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State-dependent Block of Human Cardiac hNav1.5 Sodium Channels by Propafenone

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Abstract. State-dependent blockade of human cardiac hNav1.5 sodium channels by propafenone was studied using whole-cell patch clamp techniques. Both a direct investigation using cells with inactivation-deficient sodium channels and an algorithmic approach used on cells with wild-type channels revealed a rapid binding of propafenone to the open state. This occurs approximately 4000 and 700 times faster than the binding to the resting and inactivated state, respectively. An established mathematical "gating" model was modified to represent the experimental data.

Key words: Patch clamp — Persistent currents — Quantitative modeling

Introduction

Propafenone is indicated for the treatment of supraventricular tachycardias, paroxysmal atrial fibrillation, and ventricular tachyarrhythmias. It is a high-potency blocker of the cardiac voltage-gated Na^+ channel (class Ic), as well as a less potent blocker of potassium channels and beta-adrenergic receptors.

Cardiac dysrhythmias may be caused by pathologic sodium channel function. In current models, sodium channels exist in one or several "closed" states until a sufficiently large membrane depolarization causes the channel to transition to an "open" state briefly, allowing for flow of sodium ions. Regardless of the membrane potential, the channel then rapidly transitions to one or several "inactive" states since open-channel inactivation is voltage-independent (Aldrich, Corey & Stevens, 1983). Inactivation may also occur without opening (Kuo & Bean, 1994).

A channel that fails to inactivate remains in the open state and elicits a persistent sodium current that may predispose to dysrhythmias such as long-QT syndrome (Bennett et al., 1995). In this setting, an ideal anti-arrhythmic drug would block the persistent sodium current while allowing for the normal early peak current to be reached. Ideally, the drug should not influence the channel in its resting and inactive state.

Direct investigation of the effects of various sodium channel blocking medications on the open state have been challenging because the open state persists for only approximately one millisecond. A previous study using the whole-cell patch-clamp technique investigated the characteristics of the open channel indirectly by observing the effect of repetitive stimulation (Crumb & Clarkson, 1990). A mathematical algorithm was devised to determine the pharmacokinetic rate constants describing the interaction of the open channel with a drug.

Recent developments have made direct investigation of the open channel possible because genetically altered inactivation-deficient cardiac sodium channels can now be expressed in cultured human embryonic kidney cells. In this study we applied whole-cell patch-clamp techniques to cells with inactivation-deficient human cardiac sodium channels. This allowed direct investigation of the open-channel block by propafenone. Then, the indirect method described by Crumb and Clarkson was applied to cells with wild-type human cardiac channels to find the same pharmacokinetic rate constants for comparison. We also inserted the resulting pharmacokinetic rate constants into a previously described multistate mathematical model (Kuo & Bean, 1994) to demonstrate that the kinetics of the cardiac Na^+ channel can be simulated satisfactorily.

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Materials and Methods

Site-directed Mutagenesis

The QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used to create a human cardiac mutant clone (hNav1.5–L409C/A410W) as described previously (Wang, Russell & Wang, 2004). DNA sequencing near the mutated site confirmed these mutations.

Cell Culture and Transient Transfection

Human embryonic kidney cells (HEK293t) were grown to approximately 50% confluence in DMEM (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin and streptomycin solution (Sigma, St Louis, MO), 3 mM taurine, and 25 mM HEPES (Life Technologies). They were transfected by a calcium phosphate precipitation method (Cannon & Strittmatter, 1993). Transfection of hNav 1.5-pcDNAl or hNav1.5-L409C/A410W mutant clone (5 μg) along with βi (10–20 μg) and reporter CD8-pih3m (1 μg) was adequate for current recording. Cells were replated 15 h after transfection in 35-mm dishes, maintained at 37°C in a 5% CO_2 incubator, and used after 1–4 d. Transfection-positive cells were identified with immunobeads (CD8-Dynabeads).

