Evidence for Developmental Changes in Sodium Channel Inactivation Gating and Sodium Channel Block by Phenytoin in Rat Cardiac Myocytes

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The voltage-dependent properties of the voltage-activated sodium channel were studied in neonatal (1–2 day-old) and adult rat ventricular cardiac myocytes using the whole-cell variation of the patch-clamp technique (16°C, [Na]o=15 mM, [Na]i=25 mM). The voltage dependence of the sodium conductance–membrane potential relation was similar in both neonatal and adult myocytes except for a difference in slope; the adult sodium conductance–membrane potential relation was slightly more steep. Neonatal cells also differed from adult cells by demonstrating a more negative voltage midpoint of their sodium availability curve, a slower rate of recovery from inactivation at hyperpolarized potentials, and a greater extent of slow inactivation development compared with adult cells. Phenytoin (40 μM) reduced the sodium current in a tonic and use-dependent manner in both adult and neonatal myocytes. However, phenytoin (40 μM) produced significantly more tonic block at negative holding potentials (e.g., −140 mV) in neonatal myocytes (22±5% [mean±SEM], n=14) than in adult myocytes (10±2%, n=11) (p<0.05). The amplitudes of use-dependent block obtained during trains of 1-second pulses to −20 mV were also significantly greater in neonatal myocytes than in adult myocytes when the diastolic interval was varied over a range of 0.1–1.5 seconds (p<0.05). Definition of the time courses of block development at −20 mV indicated that phenytoin had a slightly higher affinity for inactivated sodium channels in neonatal cells. In addition, the time constant of recovery from use-dependent block by phenytoin was found to be significantly longer in neonatal cells than in adult cells at membrane potentials between −160 and −100 mV (p<0.001). The marked differences in phenytoin effect on cardiac sodium channels in neonatal versus adult rat cardiac myocytes suggest that there may be significant developmental changes in the sodium channel blocking effects of class I antiarrhythmic drugs in cardiac tissue. (Circulation Research 1991;69:644–656)

Previous studies using canine cardiac Purkinje fibers and myocardium have documented the presence of developmental changes in the effects of antiarrhythmic drugs on the action potential maximum upstroke velocity (Vmax).1–5 Developmental changes in the effect of antiarrhythmic drugs on Vmax have also been observed in the developing prenatal human heart,6 as well as in a previous study7 using sodium current (INa) measurements in isolated rat myocytes. Although these studies have provided strong evidence for the presence of developmental changes in the effects of local anesthetic–like drugs on cardiac sodium channels, the molecular mechanisms underlying such changes remain poorly understood.

A primary goal of the present study was to determine whether developmental changes can be demonstrated in the effects of the class I antiarrhythmic drug phenytoin on INa under voltage-clamp conditions and to define the state-dependent basis for such developmental differences. An isolated rat myocyte model was selected for study because rat myocytes are known to undergo significant changes in both their ultrastructure and electrophysiology during
postnatal development\textsuperscript{8,9} and because both neonatal and adult rat myocytes are commonly used as cell models for characterization of the effects of sodium channel-blocking drugs.\textsuperscript{7,10-12} Phenytoin was selected for study because it has been reported to be particularly effective in the treatment of ventricular arrhythmias in young patients.\textsuperscript{13,14}

Since developmental changes in sodium channel gating could contribute to age-dependent changes in drug-channel interaction, a second goal of this study was to provide a quantitative comparison of the time- and voltage-dependent behavior of cardiac sodium channels in neonatal and adult myocytes.

**Materials and Methods**

**Isolation of Cardiac Myocytes**

*Adult myocytes.* Hearts were removed from adult rats (170–200 g) under pentobarbital anesthesia and quickly mounted by the aorta on the cannula of a Langendorff perfusion apparatus. Myocytes were isolated enzymatically using collagenase and protease enzymes using the technique described by Crumb and Clarkson.\textsuperscript{15} Harvested myocytes were suspended in minimal essential medium supplemented with Earle’s salts, t-glutamine (292 μg/ml), 3% horse serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin (Sigma Chemical Co., St. Louis, Mo.). The cell suspension was placed into culture dishes and stored in a CO\textsubscript{2} incubator at 37°C until the cells were used in experiments (within 30 hours after isolation).

*Neonatal myocytes.* Neonatal rats (1–2 days old) were killed by cervical dislocation, and hearts were removed under sterile conditions through a midline incision in the chest. The ventricles were cut into pieces 0.5–1.0 mm in diameter using a razor blade and incubated for 15 minutes at 37°C in Joklik’s medium supplemented with 50 μM CaCl\textsubscript{2}, 3.4 mM MgSO\textsubscript{4}, 12.5 mM KCl, 2 mM D-glucose, 0.7 mg/ml collagenase (type II, Worthington Biochemical Corp., Freehold, N.J.), and 0.1 mg/ml deoxyribonuclease I (type II, Sigma). The pieces of tissue were then collected, placed into a test tube containing 10 ml enzyme-free Joklik’s medium supplemented with 5% fetal bovine serum (Sigma), and vortexed for 10 seconds. The tissue was allowed to settle to the bottom of the tube, and the supernatant was collected. The latter step was repeated two times, and the supernatant solutions were combined into one tube. The supernatant, which contained a suspension of isolated cells, was then gently centrifuged to form a pellet. The supernatant was then drawn off, and the pellet of cells was resuspended in Medium 199 (Sigma) warmed to 37°C and supplemented with 0.1 mg/ml streptomycin, 100 units/ml penicillin, 3% fetal bovine serum, 10 μg/ml insulin, 10 μg/ml transferrin, 10 μg/ml sodium selenite, 1.5 μM vitamin B\textsubscript{12}, and 1 μg/ml thyroxine (all obtained from Sigma). The cell suspension was incubated for 30 minutes at 37°C in a 60-mm Falcon 3000 series culture dish to allow attachment of fibroblasts and then transferred to several 35-mm 1000 series Falcon dishes. The dishes containing the cell suspension were then stored in a CO\textsubscript{2} incubator at 37°C until used in experiments (within 48 hours after isolation).

