Biochemical and Biophysical Research Communications **276,** 204–209 (2000)
doi:10.1006/bbrc.2000.3463, available online at http://www.idealibrary.com on IDEAL®

Functional Properties of Sodium Channels Do Not Depend on the Cytoskeleton Integrity

Oscar Moran,[1] Paolo Tammaro, Mario Nizzari, and Franco Conti
Istituto di Cibernetica e Biofisica, CNR, Via De Marini, 6, I-16149 Genoa, Italy

Received August 21, 2000

Several observations suggest an interaction of the sodium channel α-subunit with the cytoskeletal structures. However, there is a wide variability in the results of experiments of heterologous expression in *Xenopus* oocytes and studies on mammalian cells are sometimes contradictory. In general, there has been no direct demonstration that ad hoc large perturbations of the cytoskeleton modify the intrinsic properties of the sodium channels expressed endogenously or heterologously in plasma membranes. We have studied in CHO cells transfected with the rat muscle sodium channel α-subunit the effects of two substances expected to produce drastic perturbations of the cytoskeletal structure: Cytochalasin-D, which depolymerizes microfilaments, and Colchicine, which inhibits the microtubules polymerization. We observed no significant differences in the voltage dependence, kinetic parameters and surface density of the expressed sodium channels after treatment of the cells with these substances. We conclude that the two known main components of the cytoskeleton do not interfere directly with the sodium channel function or with the heterologous expression of channels in the cell membrane.

The α-subunit of the sodium channel is an integral membrane protein comprising the channel pore forming and the essential structures for its gating mechanism. When expressed heterologously in *Xenopus* oocytes or in mammalian cell lines this subunit alone is correctly inserted in the cell plasma membrane allowing the selective passage of sodium currents with fairly normal voltage-dependent properties. In native system, an interaction of the sodium channel protein with cytoplasmic structures is generally expected, particularly in cells like muscle where a non-uniform distribution of the channels has a clear physiological role and should be regulated by anchoring the channel protein to cytoskeletal structures [1]. This idea is also consistent with the fact that the skeletal muscle sodium channel α-subunit contains a C-terminal PDZ-binding domain through which it could be linked to the dystrophin-actin membrane skeleton [2]. Several observations, although lacking a consistent pattern that could lead to a common explanation, suggest that such interaction might also modulate the intrinsic properties of the sodium channel and have, therefore, a physiological role distinct from that of mechanically stabilizing the presence of the channels in a particular region of the cell membrane. Most noticeably, in *Xenopus* oocytes a shift of the voltage-dependence of activation and inactivation and a hastening of the inactivation of the sodium currents mediated by exogenous rat skeletal muscle α-subunits channels is observed in patch-clamp recordings upon stretching the patch by suction of the recording pipette [3, 4], and a variety of effects on sodium inactivation have been reported for patch or whole-cell recordings from native preparations [5, 6]. A possible explanation for these effects is an alteration of the interaction of the sodium channel with cytoskeletal structures caused by the rather drastic mechanical disturbance of the cell structure inherent in the patch-clamp technique [3]. We have tested the effects of direct ad hoc challenges of a possible interaction of the sodium channel α-subunit expressed in CHO cells by prolonged exposures to agents that disrupt the two major components of the cytoskeleton, the actin microfilaments and the microtubules. In contrast to large changes in the cell morphology we find that the disruption of actin microfilaments does not affect appreciably the inactivation of sodium currents and causes barely a significant shift of the activation curve by −5 mV. Similarly minor effects are produced by the inhibition of the tubulin polymerization, which causes even more drastic morphological changes but only a slight change of the sodium inactivation kinetics without affecting the voltage-dependency of the sodium currents. Thus, in our expression system the integrity of the cytoskeleton is not at all required for the normal functioning of the sodium channels nor for

[1] To whom correspondence should be addressed. Fax: +39-010-6475500. E-mail: moran@barolo.icb.ge.cnr.it.

0006-291X/00 $35.00

their efficient and correct insertion in the plasma membrane. These results argue against the interpretation of the large effects apparently produced on the sodium currents by the mechanical stresses of the patch-clamp technique as simply due to changes the interaction of the channels with cytoskeleton components.

