Mechanism of cAMP-Dependent Modulation of Cardiac Sodium Channel Current Kinetics

Katsushige Ono, Harry A. Fozzard, and Dorothy A. Hanck

β-Adrenergic modulation is one of the most important regulatory mechanisms of ion channel function. Only recently, however, have β-adrenergic effects on cardiac Na⁺ channel activity been recognized, and some diversity of effects has been reported in different preparations. We report studies of protein kinase A-dependent phosphorylation effects on cardiac Na⁺ current using the macropatch on-cell mode voltage-clamp technique to maintain cytoplasmic composition intact. During the first 5 minutes after addition of 8-(4-chlorophenylthio)cAMP to the bath, the midpoints of both voltage-dependent availability and conductance shift in the hyperpolarizing direction an average of -7.5±2.8 mV (n=31). Moreover, these effects were not species specific; similar results were obtained in canine, rabbit, and guinea pig myocytes, and a similar shift occurred after exposure to 5 μM isoproterenol. Maximum conductance did not change, nor did single-channel conductance. The shifts of conductance and voltage-dependent availability that were induced by protein phosphorylation were distinct from and independent of the slow background shift in kinetics. We measured the background shift to be less than 0.3 mV/min and to be restricted to the channels within the patch. Pretreatment of cells with a blocker of protein kinase, N-[2-((p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (II-89), prevented the effect of 8-(4-chlorophenylthio)cAMP while not affecting the background shift in kinetics. Although clearly not the result of addition of a negatively charged phosphate to the inside face of the channel, cAMP-dependent phosphorylation affects the voltage-dependent kinetics, as expected, by an electrostatic interaction with the voltage sensor. (Circulation Research 1993;72:807–815)

KEY WORDS • sodium channel • kinetics • macropatch clamp • cAMP • isoproterenol

Cardiac excitation is critically dependent on the density and availability of voltage-gated Na⁺ channels. Na⁺ channel availability has been assumed to be primarily controlled by voltage, although recently several laboratories have published data consistent with a negative shift of voltage-dependent availability by phosphorylation using the maximum rise of the action potential upstroke (Vₘₐₓ) as an indicator of Na⁺ current (Iₐᵥ) in whole-cell recordings,5,9 and in isolated patches.10 However, not all investigators agree on this action. No change in voltage-dependent availability was reported by Murray et al11 at 36°C and by Wendt et al12 for currents recorded through perforated patches. Gintant and Liu13 observed a shift of voltage-dependent availability in isolated myocytes but no effect on Vₘₐₓ in intact fibers. We undertook to further study the effects of protein kinase A (PKA)–dependent phosphorylation on cardiac Na⁺ channels. We chose the cell-attached macropatch for these studies to avoid modification of cytoplasmic constituents, which is unavoidable during whole-cell current recordings and which could alter enzymatic actions. Because a time-dependent shift of Iₐᵥ kinetics has been reported even for recordings in cell-attached patches,14 we sought to distinguish PKA effects from time-dependent changes in kinetics and from current run-down. Our results in cardiac myocytes show that both conductance and voltage-dependent availability curves are significantly shifted in the hyperpolarized direction by cAMP and isoproterenol. This effect is mediated by cAMP-dependent phosphorylation and is independent of and distinct from the time-dependent shift of kinetics that occurs during recording. The shift in both conductance and availability can account for many of the seemingly disparate findings previously reported.