Whole-Cell Voltage Clamp

Whole-cell clamp configuration was used to record sodium currents (Hamill et al., 1981). Borosilicate micropipettes (Drummond Scientific Company) were pulled with a puller (P-87; Sutter Instruments) and heat-polished. Pipette electrodes contained 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 HEPES adjusted to pH 7.2 with CsOH. The pipette electrodes had a tip resistance of 0.5–1.0 MΩ. Access resistance was 1–2 MΩ and was further reduced by series resistance compensation. All experiments were performed at room temperature (22–24°C) under a sodium-containing bath solution with, in mM, 65 NaCl, 85 choline chloride, 2 $CaCl_2$, and 10 HEPES, adjusted to pH 7.4 with tetramethyl-ammonium hydroxide. Propafenone hydrochloride (SIGMA) was dissolved in water to make a stock solution of 10 mM. Final propafenone concentrations were made by serial dilution in bath solution. Whole-cell currents were measured by an EPC-7 (List Electronics), filtered at 3 kHz, collected and analyzed with pClamp8 software (Axon Instruments). The holding potential was set to –140 mV. Leak and capacitance were subtracted by the patch-clamp device and further by a leak-subtraction protocol (P/-4) implemented by the software.

Direct Investigation of the Open-Channel Block in Inactivation-Deficient Cells

In a simplified model, the inactivation-deficient sodium channels are thought to exist only in a resting (closed) state and an open state when cells are depolarized to +50 mV, as shown in Fig. 1.

To investigate the rate constants describing the binding of a drug to the open state (k_0 and l_0) a simple pulse protocol is applied (+50 mV for 80 ms after resting at a baseline of –140 mV). A 30 second interval between pulses was chosen to allow complete recovery from the open back to the resting state. Preliminary experiments had shown that less than 15 seconds were required for the cell to display identical current tracings without a reduction in initial peak current.

Initially, the cell is perfused with the bath solution described above. Then, the perfusion pipes are shifted to perfuse the cell with increasing concentrations of propafenone (the propafenone is diluted with the same bath solution).

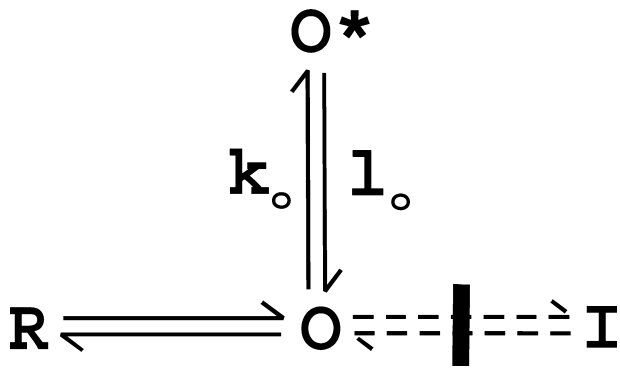

Fig. 1. Simplified pharmacokinetic model of a sodium channel including closed resting and inactivated states (*R*) and (*I*), as well as the open state (*O*). The pharmacokinetic rate constants, k_o and l_o, determine the rate at which the open channel transitions to the drug-bound state (*O**). In the inactivation-deficient channel, the path from open to inactivated state is blocked.

In truly inactivation-deficient channels, the current elicited by a flat voltage pulse should persist for the duration of the pulse when there is no exposure to propafenone. However, a minor decrease in current at +50 mV can be attributed to "slow inactivation" that is known to occur even in inactivation-deficient channels (Wang et al., 2003).

The rate constants describing the binding of propafenone to the open state (k_o and l_o) are deduced by fitting a single-exponential curve to the decaying current elicited during each voltage pulse. This is done after normalizing the traces to the control trace to account for the influence of "slow inactivation." Assuming that the perfusion provides a nearly constant concentration of drug, pseudo-first-order conditions can be assumed relating the time constant of decay of current, tau (τ), to each drug concentration ([*D*]):

$$1/\tau = k_o\,[D] + l_o \tag{1}$$

Thus, performing measurements of τ for two or more drug concentrations makes the calculation of k_o and l_o possible.

Indirect Investigation of the Blockage of Wild-type Channels

In wild-type cells inactivation occurs rapidly after the channel opens (*see* Fig. 2). Thus, the duration of the open state is too short (~ 1 ms) to apply the technique described above.

However, using an "indirect" method, k_o and l_o can be approximated after determining k_r, l_r, k_i and l_i (Crumb & Clarkson, 1990).

