Cytoskeleton modulates coupling between availability and activation of cardiac sodium channel

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Maltsev, Victor A., and Albertas I. Undrovinas. Cytoskeleton modulates coupling between availability and activation of cardiac sodium channel. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H1832–H1840, 1997.—The aim of this study was to investigate modulation of voltage-dependent steady-state activation and availability from inactivation of the cardiac Na+ channel by the cytoskeleton. As an experimental approach, we used long-lasting monitoring [63 ± 5 (SE) min] of the half-point potentials of the steady-state availability curve (V1/2A) and normalized conductance curve (V1/2Ga) in 116 rat ventricular cardiomyocytes by whole cell patch clamp at 22–24°C. Both half-point potentials shifted in the negative direction with time as an exponentially saturating change, with the shift of V1/2Ga being smaller and faster. An F-actin disrupter, cytochalasin D (Cyto-D, 20 µM), accelerated the rate of the V1/2Ga shift but decreased the range of the V1/2G shift. An F-actin stabilizer, phalloidin (100 µM), temporarily (for 28.2 ± 2.2 min, n = 15) prevented the V1/2A shift but did not influence the V1/2G shift. The best fit for the V1/2G-V1/2A relationship in untreated cells (1,021 data points measured in 51 cells) was a second-degree (2.06) power function. Cytoskeleton-directed agents modified the relationship. In Cyto-D-treated cells, the V1/2G-V1/2A relationship was shifted (by 2.5 mV) toward positive V1/2G. On the contrary, a microtubule stabilizer, taxol (100 µM), shifted the relationship toward negative V1/2G (by 12.2 mV). We conclude that coupling between availability and activation is modulated by F-actin-based and microtubular cytoskeleton.

SODIUM CHANNELS ARE responsible for the initial rapid increase in membrane permeability for Na+, which is essential for the generation and propagation of the action potential. Channel gating is determined by two fundamental channel properties: activation and inactivation. Coupling between activation and inactivation is an important characteristic of the Na+ channel, since it determines the functional response of the channels to membrane depolarization. Activation and inactivation mechanisms are the major targets in ongoing molecular studies of Na+ channel function (for review see Ref. 9). Activation is thought to involve movement of the highly charged fourth membrane-spanning segment S4 (32, 42). A hydrophobic triplet of amino acid residues in the cytoplasmic III-IV linker was suggested to form a lid that binds to a cytoplasmic region of the channel and occludes the pore, producing fast inactivation of the channel (25, 32, 41). Recently, it has been shown that mutations other than in the III-IV linker can affect inactivation as well. Complex alterations of the channel gating can be produced by mutations in the pore region (34) and in various cytoplasmic or transmembrane domains, e.g., mutations related to inherited heart diseases (4) or skeletal myopathies (28). These data suggest the importance of integrity of all channel molecule parts in the gating process. Furthermore, channel gating is determined also by the state of channel phosphorylation (33, 41), coexpression and association of the α- and β-subunits (17, 24), and factors that affect the channel environment, including membrane phospholipid composition and the cytoskeleton. Our previous studies showed that membrane partition of an ischemic metabolite, lysophosphatidylcholine, induced bursting activity of the cardiac Na+ channel and shifted activation toward negative membrane potentials (36). We also found that cytochalasin D (Cyto-D), a disrupter of the F-actin-based cytoskeleton, and antibodies to integrated proteins of the cytoskeleton, such as F-actin, β-spectrin, and ankyrin, altered gating kinetics by increasing open time, inducing a second open state, and causing prolonged bursts of openings (23, 35, 37). These findings indicated that the environment of the channel protein, in addition to the channel protein structure itself, determines the microscopic activation-inactivation coupling.

In the present study we addressed whether the cytoskeleton regulates coupling of Na+ channel activation and inactivation in terms of the relationship between steady-state activation and availability from inactivation. We took advantage of the spontaneous shift of availability and conductance curves that occurs for the cardiac Na+ channel during whole cell recording (14, 16) to characterize the relationship in a wide range of the half-point potentials of the curves. Cytoskeleton modifiers changed the relationship, indicating a modulating role for the cytoskeleton in activation-inactivation coupling.

MATERIALS AND METHODS

Isolation of Cardiomyocytes

Ventricular cardiomyocytes were enzymatically isolated from Sprague-Dawley rat hearts into Ca2+-free Eagle’s minimal essential medium with 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH adjusted to 7.3 with KOH), as previously described (37). After isolation, cells were kept for up to 12 h at 22°C in the same solution to which 0.3 mM CaCl2 was added. Only relatively small quiescent rod-shaped, cross-striated cells were used in the experiments.