**Voltage-Clamp Recording**

At the start of each experiment, a small aliquot of culture solution containing neonatal or adult myocytes was placed into a shallow bath mounted on top of an inverted microscope and perfused with an external solution of the following composition (mM): tetramethylammonium chloride 115, NaCl 25, CsCl 5, CaCl\textsubscript{2} 1.8, MgCl\textsubscript{2} 1.2, HEPES 20, and glucose 11, adjusted to a pH of 7.3 with tetramethylammonium hydroxide. Glass pipettes were pulled in two steps, and the tips were fire-polished to obtain tip openings either 3–4 μm in diameter for experiments on adult myocytes or 1–2 μm in diameter for experiments on neonatal myocytes. Glass pipettes were filled with an internal solution containing (mM) CsF 125, CsCl 20, NaF 15, bis-tris propane 10, and EGTA 5, adjusted to a pH of 7.2 with CsOH. A thermistor was placed near the cell under study, and the temperature of the external solution was cooled to 16±0.5°C with a thermoelectric device (model 806-7243-01, Cambion/Midland Ross, Cambridge, Mass.). \(I_{Na}\) was measured in the whole-cell configuration using an Axopatch 1-B amplifier (Axon Instruments, Foster City, Calif.) interfaced to an IBM PC/AT computer by a 125-kHz Labmaster board (Scientific Solutions Inc., Solon, Ohio). Generation of voltage-clamp pulse paradigms and data collection were controlled using Clampfit software (Version 5, Axon Instruments). \(I_{Na}\) was filtered at 10 kHz, digitized at sample intervals of 12–20 μsec, and stored on floppy disk. Between pulse protocols, cells were maintained at a holding potential of −140 mV to fully remove resting inactivation. Phenytoin (50 mg/ml, Elkins-Sinn Inc., Cherry Hill, N.J.) was added directly to the external solution, and cells were superfused with drug solutions for 10 minutes before defining drug effects on \(I_{Na}\).

**Evaluation of Method**

A combination of ion substitution and use of channel blockers was used to isolate \(I_{Na}\). Potassium currents were eliminated by replacement of internal and external K\textsuperscript{+} with Cs\textsuperscript{+}, and calcium currents were suppressed by substitution of internal Cl\textsuperscript{−} ions with F\textsuperscript{−} ions.\textsuperscript{16-18} No evidence for potassium or calcium currents was seen at the test potentials used. The total series resistance (\(R_s\)) for the pathway between the pipette interior and cell membrane in the whole-cell configuration was calculated from estimates of cell capacitance (\(C_m\)) and the time constant of the capacitative current’s decay (\(τ_c\)) using the equation \(\tau_c = R_s C_m\). Values of \(C_m\) were estimated from integration of capacitative current transients and had mean±SEM values of 19.5±0.6 pF for neonatal myocytes (\(n=16\)) and 92.1±11.4 pF for adult rat myocytes (\(n=16\)). Mean \(τ_c\) values were 41.3±5.2 μsec for neonatal myocytes (\(n=16\)) and 121±13.3 μsec for adult myocytes (\(n=16\)). The mean \(R_s\) for the
pathway between the pipette and cell membrane after rupture of the membrane seal was calculated to be 2.1±0.2 MΩ for experiments on neonatal myocytes (n=16) and 1.5±0.1 MΩ for experiments on adult myocytes (n=16). It was possible to electronically compensate for 60–80% of R, in these cells. The mean peak amplitudes of INa at −40 mV were 1.11±0.09 nA for neonatal myocytes (n=11) and 7.45±0.71 nA for adult myocytes (n=11). Experiments were performed in cells where the estimated voltage error attributed to uncompensated series resistance (Rf, INa) was <5 mV.

The typical format for each experiment was to monitor the INa amplitude and kinetics for stability during the initial 15-minute equilibration period after the onset of intracellular perfusion. If the current amplitude and kinetics became stable by the end of this 15-minute time period, control data were collected, followed by repetition of the same pulse protocols after a 10-minute equilibration in the presence of external solution supplemented with 40 μM (10 μg/ml) phenytoin. The ability to reverse the effects of phenytoin by changing the bath solution to a drug-free external solution after equilibration in phenytoin was not characterized.

Similar to previous reports by other investigators, we have observed time-dependent variations in channel gating characteristics as a function of time in the absence of drug. We took two steps to minimize the role of such phenomena in our experiments as far as possible. First, we avoided recording experimental data during the initial 15 minutes after the onset of intracellular perfusion, a time period when shifts in the voltage dependence of channel availability and kinetics of INa decay appeared to be largest. An exception to this 15-minute rule was made in a few pilot experiments in which the time dependence of inactivation recovery kinetics was defined (Table 3). Second, we performed all pulse protocols in neonatal and adult cells at identical (matched) time intervals after the onset of intracellular perfusion to minimize the possibility that any observed differences between the two cell types could be attributed to a systematic difference in perfusion time.

As described above, the culture medium that was used to maintain neonatal myocytes contained additional nutrients (e.g., insulin, transferrin, sodium selenite, vitamin B12, and thyroxine) not included in the adult storage medium. Inclusion of these nutrients was required to maintain neonatal cells in a viable state for more than a few hours. To determine whether a difference in these culture medium nutrients could affect the experimental results, we performed a series of eight experiments on freshly isolated neonatal cells that were stored in the adult culture medium for several hours. We found no significant differences in sodium channel properties or use-dependent block produced by 40 μM phenytoin in neonatal cells stored in either medium. For example, for neonatal cells stored in adult medium, exposure to 40 μM phenytoin produced a tonic block of 25.3±8.3% at −140 mV, a further use-dependent reduction of 34.4±3.6% during a train of 1-second pulses to −20 mV (cycle length =1.6 second), and a time constant of recovery from use-dependent block at −140 mV of 2.0±0.4 seconds (n=8). These results are not significantly different from those obtained in cells maintained in the normal neonatal storage solution (see Table 4) and indicate that differences in the storage medium do not alter the response of the cells to phenytoin.

**Curve Fitting and Statistics**

Curve fitting was performed on an IBM-PC/AT using nonlinear least-squares fitting algorithms written in the c language. Results are expressed as mean±SEM. Student’s t test was used to estimate the significance of differences between mean values for two groups of data. Differences were considered significant at p<0.05.