First, a double-pulse protocol is applied. During the first pulse, drug binds to the inactivated state as described by

$$[D] + U \overset{k_i,l_i}{\leftrightarrow} B$$

where [*D*] is the drug concentration, *U* and *B* are the fraction of unblocked and blocked channels, k_i is the rate of binding, and l_i is the rate of unbinding. The second pulse elicits a peak current proportional to the remaining unblocked channels (*U*). After applying initial conditions ($B = 0$) the rate constants can be found:

$$k_i = I_\infty/\tau_i[D] \tag{2}$$

$$l_i = 1/\tau_i - k_i[D] \tag{3}$$

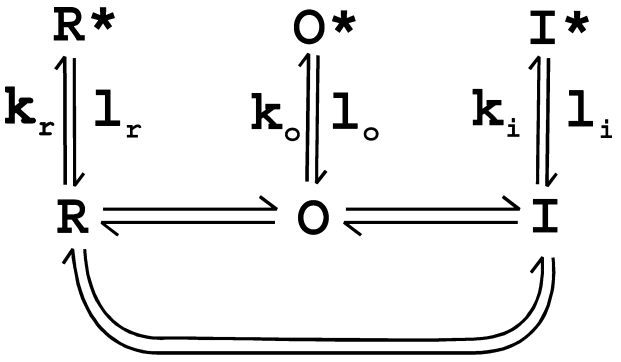

Fig. 2. Model of a sodium channel with free resting (*R*), open (*O*), and inactivated (*I*) states as well as their drug-bound counterparts (*). Constants *k* and *l* determine the rate of drug binding.

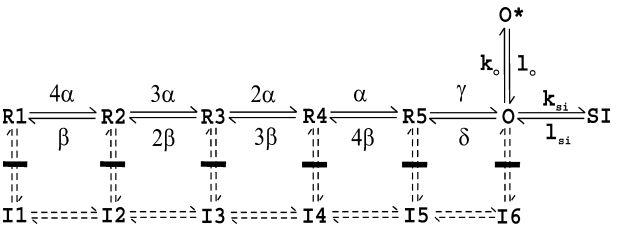

Fig. 3. A kinetic model that simulates an initial and four intermediate resting states (*R1* and *R2* through *R5*) as well as the open state (*O*) and the drug-bound open state (*O**). The inactivated states (*I1–I6*) are not accessible in this inactivation-deficient channel. However, slow inactivation is possible to the state SI. α, β, γ, and δ are: $\alpha = a_1\, e^{V/a}{}_2$, $\beta = b_1\, e^{V/d}{}_2$, $\gamma = g_1\, e^{V/g}{}_2$, $\delta = d_1\, e^{V/d}{}_2$ where *V* is in volts. The rate constants, k_o and l_o, govern binding of the drug to the open channel; k_{si} and l_{si}, describe slow inactivation.

where I_∞ is the equilibrium fraction of blocked channels and τ_i, ($= 1/\lambda_i$) is the time constant for drug binding to inactivated channels.

Next, the equilibrium level of block (R_∞) in the resting state is measured by comparing the peak currents elicited by voltage pulses (peak +50 mV, baseline −140 mV) with and without propafenone.

Finally, a train of voltage pulses is applied while applying drug. The resulting blocking rate constant λ ($= 1/\tau$) describes the envelope of decreasing peak currents and is given by

$$\lambda = \lambda_r t_r + \lambda_o t_o + \lambda_i t_i \tag{4}$$

where $\lambda_{r,o,i}$ are the rate constants of drug-binding for the resting, open, and inactive states and $t_{r,o,i}$ are the time spent in the resting, open and inactive state during the pulse train. Applying pulse trains of several frequencies enables the linear regression of λ vs. t_r. Assuming that the channel opening time t_o is uniformly 1 ms and that the rest of the pulse time is spent in the inactive state (t_i), the rate constants λ_o and λ_r can be found.

The equilibrium level of channel block at the end of a long pulse train (b_{ss}) is defined as:

$$b_{ss} = O_\infty X_1 + constant \tag{5}$$

where

$$X_1 = (1 - e^{-\lambda_o t_o})\, e^{-(\lambda_i t_i + \lambda_r t_r)} / (1 - e^{-\lambda}) \tag{6}$$

Thus, O_∞ can be determined as the slope of the plot of b_{ss} vs. X_1. The remaining rate constants can be found by using the following equations:

$$k_r = \lambda_r R_\infty / [D] \tag{7}$$

$$k_o = \lambda_o O_\infty / [D] \tag{8}$$

$$l_r = \lambda_r (1 - R_\infty) \tag{9}$$

$$l_o = \lambda_o (1 - O_\infty) \tag{10}$$

In general, the speed at which the channel is blocked by propafenone in the resting, open or inactive state can be described by the time constants tau (τ_x) as

$$\tau_x = 1/(k_x[D] + l_x) = 1/\lambda_x \tag{11}$$

where k_x and l_x are coefficients for the resting, open or inactive states.