Whole Cell Current Recordings

Whole cell recordings of Na+ current (INa) were made at 22–24°C by the patch-clamp technique (15). The patch pipettes were pulled from borosilicate glass capillaries (K 150F, WPI, Sarasota, FL). After they were heat polished, the pipettes had a tip resistance of 600–800 kΩ in standard...
solutions. Ion currents were recorded by a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA). The current was zeroed when the pipette was placed in the bath solution to correct for liquid junction potentials between the bath solution and the pipette solution. Series resistance compensation and capacitive transient cancellation were adjusted for each cell before recording to provide optimum voltage control and minimize the capacitive transient. Currents were digitized and recorded at 50 kHz onto a hard disk of a 486 computer for off-line analysis. Before digitization, currents were filtered at 10 kHz (–3 dB) using a four-pole low-pass Bessel filter. Voltage protocols and signal digitizing were performed by DigiData 1200 interface and pCLAMP 6.0 software (Axon Instruments).

\[ I_{Na} \] was measured in 116 cells obtained from 34 rats. The characteristics of the current were monitored for as long as a stable seal could be maintained, as judged by a total current (I) measured at the holding potential (V_h) = −150 mV so that I > \( V_h /100 \) µA = −1.5 nA. The duration of the whole cell experiment averaged 63 ± 5 min (maximum duration was 345 min). To obtain stable recordings for long periods, we lifted cells from the surface of the recording chamber after 345 min. To attain stable recordings for long periods, we used Cyto-D as disrupter and phalloidin as stabilizer of an F-actin-based cytoskeleton. Colchicine was used as microtubule disrupter and taxol as microtubule stabilizer. The final tubule disrupter and taxol as microtubule stabilizer. The final Na tubule disrupter and taxol as microtubule stabilizer. The final Na recording. Experiments with Cyto-D were performed each day and sonicated before each experiment. The cells were incubated with the cytoskeleton modifiers at least 4 h before \( I_{Na} \) recording. Experiments with Cyto-D were performed in relative darkness to avoid photodestruction of the Cyto-D molecule. All substances were purchased from Sigma Chemical (St. Louis, MO).

Control and Test Solutions

Solutions for whole cell experiments were selected to suppress all currents other than \( I_{Na} \). The external bath solution was composed of (in mM) 10 NaCl, 115 CsF, 20 CsCl, 2.5 MgATP, 5.0 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 5.0 HEPES (pH adjusted to 7.3 with CsOH).

We used Cyto-D as disrupter and phalloidin as stabilizer of an F-actin-based cytoskeleton. Colchicine was used as microtubule disrupter and taxol as microtubule stabilizer. The final concentration of substances in the incubation medium and pipette solution was 20 µM for Cyto-D and 100 µM for phalloidin, colchicine, and taxol. Fresh test solutions were made each day and sonicated before each experiment. The cells were incubated with the cytoskeleton modifiers at least 4 h before \( I_{Na} \) recording. Experiments with Cyto-D were performed in relative darkness to avoid photodestruction of the Cyto-D molecule. All substances were purchased from Sigma Chemical (St. Louis, MO).

Quality of Voltage Clamp

In every experiment we assessed the quality of the voltage clamp using the following criteria. The deviation from voltage \( V_{dev} \) command associated with series resistance \( R_s \) was estimated to be \( V_{dev} = 1R_s \). First, we determined the uncompensated \( R_s \) as

\[ R_s = 20 \text{ mV} / I_c \]  

where \( I_c \) is the peak value of capacitive current evoked by a square voltage pulse of 20-mV amplitude applied from −120 to −100 mV. In all experiments the uncompensated \( R_s \) was <2 MΩ. Electronic series resistance compensation \( K_s \) was imposed to a point just before oscillations occurred. The final setting of the \( K_s \) value (on an Axopatch 200A amplifier) varied from 75 to 95%. The current-voltage (I-V) relationship for peak \( I_{Na} \) was measured to determine a maximum peak of the current \( I_{(max)} \). In our experimental conditions, \( I_{(max)} \) varied from 5 to 30 nA. With \( R_s \) compensation, \( V_{dev} \) was estimated as

\[ V_{dev} = I_{max}R_s(100\% - K_s)/100\% \]  

Satisfactory voltage control was assumed if \( V_{dev} \) was <2 mV, and only these cells were included in the study. The quality of the voltage clamp was monitored throughout each experiment (about every 5 min). We terminated an experiment and disregarded all data obtained after the last quality test if \( R_s \) increased so that \( V_{dev} \) was >2 mV.

Voltage-Clamp Protocols

Steady-state voltage-dependent availability from inactivation, \( A(V) \), was determined by using a series of 1-s-long conditioning steps to various membrane potentials \( V \) followed by a test depolarization of 18-ms duration to −30 mV to assess the pool of \( Na^+ \) channels left noninactivated by the conditioning step. A 982-ms interval at a \( V_h \) of −170 mV separated the individual pulse protocols and allowed for full recovery of \( I_{Na} \). Further prolongation of the interpulse interval up to 5 s and/or membrane hyperpolarization up to −190 mV did not change the amplitude of \( I_{Na} \). Peak current \( I_{peak} \) voltage relationships (I-V curves) were obtained by applying a series of test depolarizations to various potentials from a \( V_h \) set to −150 mV to ensure full \( I_{Na} \) availability and recovery from preceding pulses during a 2-s interpulse interval. The depolarization increase followed a 3- or 5-mV step protocol.