**Results**

**Comparison of Sodium Current Gating Properties in Neonatal and Adult Cells**

To determine whether there are developmental changes in the gating behavior of rat cardiac sodium channels, we compared the kinetic and voltage-dependent properties of INa measured in voltage-clamped ventricular myocytes isolated from the hearts of neonatal and adult rats.

**Activation.** Figure 1 shows a comparison of INa measured in neonatal and adult rat myocytes. Super-
imposed tracings of \( I_{Na} \) elicited from neonatal and adult myocytes during step depolarizations over the range of \(-90 \) to \(+40 \) mV are shown in Figures 1A and 1B, and the relations between peak \( I_{Na} \) (normalized to cell capacitance \([\text{nA/}\mu\text{F}]\) ) and membrane potential are shown in Figure 1C. As illustrated in Figures 1A and 1B and Table 1, the amplitude of the peak \( I_{Na} \) (uncorrected for cell capacitance) was approximately seven times smaller in neonatal cells than in adult cells. To provide a more quantitative comparison of the voltage dependence of sodium channel opening in the two cell types, the relations between peak \( I_{Na} \) and membrane potential \( (V_m) \) were fit to the equation

\[
I_{Na} = \frac{G_{Max}(V_m-V_{Na})}{[1+\exp(V_m-V_{mid}/S)]} \tag{1}
\]

where \( G_{Max} \) is the maximal peak sodium conductance, \( V_{Na} \) is the reversal potential for \( I_{Na} \), and \( V_{mid} \) and \( S \) are the midpoint and slope factor for the relation between sodium conductance \( (G_{Na}) \) and \( V_m \) respectively. Examples of fits of Equation 1 to pooled experimental data are shown in Figure 1C. Mean values of \( V_{Na}, V_{mid}, S, G_{Max} \) and the ratio of \( G_{Max}/C_m \) for both cell types are shown in Table 1. As anticipated from the difference in current amplitudes, adult cells were found to have a significantly larger \( G_{Max} \). However, there was no significant difference in the \( G_{Max}/C_m \) ratio, suggesting that the observed difference in \( G_{Max} \) is primarily due to differences in membrane surface area. Adult cells were also found to have a slightly smaller slope factor \((p<0.002)\) (Table 1) defining the \( G_{Na}-V_m \) relation. Although this may reflect a developmental change in the voltage dependence of channel opening, it is difficult to rule out the possibility that such small differences could not also result in part from a less ideal spatial voltage control in adult cells that was due to their larger current amplitude, less spherical shape, and higher density of T tubules. Although we cannot rule out this possibility, we do not believe that series-resistance errors alone can explain the difference, since increasing the current amplitude in neonatal cells sevenfold by raising the external sodium concentration from 25 to 145 mM produced no significant change in either the midpoint or slope of the \( G_{Na}-V_m \) relation in neonatal cells (Table 1). In addition to having similar current–voltage relations, values for time-to-peak current during depolarizing steps to voltages between \(-60 \) and \( 0 \) mV were also not significantly different (Figure 1D). These results suggest that the activation properties of neonatal and adult sodium channels are relatively similar.

**Inactivation.** A comparison of inactivation gating properties in neonatal and adult cells was performed by defining the kinetics of \( I_{Na} \) decay at different potentials, the time course of recovery from inactivation at different potentials, and the voltage dependence of \( I_{Na} \) availability in both cell types. As illustrated in Figures 2A and 2B, the time course of \( I_{Na} \) decay after its inward peak in both cell types could be well described by a double exponential equation of the form

\[
I_{Na} = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + A_3 \tag{2}
\]

where \( A_1 \) and \( A_2 \) indicate the amplitude of the fast and slow components of decay, respectively, after the inward peak, \( A_3 \) is the extrapolated steady-state level of \( I_{Na} \), and \( \tau_1 \) and \( \tau_2 \) are the time constants for the fast and slow components of decay, respectively. The time courses of \( I_{Na} \) decay in both neonatal and adult cells were qualitatively similar at most potentials and contained a large rapid component, a small slow component, and a very small steady-state component \((A_3\leq1\% \) in these cells) (Figure 2). Some differences were observed, however, at selected potentials: neonatal cells contained a significantly \((p<0.05)\) larger \( A_3 \) at \(-40, -20, \) and \(-10 \) mV. Neonatal cells also had a slightly larger \( \tau_2 \) at \(-10 \) and \( 0 \) mV compared with adult myocytes \((p<0.05)\).

The steady-state inactivation \((h_s)\) curves for neonatal and adult myocytes were defined by characterizing the effect of a 5-second conditioning prepulse on the amplitude of the \( I_{Na} \) elicited by a test pulse to \(-20 \) mV. As illustrated in Figure 3, the relation between conditioning voltage and test current ampli-
tude in both cell types could be well defined by a modified Boltzmann equation of the form

$$I_{Na} = I_{Max}[1 + \exp(V_c - V_{mid})/K]$$  \(3\)

where \(I_{Max}\) is the maximum peak current obtained using holding potentials that do not inactivate sodium channels, \(V_c\) is the voltage of the conditioning prepulse, \(V_{mid}\) is the voltage for half inactivation, and \(K\) is a slope factor. Least-squares fits of Equation 3 to data indicated that \(V_{mid}\) occurred at a significantly more hyperpolarized potential in neonatal myocytes \((-97.7 \pm 2.1\) mV, \(n = 13\)) than in adult myocytes \((-88.2 \pm 1.4\) mV, \(n = 14\)) \((p < 0.001)\). \(K\) values for neonatal cells \((6.42 \pm 0.34\) mV\) and adult cells \((6.32 \pm 0.18\) mV\) were not significantly different.