Implementation of the Pharmacologic Model

A gating model for Na^+ channels that has previously been described (Kuo & Bean, 1994) was adapted by adding an open but drug-bound state that can be reached only from the open channel state. This is a statistical model in which channels must transition through five sequential closed states (*R1* through *R5*) before reaching the open state (*O*) which allows sodium current to flow. The inactive states of the original model (Kuo & Bean, 1994) were made unavailable to simulate the inactivation-deficient channel. However, one slow inactivated state was added to provide for the phenomenon of "slow inactivation" discussed above (*see* Fig. 3).

First-order kinetics describe the transitions between states using the voltage-dependent coefficients α, β, γ, and δ (in s^{-1}) which are: $\alpha = a_1 e^{V/a}{}_2$, $\beta = b_1\, e^{V/d}{}_2$, $\gamma = g_1\, e^{V/g}{}_2$, $\delta = d_1\, e^{V/d}{}_2$ where *V* is in volts. The open channel may be blocked reversibly by the drug as governed by k_o ($M^{-1}\ s^{-1}$) and l_o (s^{-1}), and slow inactivation occurs independently of propafenone as described by k_{si} (s^{-1}) and l_{si} (s^{-1}). The current flowing through the channel is calculated based on the probability that the channel is open, and based on the resistance that the open channel presents to current. This resistance can be determined from stimulating the cell with increasing voltages and measuring the resulting peak current. Mathematical simulation of the model of Fig. 3 was performed using MATLAB Simulink®.

Results

Time-dependent Block of Inactivation-deficient Channels

A square wave stimulus (+50 mV for 80 ms after resting at a baseline of −140 mV) was applied via voltage clamp to cells with inactivation-deficient sodium channels while applying increasing propafenone concentrations. Figure 4 shows the resulting family of superimposed Na^+ currents obtained from one representative cell. An attenuation of the persistent current begins around 0.1 μM and reaches approximately 90% when applying 3 μM propafenone. The peak current, on the other hand, is not influenced until the propafenone concentration exceeds 1 μM. At 3 μM, the peak is attenuated approximately 10%.

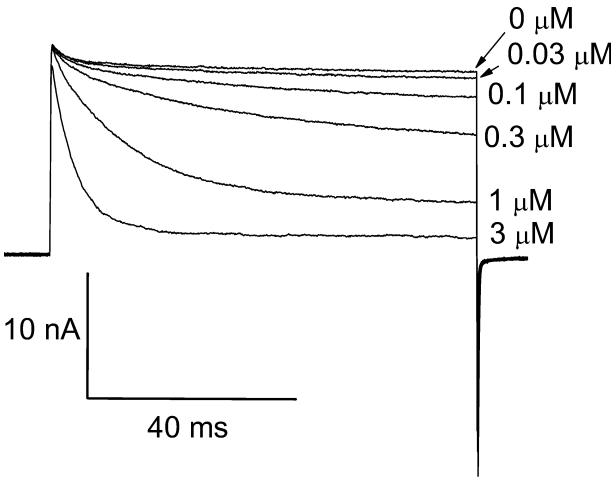

Fig. 4. Current elicited from one representative cell with inactivation-deficient channels while exposing it to increasing concentrations of propafenone. Cells were held at −140 mV. The test pulse was at +50 mV for 80 ms and was applied every 30 seconds.

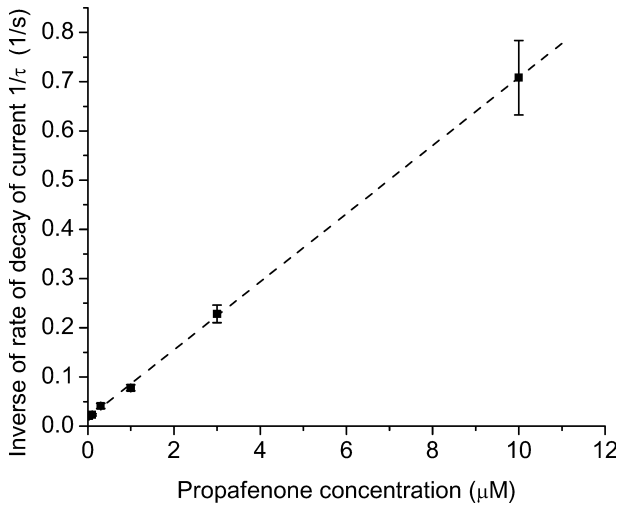

Fig. 5. Data from 9 cells with inactivation-deficient channels exposed to varying propafenone concentrations. The mean and standard error SE of the inverted decay time constants tau (τ) are shown. The slope and y-axis-intercept of the best-fit line allows calculation of the rate constants, k_o and l_o, according to equation $l/\tau = k_o\,[D] + l_o$ where $[D]$ is the concentration of propafenone.

