\), which together may explain the reduction of hH1 current in these experiments. Indeed, Na\(^+\) current increased in response to PKA stimulation when hH1 was expressed in CHO cells.\(^{10}\) Together, these studies indicate that in cells with more positive resting potentials, PKA stimulation reduces hH1 current largely as a result of the negative shift in channel availability, whereas in cells with more negative resting potentials, \(I_{Na}\) increases in response to kinase activation. These results are corroborated by data obtained in vivo in cardiac syncytial preparations. For example, in Purkinje fibers and ventricular myocardium, \(\beta\)-adrenergic stimulation has been shown to increase impulse conduction velocity,\(^{3,4}\) an effect consistent with an increase in \(I_{Na}\). On the other hand, reentrant arrhythmias due to slowed conduction have been shown to originate in damaged but viable tissue (eg, an infarct border zone), where myocyte resting potentials are frequently abnormal.\(^{1}\)

Our experimental findings reveal a previously unrecognized mechanism for kinase modulation of cardiac Na\(^+\) channels, as PKA stimulation appeared to alter trafficking of hH1 to increase the number of channels in the plasma membrane. Although we cannot totally rule out an effect of PKA to increase channel synthesis, the time course of this effect (the initial increase in hH1 current occurred within 5 to 10 minutes) strongly argues against this hypothesis, especially for a plasma membrane protein that must traverse the Golgi before membrane insertion. Moreover, we found that both chloroquine and monensin could inhibit the PKA-mediated increase in hH1 current. These agents are known to perturb intracellular vesicle trafficking and have been used extensively to study recycling of membrane proteins.\(^{17-22}\) As an important control for these experiments, chloroquine had no effect on the PKA response of K\(^+\) current derived from coexpression of Kv1.5 and Kv\(\beta\)1.3, an effect mediated by direct phosphorylation of the K\(^+\) channel \(\beta\) subunit. These results demonstrate that the PKA response in these cells was intact and that the mechanism of regulation for the 2 ion channel complexes must be different.

This distinct effect of PKA activation on the hH1 channel contributes to the increasing recognition that protein kinase stimulation can alter trafficking of membrane transporters and ion channels,\(^{11}\) in addition to previously described effects on function, expression, and synthesis. For example, modulation of the activity of the glucose transporter GLUT4 occurs by insulin-stimulated translocation of the protein from intracellular stores to the plasma membrane.\(^{31}\) Similarly for the \(\gamma\)-aminobutyric acid transporter GAT1, stimulation of protein kinase C causes relocation of transporters from cytosolic compartments to the plasma membrane.\(^{32}\) Additional studies have demonstrated that CFTR rapidly redistributes from the cytoplasm to the cell surface in multiple different cell types after cAMP stimulation.\(^{33-35}\) Other voltage-gated channels are likely subjected to similar regulation. For example, activation of PKA modulates function of the Kv1.1 channel by 2 mechanisms, which include both enhanced channel synthesis as well as redistribution of previously synthesized channels to the plasma membrane.\(^{36}\) Importantly, for at least 2 of the proteins described above (GLUT4 and CFTR), results from studies conducted in oocytes were consistent with findings in native cells, demonstrating interchangeability of mammalian and amphibian secretory pathway components and thus biochemical conservation in the regulation of these pathways. Nevertheless, as for other proteins studied, and given the inherent limitations of transient heterologous expression, additional experiments using both mammalian expression systems and cardiac ventricular myocytes are important to confirm further the effects of PKA. The recent findings by Lu et al\(^{9}\) suggesting that PKA activation increases the number of functional Na\(^+\) channels in the plasma membrane of rat cardiomyocytes indicate that a similar phenomenon likely occurs in these cells.

Our data also suggest that the hH1 channel is subjected to rapid turnover in the plasma membrane, given the prompt reduction in Na\(^+\) current on exposure to either chloroquine or monensin. Similar findings were obtained with these compounds in studies of other membrane proteins with respect to the time course and extent of reduction in cell surface expression.\(^{17,19,21,22}\) Neuronal Na\(^+\) channel isoforms can also be modulated by perturbation of channel recycling. In immature neurons, activation of Na\(^+\) channels with the neurotoxin veratridine causes rapid internalization of channels, an effect mediated by increased [Na\(^+\)]\(_{e}\).\(^{37}\) This effect is lost in adult neurons concomitant with the
development of β, coexpression, suggesting that lack of subunit anchoring in the plasma membrane may be the mechanism responsible for this effect. In our results, coexpression of the hβ2 subunit with hH1 reduced but did not abolish the PKA response to increase Na⁺ current, indicating that additional molecular mechanisms are involved.

In conclusion, we have shown that activation of PKA alters the human cardiac Na⁺ channel by both direct effects on channel function and indirect effects to alter channel recycling. These findings demonstrate a previously unrecognized mechanism whereby cardiac cells may regulate endogenous Na⁺ currents.

Acknowledgments

This work was supported by grants from the US Public Health Service (HL55665, HL02363, HL47599, and NS2387) and the American Heart Association, Tennessee Affiliate. We thank James May, Christine Williams, and Hyeon-Gyu Shin for suggestions.

References