The time course for recovery from inactivation was defined using a pulse protocol shown in Figure 4A. An 8-second conditioning pulse to \(-20\) mV was used to inactivate sodium channels, and the time course of recovery from inactivation was defined using a single test pulse evoked after a variable recovery time from 2 msec to 1.5 seconds at \(-140\) mV. A long (8-second) conditioning pulse was used to induce a definable amount of slow inactivation. The time course of recovery from inactivation in both neonatal and adult myocytes defined using this pulse protocol was well described by a double exponential function having a large fast component and a small slow component (Figure 4A). A relatively greater fraction of the total current recovered from inactivation with a slow time constant in neonatal cells \((0.24 \pm 0.02, n = 17)\) compared with adult cells \((0.17 \pm 0.01, n = 12)\) \((p < 0.005)\). In addition, the time constants for both slow and fast components of recovery were significantly larger in neonatal cells than in adult cells \((p < 0.001)\): the time constants for the fast and slow components at \(-140\) mV were \(\tau_f = 7.9 \pm 0.7\) msec and \(\tau_s = 284.4 \pm 33.4\) msec in neonatal cells compared with \(\tau_f = 4.5 \pm 0.4\) msec and \(\tau_s = 107.5 \pm 17.1\) msec in adult cells. A similar differ-

**Figure 2.** Comparison of kinetics of sodium current \((I_{Na})\) decay in neonatal and adult rat ventricular myocytes. Panel A: Current recording obtained from a neonatal rat myocyte during a 50-msec step to \(-20\) mV. \(V_{test}\), test voltage. Panel B: Current recording from an adult rat myocyte during a 50-msec step to \(-20\) mV. The arrows in panels A and B indicate zero current. The smooth curves indicate least-squares fits of Equation 2 (sum of two exponentials and a constant) to the time course of \(I_{Na}\) decay after its inward peak. The equations of fit were as follows: \(I_{Na} = -1.033\) nA \(\exp(-t/2.1\) msec\) \(-0.308\) exp\((-t/7.0\) msec\) \(-0.0016\) nA for neonate (panel A) and \(I_{Na} = -9.101\) nA \(\exp(-t/1.8\) msec\) \(-1.181\) exp\((-t/7.4\) msec\) \(-0.1283\) nA for adult (panel B). Panel C: Mean values for the relative amplitudes of the fast \((A_f)\) and slow \((A_s)\) components of \(I_{Na}\) decay plotted against different test potentials for neonatal \((n=10)\) and adult \((n=11)\) ventricular myocytes \((^p<0.05)\). Panel D: Mean time constants for the fast \((\tau_f)\) and slow \((\tau_s)\) components of \(I_{Na}\) decay plotted against different test potentials for neonatal and adult myocytes. The time constants in neonatal and adult myocytes were significantly different at \(-10\) and \(-20\) mV. The vertical bars indicate \pm SEM and are not visible when the amplitude of the SEM is smaller than the size of the symbol used to illustrate the mean value.
ence in recovery time constants was observed at other potentials between $-160$ and $-100$ mV as well (Figure 4B).

As predicted by the differences in inactivation properties, there was a significant difference ($p<0.05$) in the amount of use-dependent reduction of $I_{Na}$ definable during repetitive pulsing at interpulse intervals that were insufficient for full recovery from inactivation. When using depolarizing pulses of 1 second, $I_{Na}$ decreased by 14.7±4.9% and 7.4±3.5% ($n=33$) at cycle lengths of 1.1 and 1.6 second in neonatal myocytes, whereas $I_{Na}$ decreased by only 5.2±1.8% and 3.6±1.9% in adult myocytes at the same cycle lengths, respectively (see Figure 6). In contrast, only an insignificant (±2%) change of peak $I_{Na}$ amplitude was observed when the pulse duration was shortened from 1 second to 10 msec, suggesting that the use-dependent reductions in the absence of drug may be due to accumulation of channels in a slow inactivated state. These results indicate that there are significant developmental changes in the inactivation properties of rat cardiac sodium channels.

**Developmental Changes in Tonic and Use-Dependent Block by Phenytoin**

To determine whether there were developmental changes in the effects of phenytoin on rat sodium channels, we defined the effects of 40 μM (10 μg/ml) phenytoin on the cardiac $I_{Na}$ in both neonatal and adult myocytes. As illustrated in Figure 5, 40 μM phenytoin produced a greater reduction in peak $I_{Na}$ during a train of 1-second pulses in neonatal myocytes than in adult myocytes. When the frequency of pulsing was varied, the amplitude of the peak $I_{Na}$ was found to be reduced to a significantly greater extent ($p<0.05$) in neonatal myocytes than in adult myocytes at cycle lengths between 1.1 and 2.5 seconds (corresponding to interpulse durations of 100–1,500 msec) (Figure 6A).

As reported in previous studies, the inhibitory effect of phenytoin on cardiac sodium channels could be divided into two components: a tonic block observed during the first pulse after a long (1 minute) rest and a use-dependent block that developed dur-
In repetitive pulsing and that was strongly dependent on the stimulation cycle length (Figures 5 and 6). In the presence of 40 μM phenytoin, the levels of both tonic and use-dependent block were found to be significantly greater in neonatal ventricular myocytes than in adult ventricular myocytes (Figures 6A and 6B). After a 15-minute exposure to 40 μM phenytoin, the level of tonic block determined by application of a single pulse to -20 mV after a 1-minute rest at -140 mV was significantly greater in neonatal myocytes (21.7±4.5%, n=14) than in adult myocytes (10.0±2.1%, n=11) (p<0.05). As illustrated in Figure 6B, the level of use-dependent block at cycle lengths between 1.1 and 2.5 seconds was also significantly greater in neonatal myocytes than in adult myocytes (p<0.05). The difference in the amplitudes of use-dependent INa block between cell types observed in the presence of phenytoin was much larger than that observed under control conditions, suggesting that developmental differences in drug binding and/or unbinding during repetitive stimulation are present.

In a second series of experiments, we defined the level of use-dependent block produced by pulses of shorter duration (10 msec) in the presence of 40 μM phenytoin. In these experiments, application of a train of 30 pulses of 10 msec to -20 mV from a holding potential of -140 mV at a cycle length of 200 msec resulted in a 21.3±1.6% reduction in peak INa in neonatal cells (n=22) compared with a significantly smaller reduction of 4.7±0.6% in adult cells (n=17). The level of use-dependent block in both cell types was substantially less than that obtained when using 1-second depolarizing pulses applied at a similar interpulse interval; for example, 1-second pulses applied at a cycle length of 1.2 second reduced INa by 53.4±4.5% in neonatal cells and 38.2±4.0% in adult cells (Figure 6B). The observation that block is intensified by use of long pulses is consistent with the conclusion that phenytoin binds preferentially to inactivated channels.