MATERIALS AND METHODS

Expression system. The full-length cDNA coding for the rSkM1 sodium channel α-subunit [7] was sub-cloned between the *Eco*RI–*Xba*I sites of the expression vector pcDNA3 (Invitrogene), named μ1-pcDNA3. Chinese hamster ovary cells (CHO) were transfected by electroporation. Transfected cells were selected in the presence of 800 μg/ml of Geneticin (G-418, Sigma). Clones with resistance to the antibiotic were expanded and individually checked, and a high expression cell clone, named CHO-μ1-9, was selected. The current density and the electrophysiological properties of this cell clone were very reproducible. Cells were plated in 35 mm petri dishes and used for experiments within 3 days.

Electrophysiological recording. Sodium currents were measured using the patch-clamp technique in the whole-cell configuration [8] using an Axopatch-200 amplifier (Axon Instruments). Borosilicate glass micropipettes (Hilgemberg) were fire polished to a tip diameter yielding a resistance of 2.2 to 2.8 M when filled with the working solution. The pipettes were filled with (in mM): NaCl 25, CsF 110, EGTA 10, TEA 2, HEPES-NaOH 10; pH 7.3. The external solution had the following composition (in mM): NaCl 100, KCl 5, $CaCl_2$ 2, TEA 20, HEPES-NaOH 10; pH 7.4. Osmolarity in both solutions was adjusted to 300 mOsm/kg with mannitol. The use of intracellular Cs^+ and intra- and extracellular TEA^+ abolished most of the endogenous potassium currents. The output of the patch-clamp amplifier was filtered by the built-in low-pass 4-pole Bessel filter with a cut-off frequency of 5 kHz and sampled at 20 kHz. The cells were kept at a holding potential of -120 mV. Pulse stimulation and data acquisition used 16 bit D-A and A-D converters (ITC-16, Instrutech) controlled by a Macintosh microcomputer with the Pulse software (Heka Electronik). Linear responses were estimated from sub-threshold stimulations, partially compensated analogically, and digitally subtracted with a standard P/4 protocol. The access resistance was always less than 7 M and the cell capacity was between 7 and 20 pF, as measured by the cell capacity compensation circuit of the amplifier. The series-resistance was usually compensated between 80 and 85%. All measurements were done at a controlled temperature of 19 $\pm$ 0.5°C. Records were taken at least 6 min after establishing the whole-cell voltage-clamp, a time estimated to be long enough in this preparation to allow the removal of the ultra slow inactivation and the stabilization of the voltage-dependent properties of the channels [5, 9]. Cells yielding maximal currents higher than 4 nA were discarded to avoid large voltage-clamp errors due to uncompensated series resistance.

Chemicals. Stock solutions of 2 mM Cytochalasin-D in DMSO and 1 mM colchicine in water were maintained at 4°C and protected from light. Working solutions were freshly prepared daily by diluting the stock solutions, maintained in ice, and protected from light. All chemicals were purchased from Sigma.

Data analysis. Data were analyzed with a Macintosh microcomputer using PulseFit program (Heka Electronik) and custom software developed in the Igor environment (Wavemetrics). Statistical comparisons were done with a Student's *t* test, and statistical significance was defined by $P < 0.05$. The results were expressed as mean $\pm$ SEM (number of measures). A simplex method was used for curve fitting.