Materials and Methods

Isolation of Single Ventricular Myocytes

Ventricular myocytes were isolated from adult mongrel dogs, New Zealand rabbits, and guinea pigs. Animals were anesthetized with sodium pentobarbital (40 mg/kg i.v.). Hearts were quickly removed and rinsed in cold normal Tyrode’s solution. For preparation of canine myocytes, a portion of myocardium was enzymatically dissociated using a procedure modified from that of Salata and Wasserstrom.15 In brief, a muscle column (1.6-mm diameter) of free wall from left ventricular or from intraventricular wall was obtained by a biopsy needle (Travenol Laboratories, Deerfield, Ill.) and placed in a calcium-free cardioplegia solution at 25°C with gentle shaking for 10 minutes. Approximately 30
muscle columns were then incubated in 8 ml enzyme solution (2.5 mg/ml type 2, Worthington Biochemical Corp., Freehold, N.J.) in a plastic test tube at 37.5°C and continuously stirred with O2 bubbling. After 15 minutes, the supernatant was discarded, and the pellets of isolated cells were washed three times with 8 ml amino acid-rich (Kraftbrühe) medium. After each wash, the tube was centrifuged, and the supernatant was discarded.

Rabbit myocytes were prepared using a modification of the method of Poole et al.10 Hearts were retrogradely perfused via the aorta at 37°C with a buffer gassed with 100% O2 containing (mM) NaCl 130, HEPES 5, glucose 10, taurine 20, creatine 10, KCl 5.4, MgCl2 3.5, and NaH2PO4 0.4 (pH 7.25, adjusted with NaOH). The heart was first perfused for 4 minutes with buffer plus 0.75 mM CaCl2 and then for 4 minutes with buffer plus 0.1 mM EGTA. Finally, the heart was perfused for 10 minutes with buffer plus 0.08 mM CaCl2 plus 1 mg/ml Worthington type II collagenase plus 0.1 mg/ml type 14 protease (Sigma Chemical Co., St. Louis, Mo.). The heart was removed from the perfusion apparatus and coarsely chopped, and selected pieces were shaken at 37°C for 5-minute periods in flasks containing the collagenase solution with 1 mg/ml bovine serum albumin added. After each 5-minute incubation, tissue from one flask was filtered through a nylon gauze (pore size, 200 μm). The filtered material was centrifuged at low speed, and the harvested cells were transferred to a solution containing 150 mM potassium glutamate and 10 mM HEPES (pH 7.2).

Guinea pig cells were prepared by the method of Mitra and Morad17 In all cases, cells were stored at room temperature or refrigerated and studied within 24 hours of isolation. For study, small aliquots of cells were added to a chamber of 200-μl volume constructed on a glass coverslip coated with poly-L-lysine (molecular weight, >300,000, Sigma) and mounted on a stage of an inverted microscope (Diaphot, Nikon, Japan). Only cells with clear borders and striations were selected for study.

Solutions and Drugs

The bath solution contained (mM) potassium aspartate 150, HEPES 10, and MgCl2 2, pH 7.4, with CsOH. The pipette solution contained (mM) NaCl 280, CaCl2 1, MgCl2 1, tetraethylammonium chloride 10, HEPES 10, and nifedipine 2.5×10⁻⁴, pH 7.4, with tetraethylammonium hydroxide. Nifedipine, isoproterenol, and 8-(4-chlorophenylthio)cAMP (CPTcAMP) were obtained from Sigma. A protein kinase inhibitor relatively specific for PKA, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89),18 was kindly provided by H. Hidaka, Department of Pharmacology, Nagoya University School of Medicine (Japan) or was obtained from Calbiochem Corp., La Jolla, Calif. H-89 was used at 1 μM diluted from a stock of 10 mM in dimethyl sulfoxide (DMSO). Nifedipine and isoproterenol (with 10 mM HCl) were diluted from 10 mM stock in ethanol. CPTcAMP at 2 or 5 mM was added to the bath solution from powder just before the experiment. Control experiments (n=5) showed that ethanol at fourfold higher concentration (0.1%) than present for the experiments (0.025%) had no effect on Iiso. DMSO, when present, was at a concentration of 0.01%, and control experiments (n=3) showed no effect of DMSO when present at 0.1%.