To further examine the mechanism underlying developmental differences in phenytoin-induced block, we defined the effect of 40 μM phenytoin on the voltage dependence of INa availability. As shown in Table 2, in both cell types 40 μM phenytoin produced a hyperpolarizing shift of the midpoint of the inactivation curve, an increase in slope factor, and a reduction in the maximum level of available INa at strongly hyperpolarized potentials (INa,Max). The depression of the maximum available INa and shift of the voltage midpoint of the inactivation curve were sig-

**Figure 5.** Recordings showing inhibition of peak sodium current by 40 μM phenytoin in neonatal and adult rat ventricular myocytes. As illustrated in the inset, sodium current was recorded during a train of 30 pulses of 1-second duration to -20 mV evoked at a cycle length (CL) of 1.6 seconds. A 1-minute rest at the -140 mV holding potential preceded each pulse train. The two sets of sodium current tracings at the top show recordings obtained from a neonatal rat myocyte in drug-free external solution (panel A) and after addition of 40 μM phenytoin (panel B). The sets of sodium current tracings at the bottom show recordings obtained from an adult cell in drug-free external solution (panel C) and after addition of 40 μM phenytoin (panel D). Only the first few milliseconds of sodium current recorded during each 1-second pulse are shown. The dashed horizontal lines indicate the amplitude of peak sodium current elicited during the first pulse in control external solution. A greater block was observed in neonatal cells during exposure to phenytoin.

**Figure 6.** Plots comparing mean levels of total and tonic (panel A) and use-dependent (panel B) reduction of sodium current in neonatal (n=14) and adult (n=11) myocytes during exposure to 40 μM phenytoin. The pulse protocol used to define use-dependent block is illustrated in the inset in panel B. Peak sodium current amplitudes have been normalized to the amplitude of control sodium current obtained during a single pulse after a rest of ≥30 seconds at the holding potential. Values indicate mean±SEM; SEM bars are not visible when smaller than the size of the symbol. The mean values obtained in neonatal and adult cells were significantly different at each cycle length (CL) in both panels A and B (p<0.05).
sifically larger in neonatal cells than in adult cells (p<0.05, Table 2). The observation that phenytoin produced a larger shift in the voltage midpoint in neonatal cells (Table 2) suggests that there is a developmental difference in the extent of drug binding to inactivated sodium channels, since a drug-induced hyperpolarizing shift of this curve is believed to result from drug interaction with inactivated sodium channels.20,22 The difference in drug effect on the asymptotic current maximum (I_max) suggests that there may be developmental differences in the extent of drug blockade of open or rested channels as well.

**Time Course of Block Development**

To further test the hypothesis that there are developmental differences in phenytoin interaction with inactivated sodium channels, we characterized the time course of drug binding in neonatal and adult myocytes using a two-pulse protocol (Figure 7A). As illustrated in the inset at the top of Figure 7A, the membrane was first conditioned by a pulse of variable duration to ~20 mV. The level of block produced by this conditioning pulse was then defined using a test pulse to ~20 mV after a 300-msec interpulse interval to allow most drug-free channels to recover from inactivation. Figure 7A shows the mean time course of block development obtained in neonatal and adult myocytes.

In the absence of phenytoin, there was a small time-dependent reduction in current amplitude in both neonatal and adult cells, which reflects the development of a small amount of slow inactivation.23,24 The amplitude of slow inactivation was significantly greater in neonatal cells than in adult cells; for example, after a 9-second conditioning pulse in the absence of drug the test current was reduced by 12.7±1.6% in neonatal cells and 6.6±1.8% in adult cells (n=11) (p<0.02).

In the presence of 40 µM phenytoin, there was an initial level of block, reflecting tonic block, followed by a further time-dependent decrease in current amplitude, reflecting drug interaction with depolarized and predominantly inactivated sodium channels. In most experiments the time course of phenytoin block development could be well fit by the sum of a single exponential and a constant (i.e., by an equation of the form A_0e^(-t/τ)+A_s), and additional time-dependent components could not be resolved. However, evidence for a second small and rapid component of decay was also found in seven of 11 (64%) neonatal cells (τ=47.4±27.0 msec, range of 3.5–153 msec; A_s=3.7±1.4%, range of 1.8–21.5%) and in three of 11 (27%) adult cells (τ=13.9±10.5 msec, range of 2.3–30.6 msec; A_s=2.7±0.4%, range of 2.2–3.4%). Perhaps because of its small amplitude, this fast component could not be resolved from a fit of mean values of the block development time course (Figure 7). These results suggest that phenytoin may bind to more than one channel state during a depolarizing step, although the majority of channel block during a single long pulse may be attributed to slow drug binding to inactivated channels (i.e., A_s).

The identity of the second high-affinity state (pre-open, open, or inactivated) was not defined in these experiments. It is quite possible that drug binding to a transiently occupied state may contribute to use-dependent block observed when applying a train of short depolarizing pulses or action potentials.

As illustrated in Figure 7A, there was a large difference in the “apparent” amplitude of the slow component of channel block defined in the two cell types. However, the apparent time course of block development defined by a two-pulse protocol is not a completely accurate measure of the time course of phenytoin binding to inactivated channels, since the observed results actually reflect a combination of events including 1) time-dependent block of channels during the conditioning pulse, 2) partial recovery of channel block during the 300-msec interpulse interval, and 3) a small amount of slow inactivation of drug-free (unblocked) channels. To gain a more accurate estimate of the time course of phenytoin block development, we corrected the raw experimental data in each experiment for both the observed