Data Analysis

Peak \( I_{Na} \) values were calculated with off-line leak correction using Clampfit software (Axon Instruments). For determination of availability curves, the peak currents were normalized to the maximum peak current value and plotted against the conditioning potential \( V \). Parameters of \( A(V) \) curves were determined by fitting the data to a Boltzmann function

\[ A(V) = \frac{1}{1 + \exp( - (V - V_{1/2A} / K) )} \]  

where \( V_{1/2A} \) is the half-point of the relationship and \( K \) is the slope factor.

Normalized voltage-dependent \( Na^+ \) conductance \( G(V) \) was determined from transformations of I-V curves. The maximum \( Na^+ \) conductance \( (g_{max}) \) and reversal potential \( V_{rev} \) were estimated from a linear fit [as a slope and a cross point with test potential \( (V_h) \) axis, respectively] of an almost linear ascending portion of the I-V curve. The \( Na^+ \) conductance \( g \) at each \( V_h \) was calculated as

\[ g = I_{peak} / (V_h - V_{rev}) \]  

The data points of normalized conductance \( G = g / g_{max} \) were fitted to a third-degree Boltzmann function

\[ G(V) = \frac{1}{1 + \exp( - (a - V) / K )} \]  

The voltage dependence of steady-state activation was characterized by the half-point conductance potential \( (V_{1/2G}) \) determined for the normalized conductance curve, \( G(V_{1/2G}) = 0.5 \). \( V_{1/2G} \) was calculated as \( V_{1/2G} = a - K \cdot \ln((\frac{1}{2} - 1)) \). We used the third-degree Boltzmann function for the data analysis, since it always fit data points better than a single Boltzmann function. However, these two fitting functions yielded almost the same \( V_{1/2G} \) values for the same data set: difference <0.1 mV.

To analyze the time course of changes in voltage-dependent availability and conductance, \( V_{1/2A} \) and \( V_{1/2G} \) were plotted as a
function of experimental time. The initial slope of the negative shift (a shift rate) for $V_{1/2A}$ and $V_{1/2G}$ was determined from a linear fit to data points taken for the initial period (first 8 min) of an experiment. The time-dependent shifts of $V_{1/2A}$ and $V_{1/2G}$ were fitted with an exponential function (see Eqs. A1 and A2).

To determine the $V_{1/2G}$-$V_{1/2A}$ relationship, we measured these parameters consecutively in the same cell. Because of a minimum delay of ~2 min related to our experimental protocol, it was impossible to measure both parameters simultaneously. Therefore, for every $V_{1/2A}(t_i)$ measured at time $t_i$ ($1 < i < N$, where $N$ is total number of measurements made in the cell), we calculated the coupled $V_{1/2G}(t_i)$ from the previously measured value, $V_{1/2G}(t_{i-1})$, and the next value, $V_{1/2G}(t_{i+1})$, assuming a linear time-dependent shift of $V_{1/2G}$ between $t_{i-1}$ and $t_{i+1}$.

Origin software (version 4.1, Microcal Software, Northampton, MA) was used to fit histograms by a Gaussian function

$$f(x) = \frac{A}{\sqrt{2\pi}W} \cdot \exp \left[ -\frac{(x-Xc)^2}{2W^2} \right]$$

where $A$ is area, $Xc$ is center, and $W$ is width of the function. Other nonlinear curve fittings were done by pCLAMP (version 6.0, Axon Instruments) and StatMost (version 2.50, DataMost, Salt Lake City, UT) software. The best model describing the $V_{1/2G}$-$V_{1/2A}$ relationship was chosen from exponential, power, linear, and hyperbolic functions as judged by a smaller variance and by parameters of goodness-of-fit statistics given by StatMost software. The parameters included the correlation coefficient, the coefficient of determination, and the model selection criterion defined in APPENDIX B.

Statistical significance when comparing mean values was determined by Student’s t-test for unpaired data. If not otherwise stated, the two pools of data were considered to be significantly different at $P < 0.01$. Results are presented as means ± SE for $n$ cells.