Electrical Recording and Data Analysis

Recordings (List EPC-7, List Electronics, Darmstadt-Eberstat, FRG) were made in the cell-attached configuration19 to maintain cellular metabolic conditions intact. Bath solutions were assumed to collapse the membrane potential so that applied potential was taken as the patch potential. Large patch pipettes (macropatch) with resistances ranging between 0.2 and 0.6 MΩ were used to record from patches containing 20–250 Na+ channels. Small patch pipettes (conventional) with resistances ranging between 6 and 8 MΩ were used to record from patches with small numbers of channels. Patches were depolarized for 30–45 msec each 0.5–2 seconds from a holding potential (VH) of −150 mV.

To assess voltage-dependent channel availability, patches were conditioned at potentials between −150 and −50 mV for 500 msec and then stepped to +20 mV. Peak currents from the test steps were normalized to the peak current in a step depolarization from a VH of −150 to +20 mV. Patches were allowed to recover for 1.4 seconds between conditioning steps. Experimental protocols were controlled, and data were acquired by an AT-bus DOS-based 486 microcomputer programmed with ASYST (Asyst, Rochester, N.Y.). Channel currents were eight-pole Bessel-filtered at 4 or 2 kHz and digitized at 12 bits at 25 or 10 KHz.

For conventional patches, sweeps were leak- and capacity-corrected using the average of sweeps with no activity (nulls) at each test potential. Transitions between closed and open levels were determined using a threshold detection algorithm that required two points above half the mean amplitude of the single-unit opening. Computer-detected openings were confirmed by visual inspection, and occasional sweeps were discarded because of excessive noise. Amplitude histograms were constructed from the corrected tracings, and single-channel current was taken as the mean value from a Gaussian function fit to the amplitude histogram. For macropatches, current responses to depolarizations to voltages below threshold were averaged and scaled to leak- and capacity-correct the data from more positive potentials. Measurements were made from averages of between five and 40 individual sweeps. All experiments were performed at room temperature (20–22°C). Data are reported as mean±SD, and the significance of differences between groups was determined using Student’s t test or paired t test (p<0.05).

Results

CPTcAMP and Isoproterenol Produced a Similar Shift in Conductance and Voltage-Dependent Availability

Examples of individual sweeps for macropatch currents at −50 mV are shown in Figure 1. Even though in this patch 24 channels were estimated to be open at the peak of the current at +20 mV (using a single-channel current of 1.3 pA), note that in the control condition at this threshold potential overlapping events were rare (Figure 1A). After addition of the membrane permeant cAMP analogue CPTcAMP to
the bath, there was a dramatic increase in activity at this potential (Figure 1C). This is better appreciated in the ensemble currents (Figures 1B and 1D), which show data in the control condition and 6 minutes after the addition of CPTcAMP. The effects on \( I_{\text{Na}} \) by CPTcAMP were voltage dependent; current was augmented between threshold and 0 mV, whereas there was no obvious change in current at more depolarized potentials (Figure 1E and Table 1). \( I_{\text{Na}} \) increased, for instance, by 600% at the potential of -50 mV, by 45% at -30 mV, but only by 5% at 0 mV.

This voltage-dependent augmentation of current resulted from a shift in the voltage range over which channels activated. This can be better appreciated in the conductance transforms, which are shown in Figure 2. In addition to the shift in conductance, there was a concomitant shift in voltage-dependent availability. The voltage shift seen in Figure 2 was typical of the effect of CPTcAMP observed in all three species tested (Table 1). There was no change in maximal conductance. In addition, using a t test, or a paired t test, restricting analysis to cells in which both conductance and voltage-dependent availability were assessed, the magnitudes of the shifts for conductance and voltage-dependent availability were not statistically different (\( n=15 \)). Assuming there were no differences between measurements (conductance and voltage-dependent availability) or between species, we pooled data from all patches and estimated the magnitude of the shift produced by phosphorylation to be 7.5±2.8 mV (\( n=31 \)).