**Table 2. Effect of 40 µM Phenytoin on the Sodium Channel Inactivation Curve**

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<th>Adult</th>
<th></th>
<th>Neonate</th>
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<tbody>
<tr>
<td></td>
<td>Control (n=14)</td>
<td>40 µM Phenytoin (n=14)</td>
<td>Control (n=13)</td>
<td>40 µM Phenytoin (n=13)</td>
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<tr>
<td>I_max (nA)</td>
<td>4.33±0.39</td>
<td>4.14±0.35*</td>
<td>0.96±0.20†</td>
<td>0.84±0.19**†</td>
</tr>
<tr>
<td>Drug/control</td>
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<td></td>
<td>0.82±0.06†</td>
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<tr>
<td>V_m (mV)</td>
<td>-88.2±1.4</td>
<td>-103.2±1.8*</td>
<td>-97.7±2.0†</td>
<td>-118.1±2.53†</td>
</tr>
<tr>
<td>ΔV_m (mV)</td>
<td>15.0±0.9</td>
<td></td>
<td>-20.5±2.3†</td>
<td></td>
</tr>
<tr>
<td>Slope (mV)</td>
<td>6.31±0.17</td>
<td>7.37±0.17*</td>
<td>6.42±0.32</td>
<td>7.85±0.80</td>
</tr>
</tbody>
</table>

Values are mean±SEM. I_max, estimated maximal peak sodium current achieved at strongly hyperpolarized potentials (test voltage of ~20 mV); Drug/control, ratio of I_max in drug/control; V_m, voltage at which the sodium current is half of its maximal value; ΔV_m, amplitude of shift in V_m produced by drug; Slope, slope factor. Sodium channel parameters were obtained from least-squares fits of Equation 3 to peak sodium current values obtained during a test pulse to ~20 mV after 5-second conditioning pulses to membrane potentials ranging from −160 to −65 mV using the pulse protocol illustrated in Figure 3. A 15-second rest at −140 mV separated each iteration of the pulse protocol.

*p<0.05 compared with control in the same cell type estimated using a paired Student's t test; †p<0.05 compared with adult under the same experimental conditions estimated using a grouped Student's t test.
amount of predrug slow inactivation (by dividing drug/control INa for each time point) and for the amount of interpulse recovery from channel block (by dividing the time-dependent component of INa in drug by $\exp[-300 \text{ msec}/\tau_{\text{recovery}}]$, where $\tau_{\text{recovery}}$ was defined in each experiment by the protocol illustrated in Figure 8). When such corrected values were compared, there were significant differences in the fraction of blocked channels during depolarizing pulses of 1 msec to 9 seconds (see Figure 7B). However, the difference in the fraction of channels blocked was independent of the duration of the depolarizing pulse and approximately equal to the difference in tonic block (indicated by the y intercepts) for the two cell types. When the time course of channel block was fit with a single exponential function, neither the mean amplitude nor the mean time constant for time-dependent block development was found to be significantly different for the two cell types (neonate: $A_e=0.64 \pm 0.04$, $\tau=3.66 \pm 0.35$ seconds; adult: $A_e=0.68 \pm 0.05$, $\tau=4.08 \pm 0.18$ seconds). The estimated mean dissociation constants for drug binding to inactivated channels were $9.1 \pm 2.1 \mu M$ for neonatal cells and $16.4 \pm 4.0 \mu M$ for adult cells. The dissociation constants for the two cell types were not significantly different. These results indicate that there are no marked developmental differences in the augmentation of channel block produced by a depolarizing pulse.

**Kinetics of Recovery From Use-Dependent Block**

The pulse protocol used to determine whether there were developmental differences in the time course of recovery from use-dependent block is shown in Figure 8. Channel block was first produced by an 8-second pulse to $-20 \text{ mV}$, and the time course of recovery from block was then defined using a single test pulse evoked after a variable recovery time. In the presence of $40 \mu M$ phenytoin, recovery of INa after the conditioning pulse in both cell types was biexponential and contained a small fast component and a large slow component. There was no significant developmental difference observed in either the time constant or amplitude of the fast component of recovery, attributed to recovery of drug-free channels from inactivation (neonatal: $A_f=0.31 \pm 0.04$, $\tau_f=98.4 \pm 32.3$ msec, $n=15$; adult: $A_f=0.38 \pm 0.04$, $\tau_f=44.7 \pm 10.9$ msec, $n=14$). Although the amplitude of the slow component of recovery

![Figure 7](https://circres.ahajournals.org/doi/fig/10.1161/01.RES.69.3.652)

**Figure 7.** Characterization of the time course of phenytoin block development at $-20 \text{ mV}$. The pulse protocol used to define block development is illustrated in the inset of panel A. The time interval between each iteration of the pulse protocol was 1 minute. Panel A: Graph of the mean values of available sodium current (INa) as a function of conditioning pulse duration in the presence and absence of drug for both neonatal (n=14) and adult (n=11) cells. INa values have been normalized to the peak INa amplitude measured during a pulse to $-20 \text{ mV}$ after a 1-minute rest at the holding potential in the absence of drug. Data indicate mean±SEM; SEM bars are not visible when smaller than the symbol size. Panel B: Graph of the time course of data shown in panel A after correction for slow inactivation of unblocked current and calculated amount of interpulse recovery from phenytoin block. The smooth curves indicate least-squares best fits: $I_{\text{Na}}=0.624 \exp(-t/3.461 \text{ sec})+0.169$ for neonate, and $I_{\text{Na}}=0.674 \exp(-t/3.596 \text{ sec})+0.276$ for adult. The difference values were obtained by subtracting the mean neonate values from the adult values for each time point.

![Figure 8](https://circres.ahajournals.org/doi/fig/10.1161/01.RES.69.3.652)

**Figure 8.** The mean time course of recovery from use-dependent block by $40 \mu M$ phenytoin at $-140 \text{ mV}$ in neonatal (n=14) and adult (n=11) myocytes. The pulse protocol used to define recovery is illustrated in the inset. Sodium current (INa) values have been normalized to their steady-state rested values in the presence of drug. Data indicate mean±SEM; SEM bars are visible only when the SEM amplitude is larger than the symbol size. The smooth curves indicate least-squares fits of the time course of recovery. The best-fit equations were as follows: $I_{\text{Na}}=1-0.319 \exp(-t/1.12 \text{ msec})-0.660 \exp(-t/1.50 \text{ sec})$ for neonate, and $I_{\text{Na}}=1-0.432 \exp(-t/13.5 \text{ msec})-0.561 \exp(-t/0.55 \text{ sec})$ for adult.
from block was slightly larger in neonatal cells (A_s=0.65±0.06) than in adult cells (A_s=0.54±0.04), the difference did not achieve statistical significance. In contrast, the time constant of the slow component of recovery from block was significantly larger for neonatal cells (\(\tau_s=1.933±0.133\) seconds) than for adult cells (\(\tau_s=0.784±0.089\) seconds) (\(p<0.0001\)). A large difference in the time constant of recovery from block is expected to result in a greater accumulation of use-dependent channel block in neonatal cells during repetitive stimulation and may therefore account for most of the difference in use-dependent block observed (Figure 6B).