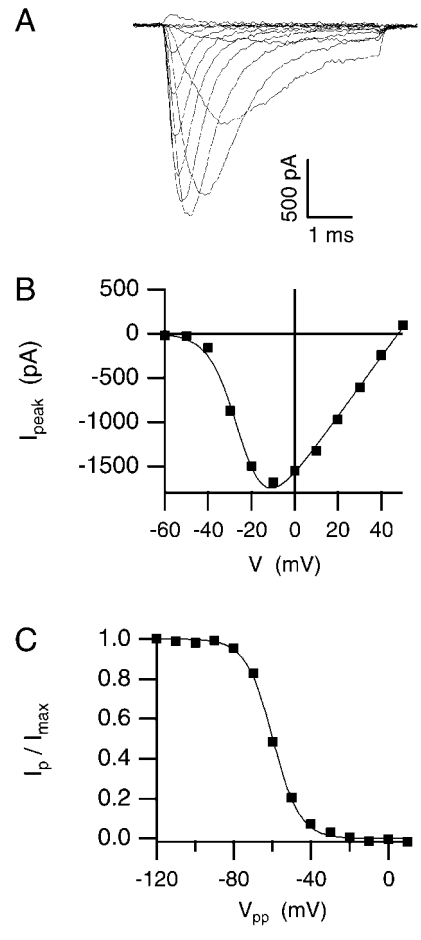

FIG. 1. Sodium currents mediated by the rat skeletal muscle sodium channel α-subunit permanently expressed in CHO cells. (A) Family of currents, recorded in whole cell configuration, elicited by 10 ms depolarizing pulses from a holding potential of -120 mV to test potentials from -60 mV to $+50$ mV (in 10 mV increment). (B) Peak currents (I_p) plotted against the test potential (V). The continuous line shows the best fit with the equation (1), yielding $V_{0.5} = -29.46$ mV, $s_a = 6.48$ mV, $E_{Na} = 45.54$ mV, $\Gamma = 37.57$ nS. (C) The inactivation curve was obtained plotting the normalized peak current (I_p/I_{max}) against the potential of a 100 ms conditioning pre-pulse (V_{pp}). The continuous line is the best fit with the equation (2), yielding $V_h = -61.23$ mV and $s_h = 7.06$ mV.

RESULTS

About 97% of the cells transfected with the sole α-subunit expressed high-density sodium currents (maximum peak values: 1.8 $\pm$ 0.2 nA, $n = 30$). The sodium current in response to a step depolarization was quite normal, characterized by a rapid increase followed by an inactivation (Fig. 1A). In most cases, the falling phase had a monophasic behavior that could be well fitted with a single-exponential function. As previously observed in transfected cells (HEK) [10], in few experiments a small fraction of the current (<3%) inactivated more slowly, but the apparent fraction of slowly inactivating channels was in any case much

TABLE 1

Effects of Cytochalasin-D and Colchicine on the Main Properties of Sodium Channels Expressed in Transfected CHO Cells: Maximum Sodium Conductance Normalized to Cell Capacitance (G_{max}); Potential of Half-Activation (V_{05}); Half-Inactivation Potential (V_h); and Inactivation Time Constant Measured at 0 mV (τ_h)

	Control	Cytochalasin-D (20 μM)	Colchicine (50 μM)
G_{max} (nS/pF)	3.157 ± 0.478 (28)	8.19 ± 2.50 (10) N.S.	4.13 ± 0.93 (12) N.S.
$V_{0.5}$ (mV)	−25.39 ± 1.03 (30)	−30.01 ± 1.98 (7) $P < 0.05$	−26.00 ± 1.98 (9) N.S.
V_h (mV)	−61.60 ± 1.08 (30)	−64.28 ± 2.00 (7) N.S.	−59.95 ± 1.26 (9) N.S.
τ_h (ms)	0.50 ± 0.03 (30)	0.56 ± 0.02 (9) N.S.	0.61 ± 0.03 (8) $P < 0.02$

Note. Control cells are compared with cells treated for 18 h with cytochalasin-D or colchicine. The data represent the means ± SEM (number of experiments). Significant changes are indicated. N.S., no statistically significant difference.

smaller than that observed when the sole α-subunit is expressed in *Xenopus* oocytes [11, 12].

The voltage-dependencies of activation and inactivation were measured using standard protocols. For the activation, sodium currents were elicited by 5 to 30 ms pulses from the holding potential to a test potential between −60 to +50 mV and peak values, I_p, were plotted against the test potential, V (Fig. 1B). The data were fitted with:

$$\frac{I_p}{I_{max}} = \frac{1}{1 + \exp[-(V - V_h)/s_h]} \quad [1]$$

where Γ is the maximum sodium conductance, $V_{0.5}$ is the half activation potential, s_a is the e-fold slope and E_{Na} is the reversal potential of the sodium current. In all experiments E_{Na} was close to that predicted for the sodium equilibrium potential by the Nernst equation.

The steady-state inactivation was characterized by plotting the peak currents elicited by a test pulse to 0 mV, preceded by a conditioning pre-pulse of 100 ms to a variable potential, V_{pp}, between −120 and 10 mV. The inactivation parameters were defined by fitting the plot with (Fig. 1C):

$$\frac{I_p}{I_{max}} = \frac{1}{1 + \exp[-(V - V_h)/s_h]} \quad [2]$$

where V_h is the half inactivation potential and s_h is the e-fold slope. The estimate of the activation and inactivation parameters of control cells ($n = 30$) are given in the second column of Table 1.