Similar results were obtained with isoproterenol in canine myocytes; conductance and voltage-dependent availability were shifted to the hyperpolarized direction by 7.3±2.3 and 6.4±1.5 mV, respectively, by 5 \( \mu \)M isoproterenol (\( n=6 \)). Current decay was faster, with the greatest change near threshold and less change apparent at more positive potentials, as would be predicted for an agent that shifted activation.20 This was similar to that seen with CPTcAMP (Figure 1). Single-channel conductance for the three species was the same: 20.1±0.8 pS (canine, \( n=6 \)), 21.6±1.0 pS (guinea pig, \( n=3 \)), and 19.9±0.4 pS (rabbit, \( n=3 \)). There was no change in single-channel current magnitude during exposure to 1 or 5 mM CPTcAMP (\( n=6 \) canine conventional patches).
TABLE 1. Na+ Current Conductance and Availability Parameters in Canine, Guinea Pig, and Rabbit Ventricular Cells in the Control Period and After Addition of 5 mM 8-(4-Chlorophenylthio)cAMP

<table>
<thead>
<tr>
<th>Species</th>
<th>Conductance</th>
<th>Availability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$G_{\text{max}}$ (nS)</td>
<td>k (mV)</td>
</tr>
<tr>
<td>Canine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.1±0.9</td>
<td>-7.0±1.0</td>
</tr>
<tr>
<td>CPTcAMP</td>
<td>2.1±0.9</td>
<td>-7.1±1.1</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.3±0.7</td>
<td>-6.7±1.9</td>
</tr>
<tr>
<td>CPTcAMP</td>
<td>2.4±0.7</td>
<td>-6.8±2.1</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1.7±1.2</td>
<td>-6.7±0.2</td>
</tr>
<tr>
<td>CPTcAMP</td>
<td>1.7±0.9</td>
<td>-7.2±0.2</td>
</tr>
</tbody>
</table>

$G_{\text{max}}$, maximal conductance; k, slope factor; $V_{1/2}$, half point of the voltage relation; n, number of cells; $I_{\text{Na}\text{-max}}$, maximal Na+ current; CPTcAMP, 8-(4-chlorophenylthio)cAMP. Values are mean±SD.

Effect of CPTcAMP and Isoproterenol Is Distinct From the Background Shift in Kinetics

A time-dependent change in kinetics that also shifts conductance and voltage-dependent availability parameters in the hyperpolarized direction is known to occur in whole-cell recordings and even in on-cell patch recordings. We estimated the rate of background shift in seven patches on canine myocytes in which paired measurements of conductance and voltage-dependent availability were made 1 minute after gigaohm seal formation and then again 10–20 minutes later. The rate of shift of each relation was calculated as the change in half points divided by the time between the two measurements. Both relations shifted at the same rate, $-0.26±0.17$ mV/min (conductance) and $-0.23±0.12$ mV/min (voltage-dependent availability).

We wanted to know whether the cAMP-dependent induced change in kinetics reflected a distinct process from this time-dependent spontaneous change of kinetics. Conductance and voltage-dependent availability were determined at 1 minute after and at 12 minutes after the gigaohm seal formation (Figure 3A). Over this time period, conductance and voltage-dependent availability shifted in the hyperpolarized direction by 2.2 and 2.4 mV, respectively. Then the patch pipette was removed from the cell, and a new pipette (patch 2) was applied onto the same cell. At 1 minute after the formation of the second gigaohm seal patch (15 minutes after the first patch formation), conductance and voltage-dependent availability curves were similar to those measured 1 minute after formation of the first patch. This experiment indicated that the shift in kinetics was restricted to the channels within the patch of membrane under the recording pipette.