RESULTS

Characterization of the Relationship Between Availability and Activation in Control Cells

Time-dependent shifts of steady-state availability and conductance curves were exponential. We monitored the time-dependent shift of $V_{1/2G}$ and $V_{1/2A}$ in 51 control cells with a mean recording time of 70.6 ± 5.2 min. The range for the shift in $V_{1/2G}$ (from −29 to −76 mV) was significantly smaller than for $V_{1/2A}$ (from −68 to −128 mV). The time-dependent changes of both parameters exhibited an exponential saturation (Fig. 1A). The time course of the shifts was obviously not parallel, because the change in $V_{1/2G}$ was smaller and saturated faster. Time constants and saturation values for the exponentials fitted to the shifts are given in Table 1. Generally, the $V_{1/2G}$ shift was saturated within ~20 min, whereas $V_{1/2A}$ shifted over 40–60 min. Although the overall shifts were different, the initial shift rate recorded within the first 8 min of the experiment was not significantly different for these parameters (Table 2). Thus we found an almost parallel shift of availability and conductance at the beginning of the recording. In some cells we observed independent changes in $V_{1/2G}$ and $V_{1/2A}$ during whole cell recording. This occurred in cells that initially (spontaneously) had a saturation value of $V_{1/2G}$ or $V_{1/2A}$. Once the parameter was found at

Fig. 1. Relationship between half-points of conductance curve ($V_{1/2G}$) and availability curve ($V_{1/2A}$) during long-term recording in whole cell patch clamp. A: representative example of simultaneous monitoring of exponential shift for $V_{1/2A}$ (*) and $V_{1/2G}$ (○). Solid lines, exponential fits with their time constants (τ). B: $V_{1/2G}$-$V_{1/2A}$ relationship derived for data points shown in A after time factor was excluded. A power function (solid line) was generated for relationship using Eq. A3. C and D: independent shifts of $V_{1/2G}$ (○) and $V_{1/2A}$ (*). One parameter did not shift during experiment, whereas another parameter shifted exponentially. $V_s$, saturation potential.
Table 1. Parameters of a single-exponential function fitted to data points of time-dependent shift of $V_{1/2A}$ and $V_{1/2G}$ curves in control cells and in cells treated with cytoskeleton modifiers

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Time Constants, min</th>
<th>Saturation Values, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_A$</td>
<td>$\tau_G$</td>
</tr>
<tr>
<td>Control</td>
<td>33.9 ± 1.9†</td>
<td>16.0 ± 0.8†</td>
</tr>
<tr>
<td>Cyto-D (20 µM)</td>
<td>16.1 ± 1.8</td>
<td>ND</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>35.3 ± 3.6†</td>
<td>16.5 ± 1.9‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 32$ for control and $n = 10$ for each cytoskeleton modifier, $\tau_A$ and $\tau_G$. Time constants; $V_{1/2A}$ and $V_{1/2G}$, saturation values of half-point of availability and conductance, respectively (see Eqs. A1 and A2). †Significantly different from each other ($P < 0.005$). ‡Significantly different from each other ($P < 0.0001$). Exponential fit was not determined (ND) for an almost linear (monotonic) shift course of $V_{1/2A}$ in cytochalasin D (Cyto-D)-treated cells.

the saturated level, it did not change, whereas another parameter shifted exponentially (Fig. 1, C and D).

$V_{1/2G}$-$V_{1/2A}$ Relationship Is Described by a Power Function

A theoretical prediction. Because we have found that $V_{1/2G}$ and $V_{1/2A}$ have an exponential time course, their relationship after the time factor is excluded can be easily shown to be a power function with a degree equal to the ratio of the time constants ($\beta = \tau_A/\tau_G$; see APPENDIX A). The relationship was obviously nonlinear, since the time constants $\tau_A$ and $\tau_G$ were significantly different ($\beta = 2$; Table 1). The suitability of the theoretical prediction to the data points of $V_{1/2G}$-$V_{1/2A}$ relationship is illustrated in Fig. 1B, which shows the power function determined in one cell from the exponential fits to the $V_{1/2G}$ and $V_{1/2A}$ time course.

Experimental evidence. We plotted data points ($n = 1,021$) measured during whole cell recordings in 51 control cells (Fig. 2). The best fit of the plot was the power function with a degree close to 2 (solid line in Fig. 2). A detailed comparison of the fits obtained by using different models is given in Tables 3 and 4. The data points were spread along the $V_{1/2G}$ axis, indicating no strict dependence between the parameters. We calculated the histogram of departures of data points from the power function fit. The histogram was fitted to a Gaussian function (Fig. 2, inset). The power function fit and parameters of the Gaussian function were used as a reference to describe effects of cytoskeleton modifiers on the $V_{1/2G}$-$V_{1/2A}$ relationship (see below).

Effects of Cytoskeleton Modifiers on the Relationship Between Availability and Activation

Effects of cytoskeleton modifiers on the time course of $V_{1/2G}$ and $V_{1/2A}$. The effects of cytoskeleton modifiers were characterized by parameters of exponential fits to the time course of $V_{1/2G}$ and $V_{1/2A}$ shifts (Table 1) and by changes in initial rates of the shifts (Table 2). The $V_{1/2A}$ shift rate was accelerated by an F-actin disrupter, Cyto-D (>2 times) but was slowed by an F-actin stabilizer, phalloidin. Whereas Cyto-D or phalloidin changed the $V_{1/2G}$ shift rate insignificantly, the $V_{1/2G}$ shift rate was accelerated by taxol, a microtubule stabilizer (Table 2). Although in the presence of phalloidin the effect of taxol was attenuated, the $V_{1/2G}$ shift rate remained significantly faster than in control cells. Cyto-D changed the time course of $V_{1/2A}$ to an almost linear (monotonic) shift (not shown). At the same time, the $V_{1/2G}$ time course remained exponential, with a time constant similar to that of control cells (Table 1). The net $V_{1/2G}$ change was less in Cyto-D-treated than in control cells by ~7.5 mV, which resulted from the difference between saturation potentials ($V_{1/2G}$; Table 1).