Effect of Cytochalasin-D

To test the effect of the disruption of microfilaments on the functional properties of the sodium currents we applied Cytochalasin-D, which depolymerizes the actin microfilament network [13]. CHO-μ1-9 cells were incubated with 20 μM Cytochalasin-D for 18 h at 37°C. The Cytochalasin-D containing solution was replaced by the standard external solution immediately before starting the electrophysiological measurements. A readily observed qualitative effect of the treatment with Cytochalasin-D was the loss of the normal fusiform shape of the cells, the great majority of which appeared spherical. However, the microfilament disruption by Cytochalasin-D did not change significantly the surface density of the expressed sodium channels estimated from the maximum conductance normalized to the cell capacitance (G_{max}; nS/pF). Also the voltage dependence of the steady-state inactivation and the time constant of inactivation (measured at 0 mV) were unchanged after 18 h of Cytochalasin-D treatment (Fig. 2A). The sole appreciable change detected in cells treated with Cytochalasin-D was a negative shift of ~5 mV in the voltage dependence of activation (see Table 1).

In few experiments, Cytochalasin-D was applied intracellularly by filling only the tip of the patch pipette with 10 μl of normal internal solution, and adding carefully on top 10 μl of internal solution containing 50 μM Cytochalasin-D. The pipette was immediately used to patch a cell in the whole-cell configuration. In this way the cell is initially dialyzed with the normal intracellular solution and only after several minutes Cytochalasin-D is actually invading the intracellular compartment. The validity of this method for a qualitative study of the delayed effect of intracellularly applied substances was tested by using intracellular solutions with different Na^+ concentrations and monitoring that indeed the value of E_{Na} changed during the first 10 min approaching that expected according to the Na^+ concentration of the solution filling the shank of the pipette (data not shown). Figure 2B shows two recordings of sodium currents, one measured 4 min after the patch formation, when probably the Cytochalasin-D had not yet diffused into the cell, and

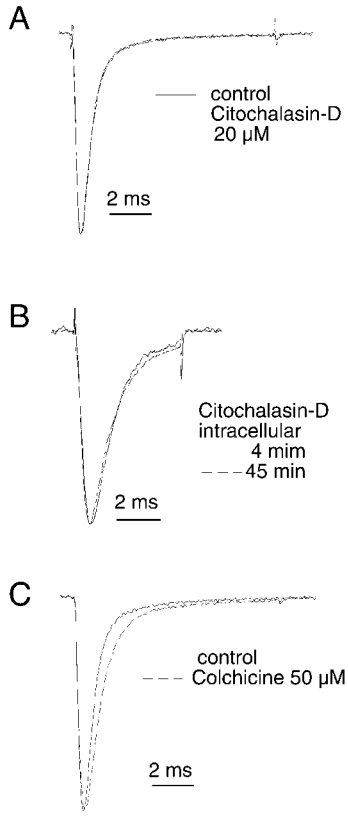

FIG. 2. Effects of disruption of cytoskeleton components on currents expressed by the sodium channel α-subunit transfected in CHO cells. (A) Currents elicited by 10 ms pulse at 0 mV from a holding potential of −120 mV in control cells (continuous line) and in cells treated 18 h with Cytochalasin-D 20 μM (broken line). The currents were scaled to the same peak amplitude. Note that the shape of the two currents is practically identical. (B) Currents elicited by 5 ms pulse at $V = V_{0.5} + 20$ mV after 4 min (continuous line) and 45 min (broken line) from the patch formation with Cytochalasin-D filled into the pipette. The two traces were scaled to the same amplitude and show no significant differences in their time courses. (C) Currents elicited by 10 ms pulse at 0 mV from a holding potential of −120 mV in control cells (continuous line) and in cells treated 18 h with colchicine 50 μM (broken line). The two traces were scaled to the same peak amplitude. The falling phase of the currents appears significantly slowed by the colchicine treatment.

the second after 45 min, when presumably Cytochalasin-D had its effect on the cytoskeleton of the cell. The second record showed a larger peak current, likely as a result of removal of slow inactivation at the hyperpolarized holding potential, so the traces have been scaled to the same peak amplitude. However, it is seen that the time course of the current is not significantly affected by the intracellular application of Cytochalasin-D.