Since the kinetics of recovery from local anesthetic-induced channel block have been previously reported to be voltage dependent,\(^{12,20-22,25}\) we also defined the time constant of recovery from block over a range of potentials between -160 and -90 mV. As illustrated in Figure 9, a similar significant difference (\(p<0.05\)) in the time constants of recovery from phenytoin block was observed over a 60-mV range in holding potentials. Interestingly, the relation between membrane potential and recovery time constant was biphasic or bell-shaped in appearance in both cell types, indicating that recovery from block is not a simple monotonic function of membrane potential, at least at voltages where sodium channel availability is less than maximal.

**Effects of Perfusion Time on Recovery Kinetics**

Since it has been proposed that the process of drug unbinding and fast inactivation may be physically coupled processes,\(^{21,22,25,26}\) one possible explanation for the observed developmental difference in the kinetics of recovery from both fast inactivation and phenytoin block is that there was a greater time-dependent hyperpolarizing shift in the voltage dependence of inactivation gating as a function of perfusion time in small neonatal cells than there was in adult cells. To assess this possibility we compared mean time constants of recovery from fast inactiva-

<table>
<thead>
<tr>
<th>Perfusion time (min)</th>
<th>Control (\tau_s) (msec)</th>
<th>Phenytoin (\tau_s) (msec)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonatal myocytes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>9±0.5</td>
<td>7.6±10</td>
<td>...</td>
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<td>19±0.5</td>
<td>8.5±0.9</td>
<td>...</td>
<td>8</td>
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<tr>
<td>(p&lt;0.0001)</td>
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<tr>
<td>46±2.6</td>
<td>...</td>
<td>2,116.3±193.2</td>
<td>8</td>
</tr>
<tr>
<td>77±7.5</td>
<td>...</td>
<td>1,693.3±151.6</td>
<td>6</td>
</tr>
<tr>
<td>(p&lt;0.0001)</td>
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<td>...</td>
<td></td>
</tr>
<tr>
<td>Adult myocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10±0.3</td>
<td>3.4±0.4</td>
<td>...</td>
<td>6</td>
</tr>
<tr>
<td>20±0.5</td>
<td>4.3±0.4</td>
<td>...</td>
<td>7</td>
</tr>
<tr>
<td>(p&lt;0.0001)</td>
<td>NS</td>
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<td>49±1.1</td>
<td>...</td>
<td>787.7±98.9</td>
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<td>78±3.1</td>
<td>...</td>
<td>839.8±111.2</td>
<td>7</td>
</tr>
<tr>
<td>(p&lt;0.0001)</td>
<td>NS</td>
<td>...</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM. Perfusion time, time since onset of intracellular perfusion with pipette solution; \(\tau_s\), fast time constant of recovery from inactivation in absence of drug at -140 mV; \(\tau_s\), slow time constant of recovery from phenytoin block at -140 mV in presence of 40 \(\mu\)M phenytoin; \(n\), number of cells; NS, not significant.

**Effects of External Sodium Ion Concentration on Phenytoin Block**

Previous reports\(^{27}\) examining drug effects on the action potential upstroke velocity in heart tissue have suggested that changes in external sodium ion concentration may alter the potency of sodium channel blockade. To determine whether the effect of phenytoin on the cardiac \(I_{Na}\) is also dependent on external sodium ion concentration, we performed a final series of experiments in neonatal myocytes perfused with an external solution containing 145 mM (versus 25 mM) external sodium. A quantitative comparison of the sodium channel-blocking characteristics of 40 \(\mu\)M phenytoin in the presence of 25 mM and 145 mM external sodium are shown in Table 4. The only significant effect produced by elevating the external...
sodium ion concentration was a reduction of tonic block by 64% ($p<0.05$).

**Discussion**

**Developmental Differences in Channel Inactivation Properties**

In comparing the gating properties of neonatal and adult rat sodium channels, we found that there were significant differences in both the kinetics and voltage dependence of sodium channel inactivation. Compared with adult cells, neonatal cells displayed a more hyperpolarized midpoint of their sodium channel availability curve (Figure 3), a slower rate of recovery from inactivation, and a greater amount of slow inactivation development during long depolarizing pulses (Figures 4 and 7).

It is unclear why neonatal sodium channels have different inactivation properties than those in adult cells. Possible explanations for the developmental differences observed in this study include the following: expression of different sodium channel subtypes, differences in interaction with other accessory proteins (e.g., β-subunits or G proteins), or developmental changes in posttranslational processes such as phosphorylation or glycosylation. Consistent with this postulate, a recent study has shown that injection of unfractionated RNA into *Xenopus* oocytes can both increase the kinetics of $I_{Na}$ decay as well as produce a depolarizing shift of the sodium channel availability curve, indicating that expression of proteins other than the α-subunit can alter sodium channel inactivation gating. This suggests that developmental changes in the expression of mRNA species other than those encoding the α-subunit could conceivably account for developmental changes in sodium channel inactivation gating.

In contrast to channel inactivation, the time and voltage dependence of $I_{Na}$ activation were very similar in the two cell types, with the only notable difference being a small but significant difference in the steepness of the $G_{Na}$-V_m relation (Table 1).

**Comparison of Our Results With Other Studies**

In our experiments we found that phenytoin was more potent in producing both tonic and use-dependent block in neonatal cells and that the kinetics of recovery from use-dependent block in neonatal cells were slower in neonatal rat myocytes than in adult myocytes. These results are qualitatively similar to results of Hering et al., who found that mexiletine was a more potent blocker of $I_{Na}$ in neonatal rat myocytes than in adult cells. In a preliminary series of experiments we have found that lidocaine is also a more potent sodium channel blocker in neonatal cells than in adult cells. Taken together, these results suggest the presence of a developmental pattern for rat sodium channels, with class I antiarrhythmic drugs being more potent blockers of neonatal sodium channels than adult sodium channels. Our observation of a slower rate of recovery from phenytoin block at hyperpolarized potentials is also qualitatively consistent with the observation of slower recovery from lidocaine-induced depression of $V_{max}$ in neonatal canine ventricular muscle.