Phalloidin produced an apparent uncoupling of availability and activation observed as asynchronous shifts in the time course of $V_{1/2G}$ and $V_{1/2A}$ (Fig. 3). In the majority of myocytes (15 of 22 cells, 68.2%), phalloidin temporarily (for 28.2 ± 2.2 min) prevented the time-dependent shift in $V_{1/2A}$ (arrows in Fig. 3). After the delay, however, the exponential shift in $V_{1/2A}$ had the

Table 2. Effect of cytoskeleton modifiers on the initial rates of time-dependent shift of $V_{1/2A}$ and $V_{1/2G}$

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>$n$</th>
<th>$dV_{1/2A}/dt$</th>
<th>$dV_{1/2G}/dt$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51</td>
<td>-0.254 ± 0.043</td>
<td>-0.296 ± 0.023</td>
</tr>
<tr>
<td>Cyto-D (20 µM)</td>
<td>14</td>
<td>-0.220 ± 0.060</td>
<td>-0.666 ± 0.080*</td>
</tr>
<tr>
<td>Phalloidin (100 µM)</td>
<td>22</td>
<td>-0.230 ± 0.033</td>
<td>-0.194 ± 0.024*</td>
</tr>
<tr>
<td>Phalloidin + taxol (100 µM)</td>
<td>13</td>
<td>-0.450 ± 0.067*</td>
<td>-0.160 ± 0.056*</td>
</tr>
<tr>
<td>Taxol (100 µM)</td>
<td>12</td>
<td>-0.671 ± 0.085*</td>
<td>-0.256 ± 0.074</td>
</tr>
</tbody>
</table>

Values are means ± SE in mV/min; $n$, number of cells. $dV_{1/2A}/dt$ and $dV_{1/2G}/dt$, time-dependent shift in $V_{1/2A}$ and $V_{1/2G}$, respectively. Significantly different from control cells: *$P < 0.0001$, †$P < 0.006$, ‡$P < 0.012$. Significant increase compared with control cells: *$P < 0.02$, †$P < 0.01$. The best fit of the plot was the power function determined in one cell from the exponential fits to the $V_{1/2G}$ and $V_{1/2A}$ time course.
same time constant in phalloidin-treated and control cells (Table 1). The exponential change in V_{1/2G} always occurred immediately after disruption of the membrane patch. Taxol did not potentiate the phalloidin effect. In myocytes treated with taxol + phalloidin, the delay (35.3 ± 11.2 min) of the onset of the exponential V_{1/2A} shift was similar to the delay observed in myocytes treated with phalloidin alone. The percentage of cells (8 of 13, 61.5%) showing the delay of the V_{1/2A} shift also remained unchanged.

Effects of cytoskeleton modifiers on the V_{1/2G}-V_{1/2A} relationship. To examine the cytoskeleton modulation of the V_{1/2G}-V_{1/2A} relationship, we compared the relationships obtained when myocytes were treated with cytoskeleton disrupters (Fig. 4) and with stabilizers (Fig. 5). The reference line of the power function fit established in control cells (Fig. 2) is shown in the plots to visualize the modulation effect. The position of data points was clearly shifted above the reference line in Cyto-D-treated cells (Fig. 4A). To quantify the modulation effects of cytoskeleton modifiers on the V_{1/2G}-V_{1/2A} relationship, we calculated the histograms (insets in Figs. 2, 4, and 5) of departure of data points from the reference line along the V_{1/2G} axis. The parameters of a Gaussian function fitted to the histograms [center (Xc) and width (W); Fig. 4A, inset] in cells treated with cytoskeleton modifiers were compared with those of control cells (Fig. 2, inset). Whereas the center of distribution in control cells was at zero, Xc values were significantly shifted (by 2.5 mV) toward more positive values in Cyto-D-treated cells. A microtubule disrupter, colchicine, however, did not potentiate the Cyto-D effect (Fig. 4B).

In cells treated with the F-actin stabilizer phalloidin, the position of data points in the V_{1/2G}-V_{1/2A} relationship was almost unchanged (Fig. 5A). However, data points were more closely localized at the reference line than in control cells. This "stabilization" effect was reflected by a significant reduction in Gaussian width (W = 3.42 mV, i.e., less than one-half that in control cells). Taxol, a microtubule disrupter, dramatically shifted data points below the reference line (Fig. 5B). The taxol effect was characterized by a >12-mV shift in position of the Gaussian center toward negative V_{1/2G}. In the presence of phalloidin, the effect of taxol was attenuated, with a shift in V_{1/2G} of only 2.6 mV (Fig. 5C).