Effects of Colchicine

We attempted to disrupt the microtubules by incubating the cells with a solution containing 50 μM colchicine for 18 h. This treatment resulted in a dramatic change in shape (qualitatively similar, but more pronounced of that produced by Cytochalasin-D) and a drastic decrease in number of surviving cells. The sodium currents in cells incubated in 50 μM colchicine for 18 h showed fairly normal kinetics and voltage dependence of activation (Fig. 2C, Table 1). The sole significant change produced by the treatment with colchicine was a slight slowing of inactivation (Fig. 2C). The time constant of inactivation at 0 mV was 20% larger in cells treated with colchicine, when compared with controls (Table 1).

Occasional observations of petri dishes with cells kept in colchicine solution for 7 days showed a dramatic increase of the cell mortality and a much larger membrane surface of the few surviving cells. In few experiments on these cells we found that the prolonged colchicine treatment resulted in a roughly three-fold increase of the density of expressed sodium channels, indicating that the surviving cells continue to synthesize and dock correctly these membrane proteins although perhaps at an “abnormal” rate. The sodium currents measured in these cells reached peak values of more than 20 nA, saturating the patch-clamp amplifier and not measurable to proper quantitative characterization.

DISCUSSION

Our present work shows that the depolymerization of two of the main cytoskeleton components, actin and tubulin, causes only barely appreciable changes of the functional properties of the sodium channels heterologously expressed in CHO cells. We also observed that the sodium currents for unit membrane, reflecting the density of expressed channels, is rather insensitive to treatment with colchicine or Cytochalasin-D, suggesting that actin and tubulin are not essential for the transfer of the sodium channel α-subunit to the plasma membrane. However, the few occasional measurements on cells exposed for 1 week to colchicine, and showing a higher than normal sodium channel density suggest that the integrity of the microtubules is an important factor for the regulation of the correct density of channels in the membrane.

In cells treated with Cytochalasin-D, the activation curve is only slightly shifted towards more negative values. Shifts in the same direction of both $V_{0.5}$ and V_h were reported as a consequence of membrane-patch excision in myocardiocytes [6, 14, 15], chromaffin cells [16], and neuroblastoma cells [17], or as an effect of the giga-seal formation in cell-attached recording from myoballs [18]. Differently, Fernandez and co-workers [19] did not find any difference in measurement from whole-cells or from outside-out excised patches from rat clonal pituitary cells. The shift of $V_{0.5}$ that we observe in CHO cells as a consequence of Cytochalasin-D treatment is very small and not accompanied by any

significant shift of the inactivation curve as reported for those preparations. If we assume, as suggested [14, 15, 18], that the excision destroys the actin cortex (the portion of microfilaments anastomized in the peripheral part of the cytoplasm), it is expected that Cytochalasin-D produces the same effects and our present result indicate that the gating differences observed with different patch configurations are unlikely a direct consequence of the rupture of cytoskeleton elements.

In the expression of sodium channel α-subunits in frog oocytes, large parallel shifts of both activation and inactivation are observed as an effect of "aging" of the patch [12, 20] and suggested to result from the disrupting of the cytoskeleton upon membrane stretching, since they are accelerated by the patch excision [3, 12] or simply by increasing the suction in the patch pipette [4]. The parallel shifts may be easily explained if the disruption of the cytoskeleton causes changes of the surface membrane potential altering the electric field effectively acting on the voltage sensors of the channels. Another effect of the patch formation observed for the oocyte expression system is the marked change in the modal behavior of the sodium channel. When the α-subunit alone is expressed in oocytes, a significant fraction of the channels inactivates in a slow mode, with a time constant that is roughly 10-fold slower than that of normal sodium inactivation. The propensity of the channels to inactivate in the slow mode is strongly reduced by patch excision or by suction in the patch pipette, causing the sodium currents to inactivate almost exclusively in the normal fast mode. Such changes were never observed in the heterologous expression of the sodium α-subunit in mammalian cells yielding currents that do not have the abnormally large slow inactivation component observed in oocytes. It is possible that the exacerbation of the modal behavior of the sodium channels expressed in oocytes is due to a channel–cytoskeleton interaction that is peculiar of these cells. In that case the changes in modal properties as due to cytoskeleton perturbations would not be in conflict with our finding, but the changes would have scarce physiological significance.