What is less clear is whether a similar pattern of developmental changes in drug sensitivity occurs in different species, cardiac cell types, and experimental conditions. For example, opposite developmental changes in the time constant for recovery from lidocaine block of $V_{max}$ have been reported to occur in canine ventricular myocardium and canine Purkinje fibers, suggesting the possibility that there could be significant tissue-specific differences. Such tissue differences could possibly explain why we found a significant developmental difference in phenytoin effect on $I_{Na}$ in rat myocytes, whereas a previous study defining the effects of phenytoin on $V_{max}$ in canine Purkinje fibers found no significant developmental change in phenytoin action. Further experiments using direct measurements of drug effect on $I_{Na}$ in isolated Purkinje fibers are needed to confirm this hypothesis.

**Differences in Channel Inactivation May Underlie Differences in Use-Dependent Block**

In our study we found that neonatal cells had both a significantly slower rate of recovery from fast
inactivation and channel block by phenytoin. The similar directional change in both parameters suggests that the process of recovery from channel block and recovery from channel inactivation may be physically coupled mechanisms, as previously postulated by the modulated receptor model.\textsuperscript{22,25,26} According to this model, the rate of recovery from use-dependent channel block at hyperpolarized potentials occurs chiefly over the pathway ID→RD→R:

\[
\begin{array}{c}
\text{ID} \\
\text{RD} \\
\text{D}
\end{array}
\begin{array}{c}
\text{R} \\
\text{D} \\
\text{D}
\end{array}
\begin{array}{c}
l_r \\
k_r \\
\alpha^*_r \\
\beta^*_r
\end{array}
\begin{array}{c}
\text{ID} \\
\end{array}
\]

where D indicates drug, ID is the inactivated-blocked state, RD is the rested-blocked state, R is the rested drug-free state, $\alpha^*_r$ and $\beta^*_r$ represent the rate constants governing closed-channel inactivation for drug-associated channels, and $k_r$ and $l_r$ represent binding and unbinding rate constants, respectively, governing drug interaction with rested channels. Mathematical models\textsuperscript{22,25} based on the modulated receptor hypothesis predict that changes in the kinetics of recovery from channel inactivation should also result in directionally similar changes in the rate of recovery from channel block as long as the ID→RD transition is the rate-limiting step for channel unblocking (i.e., the ID→RD step is much slower than the RD→R step), as has been proposed to be true for many drugs.\textsuperscript{22,25,26} Our observation of both a slower rate of recovery from channel inactivation and slower rate of recovery from use-dependent block by phenytoin in neonatal versus adult cells therefore seems consistent with the predictions of the modulated receptor model. The modulated receptor model may also account for the bell-shaped voltage dependence for phenytoin recovery time constants (Figure 9), if it is assumed that the voltage dependence of the time constant for recovery from fast inactivation is also bell-shaped with a more positive peak. Although we did not define the voltage dependence of recovery from fast inactivation under drug-free conditions at potentials sufficiently positive to resolve the presence of a peak (\(\tau_R\) in Figure 4B), previous studies have provided evidence for a bell-shaped voltage dependence of \(\tau_R\) (or \(\tau_B\)) with an apparent peak near \(-90\) mV in both cat atrial myocytes (at 13–17°C)\textsuperscript{30} and rat papillary muscle (at 25°C).\textsuperscript{31} The observation of a more hyperpolarized peak for the phenytoin unblocking time constant (e.g., near \(-120\) mV in Figure 9) is consistent with the conclusion of a previous study that phenytoin produces a hyperpolarizing voltage shift in inactivation kinetics for drug-associated channels of \(-30\) mV.\textsuperscript{32} Therefore, it seems reasonable to speculate that the primary molecular mechanisms responsible for the observed developmental differences in use-dependent block by phenytoin is a difference in channel inactivation gating rather than a developmental change in the structure of the local anesthetic–receptor site. It also seems plausible that the same modulated receptor model could also account for the much more shallow voltage dependence of recovery kinetics that we have observed for other drugs (e.g., cocaine and RAC109).\textsuperscript{15,33} if it is assumed that the rate of drug escape from rested–closed channels (RD→R transition) is relatively voltage independent and slow for these drugs, so that it becomes a rate-limiting step for channel unblocking at hyperpolarized potentials. More studies are needed to further test this hypothesis.

In addition to differences in the rate of recovery from phenytoin block, we also found a marked difference in the amplitude of tonic block as well (Figure 6, Tables 2 and 4). Differences in tonic block observed at strongly hyperpolarized potentials (e.g., $I_{Na}$ in Table 2) are unlikely to be due to differences in channel inactivation properties, suggesting that there may be more than one change in sodium channel properties that occurs during development. Since the molecular mechanisms underlying tonic block have not been well defined, it is not clear whether the observed difference in tonic block reflects a difference in nonspecific effects of the drug on the channel, differences in drug affinity for the rested conformation of a state-dependent receptor, or a developmental change in drug binding to a second receptor site responsible for producing tonic block. Our observation that there was a similar difference in the levels of both tonic block and steady-state channel block achieved during long pulses to \(-20\) mV (Figure 7B) may be accounted for in terms of either a modulated receptor hypothesis, by postulating developmental changes in drug affinity for both rested and inactivated channels ($K_{ma}$ and $K_{ad}$), or a plural receptor mechanism,\textsuperscript{34} in which a developmental change in a state-independent binding site responsible for producing tonic block is assumed. Further studies are needed to distinguish between these two possibilities.

In summary, the results of this study indicate that there are developmental changes in both sodium channel inactivation gating and sensitivity to channel blockade by phenytoin that occur in the postnatal rat heart. These findings suggest that the electrophysiological and pharmacological properties of sodium channels defined in cells at one stage of development should not be assumed to be identical to the properties of sodium channels in cells at another stage of development.

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References


KEY WORDS: sodium channel • cardiac myocytes • neonates • phenytoin • developmental pharmacology
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