Slope factors of G(V) and A(V) curve were not influenced by cytoskeleton modifiers. The slope factor for the G(V) curve increased during whole cell recording from K(t = 0) = 5.65 ± 0.28 mV (measured in 51 cells) to a steady-state level of 7.18 ± 0.14 mV after 40 min of recording. The time course of the change was well described by an exponential function, with a time constant of the best fit to all data points of 15.8 min (not shown). The slope factor for the A(V) curve did not change during the experiment: 5.59 ± 0.09 mV at the beginning of the experiment and 5.61 ± 0.09 mV after 60 min of whole cell recording. Cytoskeleton modifiers

Table 3. Parameter values of best fits using different models for data points in V_{1/2G}-V_{1/2A} relationship

<table>
<thead>
<tr>
<th>Model</th>
<th>Function f(x)</th>
<th>P_0</th>
<th>P_1</th>
<th>P_2</th>
<th>P_3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power</td>
<td>P_1 \cdot x - P_2 \cdot e^{-P_0 + P_3}</td>
<td>2.064</td>
<td>8.70 \times 10^{-3}</td>
<td>-128.9</td>
<td>-68.89</td>
</tr>
<tr>
<td>Exponential</td>
<td>P_1 \cdot \exp[(x - P_2)/P_0]^3 + P_3</td>
<td>28.70</td>
<td>0.3327</td>
<td>-212.9</td>
<td>-76.80</td>
</tr>
<tr>
<td>Hyperbolic</td>
<td>P_1 / (x - P_2 + P_3)</td>
<td>-2.552</td>
<td>-33.4</td>
<td>-98.01</td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>P_2 \cdot x + P_3</td>
<td>0.585</td>
<td>0.257</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P_0, P_1, P_2, and P_3, parameter values (see Fig. 2).

Table 4. Comparison of different models describing data points in V_{1/2G} - V_{1/2A} relationship in Fig. 2

<table>
<thead>
<tr>
<th>Model</th>
<th>Variance</th>
<th>Goodness-of-Fit Statistics</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>r</td>
</tr>
<tr>
<td>Power</td>
<td>13.618</td>
<td>0.8817</td>
</tr>
<tr>
<td>Exponential</td>
<td>13.759</td>
<td>0.8804</td>
</tr>
<tr>
<td>Hyperbolic</td>
<td>13.930</td>
<td>0.8788</td>
</tr>
<tr>
<td>Linear</td>
<td>17.914</td>
<td>0.8409</td>
</tr>
</tbody>
</table>

Best model was a power function. Choice of best model was judged by a smaller variance of residues and by parameters of goodness-of-fit statistics: correlation coefficient (r), coefficient of determination (COD), and model selection criterion (MSC) (see APPENDIX B). Parameters of models are shown in Table 3.
DISCUSSION

An important role for the cytoskeleton in the modulation of gating for \( \text{Na}^+ \) channels of different types has been previously described (8, 23, 29, 35, 37). In the present study, using the spontaneous shift of voltage dependence of activation and inactivation gating (16) as a model, we established that 1) the half-points of steady-state activation and availability curves for cardiac \( \text{Na}^+ \) channels are coupled by a second-degree power function and 2) the relationship is modulated by F-actin- and tubulin-based cytoskeleton.

Time-Dependent Shift of Availability and Activation Are Modulated by the Cytoskeleton

We showed for the first time that the time-dependent shifts of voltage dependence for availability and activation of cardiac \( \text{Na}^+ \) channels were not monotonic, as reported previously (16) but, rather, a slow \((t = 10–60 \text{ min})\) exponentially saturating change that was smaller and quicker for the activation curve. This appears to be an important property of the cardiac \( \text{Na}^+ \) channel. Less shift in the activation process than in availability was also observed for canine cardiac \( \text{Na}^+ \) channels in cell-free and cell-bound patches, as judged by the relatively normal threshold for channel activation (6). The smaller and faster shift for the activation curve has been recently found by Wang et al. (38) for a cardiac isoform of the \( \text{Na}^+ \) channel but not for the isoform of skeletal muscle. The attainment of saturation implies that a new equilibrium of the important factors determining \( \text{Na}^+ \) channel voltage gating is established during whole cell dialysis. In the present study we showed that F-actin- and tubulin-based cytoskeleton is an important factor determining the position of the steady-state activation and availability curves of cardiac \( \text{Na}^+ \) channels. In particular, the stabilization of the position

![Fig. 4. Effect of cytoskeleton disrupters on \( V_{1/2A} - V_{1/2G} \) relationship. A: cytochalasin D (Cyto-D, 20 \( \mu \text{M} \)) shifted relationship to more positive \( V_{1/2G} \) values (243 data points measured in 14 cells). B: colchicine (100 \( \mu \text{M} \)) did not potentiate Cyto-D effect (124 data points measured in 4 cells). Effect of cytoskeleton modifiers is shown in comparison to position of parabolic curve (solid line) representing best fit for relationship in control cells (same line as in Fig. 2). Insets: histograms of \( V_{1/2G} \) departure from reference line. Histograms were calculated with a bin value of 1.2 mV for overall data points in each plot and fitted by a Gaussian function (solid line) given by Eq. 6, with values of \( X_c \) and \( W \) shown in plots.