Approximately 50% of the cells investigated displayed an unusual but stable and reproducible increase in the peak current when switching from the control perfusion (no propafenone) to 0.03 μM propafenone. However, successive increases in propafenone concentration from 0.03 to 3 μM led to the expected response in all cells, as shown in Fig. 4.

The small decrease in current that occurred in absence of propafenone (control trace) could be attributed to "slow-inactivation." In the averaged control traces of 8 cells the current decreased by 8.7 ± 1.4 % (mean ± SE).

The kinetic parameters describing the binding of propafenone to the open state were calculated by the method outlined in the Methods above after normalizing to account for the slow inactivation. Figure 5 shows the results using measurements from 8 cells yielding

$$k_o = 73.0 \pm 6.4\ s^{-1}\ \mu M^{-1}\ (\text{mean} \pm \text{SE}),$$
$$l_o = 24.5 \pm 7.0\ s^{-1}\ (\text{mean} \pm \text{SE}).$$

The time constant τ_O, with which propafenone binds to the open channel, is included in the left-hand column of Table 1 below and the equilibrium dissociation constant ($K_D = l_o/k_o$) is included in Table 2 below.

For an estimate of the rate constants governing slow inactivation, the control current traces were fitted to an exponential decay function with an offset. This yielded the time constant of slow inactivation ($\tau_{O \to SI}$),

$$\tau_{O \to SI} = 24.4 \pm 1.1\ \text{ms}\ (\text{mean} \pm \text{SE})$$

and the corresponding rate constants,

$$k_{si} = 4.1 \pm 1.32\ s^{-1}\ (\text{mean} \pm \text{SE}),$$
$$l_{si} = 36.9 \pm 1.32\ s^{-1}\ (\text{mean} \pm \text{SE}).$$

Comparison of Blocked Inactivation-Deficient Channels with Unblocked Wild-Type Channels

Cells with wild-type sodium channels were exposed to control solution and were subjected to the same square-wave stimulation protocol as described for the previous experiment. The resulting current traces for 13 cells were averaged and displayed in Fig. 6. For comparison with inactivation-deficient channels exposed to propafenone, the same square-wave stimulus was applied to 8 cells with inactivation-deficient channels while increasing the propafenone concentration. Two traces corresponding to 3 and 10 μM propafenone are superimposed on this plot.

The time constant of inactivation of wild-type sodium channels ($\tau_{O \to I}$) was calculated for comparison to the inactivation of the inactivation-deficient channels above:

$$\tau_{O \to I} = 0.62 \pm 0.03\ \text{ms}\ (\text{mean} \pm \text{SE}).$$

Indirect Investigation of the Blockage of Wild-Type Channels

As described in the Methods above, a double-pulse protocol was applied to determine the dynamics of drug-binding to the inactivated state of the channel. Figure 7 shows the results of the double-pulse experiment. A double-exponential fit to the curve was

Table 1. Comparison of time constants (τ) representing the speed at which 3 μM propafenone binds to channels in the resting (*r*), open (*o*) and inactive (*i*) states

	Direct investigation using inactivation-deficient channels	Indirect method of Crumb and Clarkson using wild-type channels	Ratio to τ_o (method of Crumb and Clarkson only)
τ_r	—	23.6967 (s)	4123
τ_o	0.0043706 (s)	0.0057471(s)	1
τ_i	—	3.8865 (s)	676

The direct investigation using inactivation-deficient channels is shown in the left column (only τ_o was determined). The results of the indirect method of Crumb and Clarkson are in the middle column.