In a second type of experiment, as shown in Figure 3B, when CPTcAMP was applied in the bath, the conductance and voltage-dependent availability curves shifted to a greater extent. However, more importantly, when a second pipette was applied to the same cell, rather than reflecting the original half points, conductance and availability were close to those measured after the cAMP effect had stabilized. Two additional experiments using the same protocol showed similar results. Mean values were $5.3±1.4$ mV and $6.8±1.5$ mV ($n=3$). Moreover, the measurements with the second pipette were always similar to or slightly to the right of the last measurement with the first pipette. These observations provide evidence that cAMP-dependent change in kinetics is distinct from the time-dependent changes, in that the background change in kinetics was restricted to the membrane area under the patch and was additive to the cell-wide changes induced by cAMP.

Shifts in Kinetics Make PKA Effects Both Holding Potential and Test Potential Dependent

The similar shift in kinetics produced by CPTcAMP and isoproterenol can produce an increase in $I_{\text{Na}}$, a decrease in $I_{\text{Na}}$, or no effect, depending on the selection of the holding and test potentials in the voltage-clamp protocol. Figure 4 illustrates these three cases after addition of 5 μM isoproterenol to the bath for patches representative of the effects seen ($n=7$). Figure 4A shows the time-dependent course of the effect of isoproterenol on $I_{\text{Na}}$ when this was monitored by currents elicited from a $V_{H}$ of $-150$ mV to a test potential ($V_{T}$) of $-50$ mV. Because of the slow background shift in kinetics, peak $I_{\text{Na}}$ at this $V_{T}$ (on the steep portion of the conductance relation) increased over the control recording period. After the addition of 5 μM isoproterenol to the bath, there was a large enhancement of $I_{\text{Na}}$, which occurred over 5 minutes, and the decay of the ensemble current accelerated (Figure 4A, inset). These effects were similar to the effects of CPTcAMP with the same experimental protocol (Figure 1A, inset) and were what was expected from the shift in activation.

However, addition of 5 μM isoproterenol changed the peak current in the opposite direction when the currents were elicited with a $V_{H}$ of $-90$ mV to a $V_{T}$ of $+30$ mV (Figure 4B). With this $V_{H}$, which was on the steep portion of the availability relation, the peak current decreased during the control period, reflecting the background change in availability at this $V_{H}$. At approximately 5 minutes after the addition of 5 μM isoprotere-
Figure 2. Graph showing Boltzmann fits to fractional conductance (right curves) and voltage-dependent availability (left curves) in a rabbit myocyte patch. Relative conductance values were calculated as follows: fractional conductance=$I_{Na}/\left[G_{ma}(V_i-V_{m})\right]$, where $I_{Na}$ represents the current amplitude at the test potential ($V_i$) and $G_{ma}$ is the maximal conductance value of each current–voltage relation extrapolated through the pseudo–reversal potential ($V_{ma}$) estimated by linear regression of currents at the most positive potentials. Control data (O) and data during exposure to 8-[(4-chlorophenylthio)AMP (CPTcAMP; ●) are shown. Normalized conductance data and peak currents measured in voltage-dependent availability protocols were fit by a Boltzmann distribution as follows: fraction=1/[1+e($V_i-V_{ma}$)]. where the normalized data (fraction) were expressed as a function of voltage ($V_i$), the test potential in the case of conductance, and the conditioning potential in the case of voltage-dependent availability. Parameters were estimated by nonlinear regression on a 486-based microcomputer were the half point of the relation ($V_{ma}$) and the slope factor (k), both expressed in millivolts. Control data (O) and data during exposure to CPTcAMP (●) are shown. It should be noted that only for ease of plotting are data expressed as fraction of maximum. Effects of CPTcAMP on currents at positive potentials were small (see Table 1). In this case, peak current at +20 mV was −88.2 pA for control data and −89.0 pA for data during exposure to CPTcAMP. The conductance half-point value shifted in the hyperpolarizing direction from −43 mV for control data to −49 mV after addition of 5 mM CPTcAMP. The slope factor was −6.9 mV for control data and −6.6 mV after addition of CPTcAMP. The half-availability value changed from −92 mV for control data to −102 mV after addition of 5 mM CPTcAMP. The slope factor was 5.8 mV for control data and 6.9 mV in the presence of CPTcAMP. Patch No. 90112042 was from a rabbit myocyte.