![Fig. 5. Effect of cytoskeleton stabilizers on \( V_{1/2A} - V_{1/2G} \) relationship. A: phalloidin (100 \( \mu \text{M} \)) did not shift relationship (441 determinations in 22 cells). B: taxol (100 \( \mu \text{M} \)) significantly shifted relationship toward negative \( V_{1/2G} \) values (251 determinations in 12 cells). C: phalloidin + taxol reduced taxol effect (284 determinations in 13 cells). Reference line is parabolic curve representing best fit for relationship in control cells (same line as in Fig. 2). Insets: histograms of \( V_{1/2G} \) departure from reference line. Histograms were calculated with a bin value of 1.2 mV for overall data points in each plot and fitted by a Gaussian function (solid line) given by Eq. 6, with values of \( X_c \) and \( W \) shown in plots.](http://ajpheart.physiology.org/)

![Downloaded from http://ajpheart.physiology.org/ by 10.220.33.3 on May 24, 2017](http://ajpheart.physiology.org/)
for the availability curve by phalloidin represents the first report of the time-dependent shift prevention during cell dialysis. On the contrary, F-actin disruption by Cyto-D accelerated the shift rate. This is in agreement with the observation that the voltage dependence of Na\(^+\) channel gating does not change in nystatin-perforated cardiomyocytes, where the cytoskeleton remains intact (40). Conversely, a pronounced V\(_{1/2A}\) shift was found in the cell-attached patch configuration (6), where cytoplasm remained intact, but the cytoskeleton integrity might be damaged by plasma membrane aspiration into the patch pipette.

V\(_{1/2G}\)-V\(_{1/2A}\) Relationship Is Modulated by Cytoskeleton

The main finding of the study was that the relationship of the steady-state activation and availability is strongly modulated by cytoskeleton modifiers. An important result was that F-actin modifiers influenced availability and activation but tubulin modulates only activation shift. Another interesting observation was that phalloidin alone did not change the activation, but it almost completely abolished the activation shift induced by taxol (−12.5 mV), indicating an important interplay of different cytoskeleton types in Na\(^+\) channel modulation. The finding of modulation of Na\(^+\) channel gating by microtubules is in line with previous reports showing the presence of tubulin along the plasma membrane in rat papillary muscle (39) and a modulating role of microtubules in the function of plasma membrane proteins such as insulin receptors (12), G-proteins (26), and Ca\(^{2+}\) channels (18).

Our finding that stabilization of F-actin by phalloidin influenced availability but not activation is consistent with single channel data. We previously reported that Cyto-D and antibodies to F-actin modulate channel gating by increasing open time and causing frequent bursts of channel openings (23, 35, 37). The changes mainly related to inactivation state but not to activation. Thus the data of single channel studies together with the present data show a degree of independent changes modulated by the F-actin cytoskeleton on the level of microscopic and macroscopic parameters in inactivation.

Possible Mechanisms of Cytoskeleton Influence on Na\(^+\) Channel Steady-State Activation and Availability

The shift of voltage dependence of Na\(^+\) current gating could not be explained by a simple membrane voltage shift, as a surface charge effect, or by alterations in Donnan potentials, because the shifts for availability and activation curve were not parallel. We have found that, in addition to the effect on steady-state activation and availability, Cyto-D significantly slowed I\(_{Na}\) inactivation (23). Furthermore, when only one mechanism is considered, it is difficult to explain asynchronies in availability and activation shifts. We found that availability shift was prevented by phalloidin but accelerated by Cyto-D. At the same time, V\(_{1/2G}\) undergoes a time-dependent shift immediately after establishment of the recording configuration. This resulted in a coupled shift of V\(_{1/2A}\) and V\(_{1/2G}\) in the case of the Cyto-D effect, but phalloidin decoupled these parameters. These data can be explained assuming a complex cytoskeleton modulation of activation and inactivation by mechanisms of a different nature. On the basis of the results of Na\(^+\) channel mutations, it has been shown that a critical structure for Na\(^+\) channel inactivation is a cluster of hydrophobic residues in the intracellular III-IV linker localized at the cytoplasmic site of the plasma membrane (25, 32, 41). Being close to the cytoplasmic site of the plasma membrane (7), the inactivation gate can be influenced by integrity of the plasma membrane-attached cytoskeleton network, where F-actin is a main structural component (5). Most models of the Na\(^+\) channels propose that the S4 segment of the Na\(^+\) channel protein participates in sensing the membrane potential during the gating process (32, 42). The influence of the cytoskeleton on this voltage sensor could be via direct cytoskeleton attachment to Na\(^+\) channel protein (1), or, indirectly, by plasma membrane phospholipids (36), which, in turn, are linked to the cytoskeleton (2).