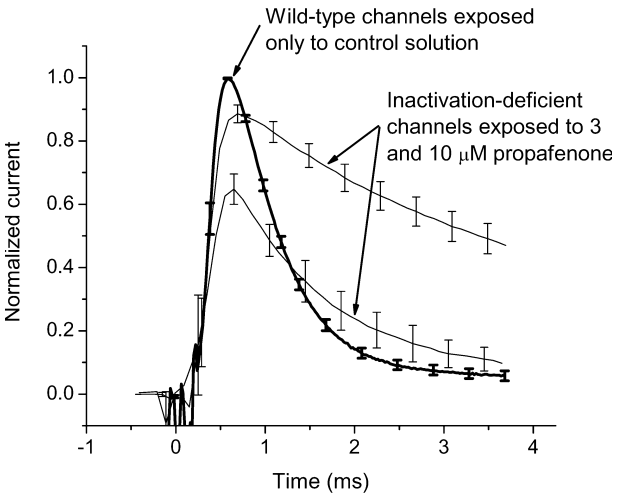

Fig. 6. Thirteen cells with wild-type hNav1.5 channels exposed only to control solution were compared to 8 cells with inactivation-deficient hNav1.5 channels exposed to 3 and 10 μM propafenone. The propafenone attenuates the persistent current of the inactivation-deficient channels at these clinically-relevant concentrations. However, an increasing attenuation of the peak current also occurs. All currents were normalized to the current peak values when exposed to control solution. Mean and standard error are displayed.

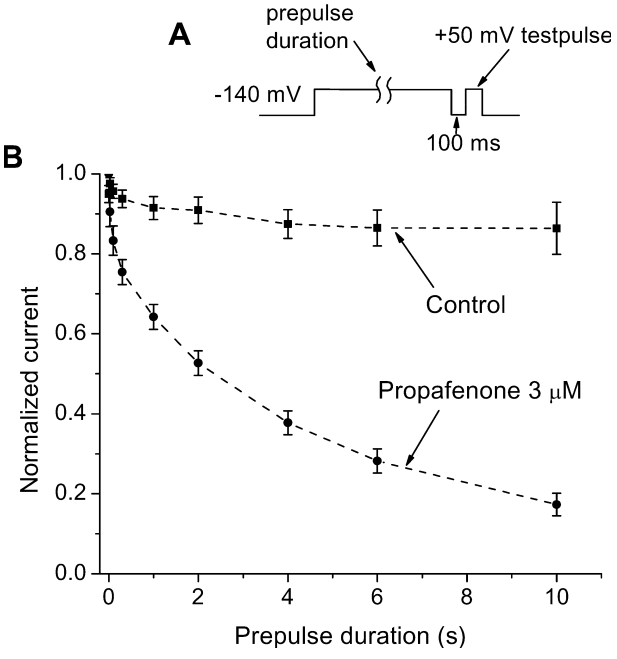

Fig. 7. Stimulation of 6 cells (mean with SE) with a double-pulse protocol (*A*) to demonstrate the binding of propafenone to the inactivated state. During the first pulse, channels are held in the inactivated state and bind to propafenone. The brief inter-pulse interval (100 ms) allows drug-free inactivated Na^+ channels to recover while most drug-bound inactivated channels remain blocked. A reduction in peak current (shown in *B*) occurs as the prepulse duration is increased, illustrating the relatively slow binding of propafenone to the inactivated state.

applied yielding a fast and a slow component. In the presence of propafenone (3 μM), inactivated channels bind slowly with a time constant of ~ 4 seconds. The corresponding rate constants were calculated from this time constant:

$$k_i = 0.0758 \pm 0.0063\ s^{-1}\ \mu M^{-1} (\text{mean} \pm \text{SE}),$$

$$l_i = 0.0299 \pm 0.006\ s^{-1}\ (\text{mean} \pm \text{SE}).$$

Next, the equilibrium level of blockade (R_∞) of the resting state was assessed. Again, an unexpected increase in peak current was observed in approximately 50% of all cells when switching from no propafenone to 0.03 μM propafenone. However, as the propafenone concentration was increased from 0.03 to 10 μM the expected uniform decrease in peak current occurred. Because there was no significant drop in peak current while increasing the drug from 0.03 μM until crossing a threshold of approximately 0.3 μM, the peak at 0.03 μM propafenone was taken to be the control to calculate the resting block. By switching between 0.03 and 3 μM repetitively in 3 cells (11 measurements), the resting block (R_∞) was found to be 9.9 ± 4.9 %.