To address whether cAMP changes in $I_{Na}$ were direct or secondary to phosphorylation by PKA and whether the background shift in kinetics reflected changes in phosphorylation, the effect of protein kinase inhibitor on the changes of $I_{Na}$ by CPTcAMP were studied. Figure 5C shows the effects of CPTcAMP when the cell was pretreated with H-89 for 60 minutes before and during the experiment. In contrast to untreated cells, $I_{Na}$ was unchanged by CPTcAMP when the PKA effect was prevented by H-89. The slow increase in $I_{Na}$ reflected the background shift in conductance, and the decrease in $I_{Na}$ reflected the background shift in voltage-dependent availability. Similar results were observed in five patches. The background shift was unaffected by H-89, which provides strong evidence that the time-dependent shift of kinetics is independent of channel phosphorylation.

**Discussion**

**Cell-Attached Macropatch-Clamp Method**

Our experiments were performed with large pipettes in the cell-attached recording mode.21 This method is particularly useful for the studies of regulation of the channel by neurotransmitters and hormones, because the intracellular milieu remains intact. Large patch pipettes permit recording from many Na+ channels within the patch area, so that the current ensemble constructed from only a few sweeps or even from a single sweep displays whole-cell-like Na+ currents. Although clamp speed may not be suitable for following extremely fast transients, this technique has the benefit that it does not restrict investigation to small and/or spheroidal-shaped cells nor does it require use of extremely low Na+ concentrations to control the currents. It should be noted that the choice of pipette solution here, high ionic strength in combination with a modest divalent cation concentration, produced an environ-


dent availability and conductance curve, respectively. These findings were confirmed in seven patches.

We further investigated the voltage-dependent effect of cAMP and isoproterenol using a two-pulse protocol as shown in Figure 5A, which was designed to monitor the time course of the shift in conductance and voltage-dependent availability in the same patch. The patch was held at −150 mV (fully available) and depolarized to −30 mV (less than fully activated). This choice of $V_{hi}$ and $V_{fi}$, therefore, monitored the change in the voltage dependence of activation (P1 in Figure 5A). After this test pulse, the patch was held at −90 mV for 235 msec, and then a test pulse to +20 mV was given. This combination of a $V_{hi}$ on the steep portion of the availability curve and a $V_{fi}$ where channels were fully activated monitored the change of the voltage-dependent availability curve (P2 in Figure 5A). In Figure 5B, during the control period, the current from the hyperpolarized $V_{hi}$ (I$_{p1}$) increased slightly as the background shift in conductance produced more fully activated currents at −30 mV. At the same time the current from the depolarized $V_{fi}$ (I$_{p2}$) decreased slightly as the background shift in voltage-dependent availability made channels somewhat less available at a $V_{fi}$ of −90 mV. During 5 minutes after the introduction of 2 mM CPTcAMP to the bath, I$_{p1}$ increased, whereas I$_{p2}$ decreased, demonstrating the concomitant shift in conductance and voltage-dependent availability.

The size of the peak current was not altered by isoproterenol with this choice of $V_{hi}$ and $V_{fi}$. In addition, the change in $I_{Na}$ produced by 5 μM isoproterenol was variable and small when the effect was monitored from a $V_{hi}$ of −90 mV to a $V_{fi}$ of −50 mV (not shown). This was undoubtedly because a $V_{hi}$ of −90 mV and a $V_{fi}$ of −50 mV were close to the midpoints of voltage-depen-
ment in which channels activated and inactivated at rather negative potentials (Table 1). Competition between monovalent and divalent ions, as well as the choice of divalent cations, and their concentrations can markedly alter the voltage range of cardiac Na⁺ channel kinetics. Recordings were often stable for at least 20 minutes, and the background shift in kinetics was quite slow, less than 0.3 mV/min, which is at the low end of the range previously reported for cell-attached recordings and whole-cell recordings.