A reduction of the peak sodium current and a slowing of inactivation have been described as a result of treatment of cardiac myocytes with Cytochalasin-D [20, 21]. No such changes were observed in our experiments with CHO cells expressing the skeletal muscle α-subunit. The difference may be explained by the particular properties of cardiomyocytes: as other muscle cells they likely have a particularly high content of actin heavily involved in chemical reactions that be a major component of the cell metabolism; it is plausible that the depolymerization of actin leads to major changes affecting some of the intracellular factors that modulate sodium channels.

In our preparation, the treatment with colchicine did not cause any significant change in the voltage dependence but produced a small increase of the time constant of inactivation. This may indicate a possible interaction of the microtubules with the intracellular domain of the sodium channel involved in the inactivation process. However this modest change of inactivation kinetics is substantially different from the change of slow mode properties observed in the oocyte preparation where the inactivation phase of the current is clearly biexponential and where the slow mode is about 10-fold slower than the normal mode.

We conclude that per se the destabilization of microfilaments or microtubules produces only minor effects on the functional properties of the sodium currents expressed by the α-subunit of rat muscle sodium channel expressed by transfected CHO cells, and that larger and controversial effects that indirectly suggest for other preparations an important interaction of sodium channels with cytoskeletal structures, are more likely involving other preparation-specific cytoplasmic factors.

ACKNOWLEDGMENT

This work was supported by Telethon Grant 926.

REFERENCES

1. Almers, W., Stanfield, P. R., and Stühmer, W. (1983) *J. Physiol.* **336,** 261–284.
2. Gee, S. H., Madhavan, R., Levinson, S. R., Caldwell, J. H., Sealock, R., and Froehner, S. C. (1998) *J. Neurosci.* **18,** 128–137.
3. Shcherbatko, A., Ono, F., Mandel, G., and Brehm, P. (1999) *Biophys. J.* **77,** 1945–1959.
4. Tabarean, I. V., Juranka, P., and Morris, C. E. (1999) *Biophys. J.* **77,** 758–774.
5. Wend, D. J., Starmer, C. F., and Grant, A. O. (1992) *Am. J. Physiol.* **263,** C1234–C1240.
6. Nilius, B. (1988) *Biophys. J.* **53,** 857–862.
7. Trimmer, J. S., Cooperman, S. S., Tomiko, S. A., Zhou, J., Crean, S. M., Boyle, M. B., Kallen, R. G., Sheng, Z., Barchi, R. L., Sigworth, F. J., Goodman, R. H., Agnew, W. S., and Mandel, G. (1989) *Neuron* **3,** 33–49.
8. Hamill, O., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. (1981) *Pflüger's Arch.* **391,** 85–100.
9. Cummins, T. R., and Sigworth, F. J. (1996) *Biophys. J.* **71,** 227–236.
10. Ukomadu, C., Zhou, J., Sigworth, F. J., and Agnew, W. S. (1992) *Neuron* **8,** 663–676.
11. Ji, S., Sun, W., George, A. L., Jr., Horn, R., and Barchi, R. L. (1994) *J. Gen. Physiol.* **104,** 625–643.
12. Moran, O., Nizzari, M., and Conti, F. (1998) *in* Neuronal Circuits and Networks (Torre, V., and J. Nicolls, Eds.), pp. 3–19 Springer Verlag, Berlin Heidelberg.

13. Cooper, J. A. (1987) *J. Cell Biol.* **105,** 1473–1478.
14. Chachelin, A. B., De Peyer, J. E., Kokubun, S., and Reuter, H. (1983) *J. Physiol.* **340,** 389–401.
15. Kolhardt, M. (1990) *Am. J. Physiol.* **259,** C599–C604.
16. Frenwick, E., Marty, A., and Neher, E. (1982) *J. Physiol.* **331,** 599–635.
17. Moran, O., and Conti, F. (1990) *Eur. Biophys. J.* **18,** 25–32.
18. Fahlke, C., and Rüdel, R. (1992) *Pflüger's Arch.* **420,** 248–254.
19. Fernandez, J. M., Fox, A. P., and Krasne, S. (1984) *J. Physiol.* **356,** 565–585.
20. Maltsev, V. A., and Undrovinas, A. I. (1997) *Am. J. Physiol.* **273,** H1832–H1840.
21. Undrovinas, A. I., Shander, G. S., and Makielski, J. C. (1995) *Am. J. Physiol.* **269,** H203–H214.