The remaining algorithm relies on the response of the sodium channel to repetitive stimulation: a train of voltage pulses (+ 50 mV for 20 ms) was applied at frequencies ranging from $1/8^{th}$ to 5 pulses per second (Hz). Even at the highest frequency (5 Hz) there was no reduction in peak current in the absence of propafenone. However, applying propafenone (3 μM)

Table 2. Comparison of equilibrium dissociation constants obtained from the direct method involving the inactivation-deficient cells and the indirect method involving the method of Crumb and Clarkson

	Equilibrium dissociation constants of propafenone $K_D = l/k$ μM
Resting state (Crumb and Clarkson)	27.1
Open state (Crumb and Clarkson)	0.405
Open state (direct method)	0.336
Inactivated state (Crumb and Clarkson)	0.395

to the cell resulted in pronounced reductions in peak current as shown for a representative cell in Fig. 8.

The decay of the peak current in Fig. 8 results from the interaction between the channel and propafenone as the channel repetitively cycles through the resting, open, and inactive states. The open state was assumed to exist for 1 ms, the inactivated state for the 19 ms (pulse width 1 ms), and the resting state varies according to pulse frequency. Thus, the algorithm described in the Methods above was used to solve for the rate constants of the open and inactive states in 9 cells:

$$k_o = 51.1 \pm 8.4\ s^1\ \mu M^{-1} (\text{mean} \pm \text{SE}),$$
$$l_o = 20.7 \pm 8.5\ s^1\ (\text{mean} \pm \text{SE}),$$
$$k_r = 0.0014 \pm 0.0003\ s^1\ \mu M^{-1} (\text{mean} \pm \text{SE}),$$
$$l_r = 0.038 \pm 0.008\ s^1\ (\text{mean} \pm \text{SE}).$$

Thus, the time constants tau (τ) that describe the speed at which each state is bound by propafenone can be calculated using Eq. 11. They are shown in the middle column of Table 1. Table 2 contains the equilibrium dissociation constants (K_D) for the resting, open and inactivated states.

Simulation of the Inactivation-Deficient Channel Using a Mathematical Model

The mathematical model presented above (*see* Methods and Fig. 3) was adapted by inserting the coefficients k_o, l_o, k_{si}, and l_{si}, that had been obtained from the experiment with the inactivation-deficient channels above. In Fig. 9, the performance of the model was compared to the experimental data from which the coefficients were derived. The model was driven by the same square wave used to generate Fig. 4, while increasing the concentration of propafenone. Superimposed are the averaged and normalized currents from 8 cells.

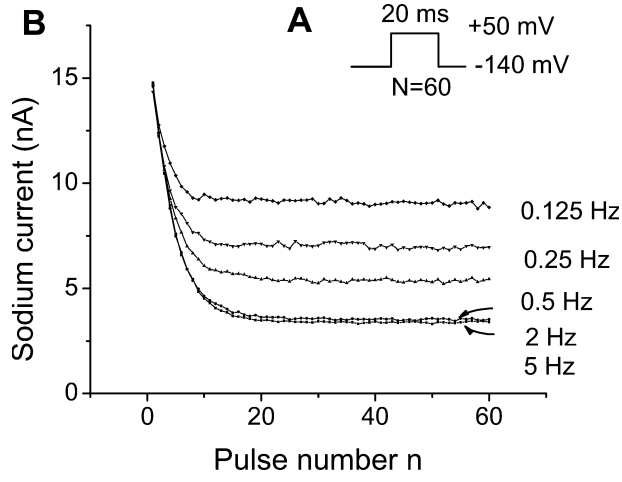

Fig. 8. Repetitive stimulation of one representative cell with a pulse train (*A*) to demonstrate use-dependent blockade of sodium channels by 3 μM propafenone (*B*). The rate of current decay allows calculation of the binding coefficients for the open and resting state.

Several parameters are necessary in the model, but were not directly derived by the experiments above (α, β, γ, and δ). By influencing the speed of transition from one state to another, these parameters determine the rising phase of the current and also influence the peak current. Repetitive approximation was performed to minimize the mean-squared error between the experimental and the simulated current traces. A mean-squared error (MSE) of 0.00048 (normalized current squared) was achieved with the following parameters (in ms^{-1}):

$$\alpha = 1.6\ e^{V/40},$$
$$\beta = 0.3\ e^{-V/40},$$
$$\gamma = 50\ e^{V/100},$$
$$\alpha = 0.6\ e^{-V/25},$$

where V is in millivolts.

The model also displays a reduction in the peak current when the propafenone concentration exceeds 0.3 μM. This occurs despite the fact that this model was modified to neglect any possibility of a resting blockade. At 3 μM the attenuation of the peak current of the model and of the actual experimental data ($n = 8$) is 12% and 11 ± 2.7% (mean ± SE), respectively.