Physiological Significance

Our detailed analysis of the V\(_{1/2G}\)-V\(_{1/2A}\) relationship indicates that cytoskeleton disruption by Cyto-D modified the coupling of steady-state activation and availability by shifting the relationship toward positive V\(_{1/2G}\) values (Fig. 4A). This result suggests a reduction of cell excitability in conditions affecting cytoskeleton integrity. Particularly, ischemia leads to cytoskeleton disruption (31, 43), and energy depletion induces disintegration of cytoskeleton structure of F-actin filaments (19). On the other hand, a large shift of the V\(_{1/2G}\)-V\(_{1/2A}\) relationship toward negative V\(_{1/2G}\) in cells with modified microtubules (Fig. 5B) may decrease the threshold of I\(_{Na}\) activation and produce premature excitations. Moreover, the shift in the relationship can result in a sustained inward I\(_{Na}\) ("window" current) because of a larger overlap of the steady-state activation and availability curves. It has been recently shown that the sustained inward I\(_{Na}\) leads to excitation abnormalities of the myocardium, such as prolongation of action potential, long Q-T syndrome, and arrhythmias (4, 13). From this point of view, pharmacological interventions targeting the cytoskeleton can potentially lead to cardiac arrhythmias. Different cytoskeleton-directed anti-tumor agents, including taxol, produce a variety of cardiac disturbances, including ventricular tachycardia (27). Our data indicate that the cardiotoxic effect of the drugs can be related to alterations of the Na\(^+\) channel gating.

In conclusion, we have shown that coupling between Na\(^+\) channel voltage-dependent availability and activation is modulated by F-actin-based and microtubular cytoskeleton. The cytoskeleton modulation of cardiac Na\(^+\) channel gating could be important in maintaining normal myocyte excitability.
APPENDIX A

The exponentially saturating shifts of $V_{1/2A}$ [$V_{1/2A}(t)$] and $V_{1/2G}$ [$V_{1/2G}(t)$] can be represented by

$$V_{1/2A}(t) = (V_{1/2A}_0 - V_{1/2A}_s) \cdot \exp(-t/\tau_A) + V_{1/2A}_s \quad (A1)$$

$$V_{1/2G}(t) = (V_{1/2G}_0 - V_{1/2G}_s) \cdot \exp(-t/\tau_G) + V_{1/2G}_s \quad (A2)$$

where $t$ is time of whole cell recording, $\tau_A$ and $\tau_G$ are time constants, $V_{1/2A}$ and $V_{1/2G}$ are saturation levels, and $V_{1/2A}_s$ and $V_{1/2G}_s$ are initial values (at $t = 0$). At any given time (eliminating t from Eqs. A1 and A2 by simple transformations), the $V_{1/2C}$-$V_{1/2A}$ relationship can be written as a power function

$$V_{1/2A} = V_{1/2A}_s + (V_{1/2G}_0 - V_{1/2G}_s) \cdot (V_{1/2G}_0 - V_{1/2A}_s)^{\beta} \quad (A3)$$

where $\beta$, a degree of the power function, equals the ratio of the respective time constants ($\tau_A/\tau_G$).

APPENDIX B

The choice of the best model describing the $V_{1/2C}$-$V_{1/2A}$ relationship was judged by a smaller variance and by parameters of goodness-of-fit statistics, including coefficient of determination (COD) and the model selection criterion (MSC). The COD gives the fraction of the total variance accounted for by the tested model and is defined by

$$COD = \frac{\sum(y_i - \bar{y})^2 - \sum(y_i - \bar{Y})^2}{\sum(y_i - \bar{y})^2} \quad (B1)$$

where $y_i$ is the observed data set ($1 \leq i \leq n$) and $Y_i$ represents calculated values corresponding to $y_i$ values. Because different models have a different number of parameters, the MSC was used to represent the information content of a given set of parameter estimations by normalizing the COD to the parameter number. The MSC is defined by

$$MSC = \ln \frac{\sum(y_i - \bar{y})^2}{\sum(y_i - \bar{Y})^2} - 2p/n \quad (B2)$$

where $p$ is the number of parameters used by a model and $n$ is the number of data points.

We thank Beth Malleis and Nidas Undrovinas for assistance in cell preparation and Dr. J. C. Makieksi and Dr. H. N. Sabbah for helpful discussions and critical reading of the manuscript.

Financial support was provided by National Heart, Lung, and Blood Institute Grant HL-53819 (A. I. Undrovinas). Preliminary data for this study were reported in abstracts (20–22).

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Received 25 November 1996; accepted in final form 9 J une 1996.

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