Consequences of cAMP-Dependent Phosphorylation

Several laboratories have reported effects of cAMP-dependent phosphorylation on cardiac I_{Na}, which were produced by addition of isoproterenol or permanent cAMP analogues. Although the description of results appears somewhat diverse at first glance, most previous studies using whole-cell currents are consistent with the action of phosphorylation reported here. In the earliest studies using whole-cell recordings, investigators reported a reduction of I_{Na}, which is the effect most evident when V_{H} produces voltage-dependent availability less than unity (e.g., see Figure 5B). These investigators recognized that the inhibition of I_{Na} was secondary to the negative shift of availability. More recently published whole-cell current data confirmed the negative shift of voltage-dependent availability, although Murray et al., recording at 36°C, and Wendt et al., recording through perforated patches, did not observe a shift in availability. On the other hand, a single-channel study, in which holding potentials were chosen to partially inactivate channels, also reported a reduction in activity after exposure to cAMP. In most studies in more intact preparations, with V_{max} used as an index of I_{Na}, depression of V_{max} was apparent when preparations were partially depolarized. Under these conditions, investigators have reported a shift of the voltage dependence of inactivation to more negative potentials. However, in one study no effect of isoproterenol in either normally polarized or partially depolarized Purkinje fibers was apparent. It is probably not surprising that the concomitant shift of activation was not detected in any of these experiments, since V_{max} occurs at different potentials depending on its magnitude.

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Time-dependent and cAMP-dependent changes in kinetics of the Na⁺ current (I_{Na}). Panel A: Time-dependent shift of fractional conductance and voltage-dependent channel availability in the hyperpolarized direction in two different patches on the same cell. The conductance curve shifted 2.2 mV, and the voltage-dependent availability curve shifted 2.4 mV in the hyperpolarized direction over 11 minutes (a [ ] and b [ ], respectively). However, a second patch made on the same cell showed similar conductance and voltage-dependent availability characteristics (dotted curve, c [ ]) to the initial measurements in the first patch (a). Patches were Nos. 900426A2 and 900426A3. Panel B: Fractional conductance and voltage-dependent availability (a[ ]) shifted in the hyperpolarized direction after exposure to 8-(4-chlorophenylthio)cAMP (b [ ]). Unlike the case where fractional conductance and voltage-dependent availability shifted over time, characteristics of conductance and voltage-dependent availability remained shifted in a second patch (dotted curve, c [ ]). Patches were Nos. 900424M1, 900424M2, and 900424M3 from canine myocytes.
In general, the shift in conductance has been unrecognized, although augmentation of \( I_{Na} \) has been reported by some investigators.\(^6\) This is probably because the range of potentials tested was often very limited. However, Matsuda et al\(^6\) reported data over the entire voltage range over which \( Na^{+} \) channels activate and did not observe a significant shift of either conductance or voltage-dependent availability. It should be noted that these investigators based their conclusions on statistics derived from grouped data. Given the small shift induced by PKA phosphorylation, it may have been the case that differences between cells masked the phosphorylation-induced shift. Although it is sometimes argued that effects so small that they cannot survive grouping must not be significant, this is certainly not the case here. Both conductance and channel availability are quite voltage dependent, changing e-fold in 5–8 mV. A small shift in the voltage range over which channels activate can, therefore, profoundly affect currents at those potentials (e.g., see Figure 1E). Similarly, even small changes in the range of potentials over which channels voltage-dependently inactivate can markedly affect \( I_{Na} \) when \( V_{m} \)s produce availability less than unity. On the other hand, their conductance data are quite similar to the data reported here, given that the evaluation of maximal conductance was difficult because the midpoint of conductance under their experimental conditions was approximately −24 mV. Maximal conductance, therefore, occurred over a potential range where currents were small, very near reversal for the current. Indeed, currents above 20 mV converged.

Not all the reported actions of cAMP on cardiac \( Na^{+} \) channels can be explained by the parallel shift of activation and inactivation. Contrary to the expectation of an action mediated by a second messenger pathway, in excised cell-free patches an effect of cAMP persists,\(^10\) and application of the catalytic subunit of PKA to the intracellular face of excised patches produced kinetic effects opposite those reported here and by others.\(^24\) In addition, in one report the extracellular application of nonpermeant cAMP was observed to induce kinetic shifts.\(^25\) It should also be noted that other direct effects of GTP-binding proteins on the \( Na^{+} \) channel, secondary to \( \beta \)-adrenoreceptor stimulation, have been reported.\(^6\)\(^7\) Although we did not observe any effects that could be attributed to such direct actions, our experiments were not designed to critically examine such actions.

Recently, Li et al\(^26\) described \( I_{Na} \) from cells transfected with the \( \alpha \)-subunit of the rat brain II A \( Na^{+} \) channel. They observed a marked reduction in \( I_{Na} \) after

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**Figure 4.** \( Na^{+} \) current (\( I_{Na} \)) after application of 5 \( \mu M \) isoproterenol. Insets show the average currents (tracings a–e) for six to 41 sweeps at 1 Hz as indicated by filled circles and horizontal bars. Panel A: Graph showing \( I_{Na} \) augmentation by isoproterenol at −50 mV when holding potential was −150 mV. Patch was No. 90013013. Panel B: Graph showing \( I_{Na} \) reduction by isoproterenol at +30 mV when holding potential was −90 mV. Patch was No. 90020611 from a canine myocyte. Panel C: Graph showing that \( I_{Na} \) did not change after exposure to isoproterenol when the test potential was +30 mV and holding potential was −150 mV. Note larger number of channels in the patch so that only a single sweep was needed for each of the five currents shown in the inset. Patch was No. 91070911.
exposure to cAMP, which is consistent with our data if their $V_H$ produced less than full availability, as suggested by their steady-state availability relations. Alternatively, this effect could be secondary to differences between channel isoforms.

**Molecular Basis of the Effect of Phosphorylation**

Of course it is not possible to conclude from these experiments that phosphorylation of the Na$^+$ channel protein itself by PKA accounts for the effects observed. However, all of the Na$^+$ channels thus far cloned, with the exception of the eel electroplax channel, have consensus sequences for phosphorylation, and PKA-dependent phosphorylation has been demonstrated biochemically for rat brain Na$^+$ channels. Although the phosphorylation of the channel would be expected to increase the negative charge on the cytoplasmic surface by simple addition of charged phosphate to the Na$^+$.
channel, this would be expected to produce a parallel shift in conductance and voltage-dependent availability in the opposite direction from that observed. In neuronal cells, shifts of the conductance and availability of delayed rectifier K⁺ channels toward positive potentials have been reported after channel phosphorylation. Clearly, the action on Na⁺ channels is more complex. It is of interest that the mechanism of modulation by isoproterenol and cAMP of cardiac dihydropyridine-sensitive (L-type) Ca²⁺ channels appears to be similar to that described here for cardiac Na⁺ channels. Bean and Tiaho et al. demonstrated a shift of both voltage-dependent availability and conductance in the hyperpolarized direction by cAMP-dependent modulation. This action accounts for the large increases in the Ca²⁺ current often reported after β-adrenergic stimulation. The similarity of molecular structure of the α-subunits of the Na⁺ and Ca²⁺ channels thus far cloned, which includes possible phosphorylation sites, suggests that elucidation of the molecular events produced by phosphorylation to alter voltage-sensing function (site or sites of phosphorylation and the contribution to the voltage-dependent gating) may be similar for the two channel types.

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