Discussion

The main new experimental result presented here is that the blockade of human cardiac hNav1.5 sodium channels by propafenone occurs most rapidly during the open state. This was demonstrated directly as a time-dependent block in cells with inactivation-

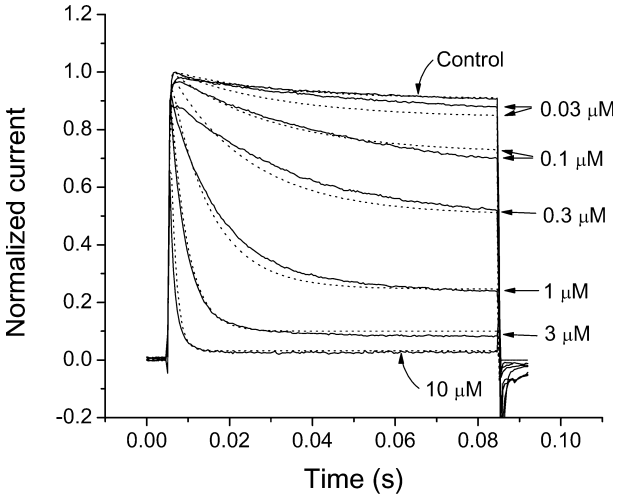

Fig. 9. Comparing the model (*dotted lines*) of figure 3 to the experimental data (*solid lines*) from inactivation-deficient channels. Data from 8 cells were averaged as increasing concentrations of propafenone were applied.

deficient channels. Using an indirect method to measure resting-, open-, and inactive-channel block (Crumb & Clarkson, 1990) in cells with wild-type channels, similar results were found. Table 1 compares the time constants derived from both methods: the rate of binding to the open state is approximately 4000 times faster than that to the resting state and approximately 700 times faster than to the inactive state. This finding stands in contrast to previously published experiments in which predominantly inactive-state binding was reported in ventricle cells of guinea pigs (Schreibmayer & Lindner, 1992). Experiments with cardiocytes of rats also supported their conclusion (Kohlhardt & Fichtner, 1988). Table 2 compares the equilibrium dissociation constants (K_D) and shows that K_D is quite similar in the open and inactivated states.

In the context of cardiac electrophysiology, where an action potential may last for 200–400 ms, the channel may only spend 0.5–0.25% of the depolarization time in the open state (presumed open time = 1 ms). The rest of the time is spent in the inactivated state. Thus, the open state is blocked more quickly by propafenone (approximately 700 times faster than to the inactivated state), but much more time is spent in the inactivated state (200–400 fold more time). Therefore, the clinical profile of propafenone may be influenced heavily by both the open-channel and inactivated-channel blocking ability.

When exposed to propafenone, the electrical behavior of cells with inactivation-deficient channels begins to resemble the behavior of cells with wild-type channels. Figure 4 shows that the pathologically persistent current resulting from a long square-wave stimulation begins to taper at propafenone concentrations around 1 μM. This is in the range of reported therapeutic plasma concentrations (0.17–2.8 μM) (Connolly et al., 1983). Ideally, a drug used to treat pathologically persistent sodium current should attenuate mainly the persistent current, while leaving the magnitude of the initial peak current intact. However, at the concentration needed to attenuate the persistent current considerably, propafenone does cause a decline in the peak current as well (12% at 3 μM).

Cells with inactivation-deficient cardiac Na^+ channels have just recently become available (Grant et al., 2000). Before this, an alternative method had been used for the indirect investigation of open channel blockade by cocaine in normal guinea-pig sodium channels (which inactivate rapidly) using a series of experiments including repetitive stimulation (Crumb & Clarkson, 1990). When we applied this series of experiments to cells expressing normal human hNav1.5 cardiac channels, the resulting coefficients governing the interaction of propafenone with the open channel (k_o and l_o) were similar to those derived from the direct experiment on the inactivation-deficient channels. In addition, this method yielded rate coefficients for the interaction of propafenone with the channel in its resting and inactivated state.

Thus, the method described by Crumb and Clarkson, which includes underlying simplifying assumptions (e.g., the one millisecond opening time), yielded compatible results in our case and was useful for evaluation of the very short open time of the normal human cardiac sodium channel.

An unexpected finding concerning an increase in peak current in cells when exposed to very low concentrations of propafenone required modifications in the data analysis: In the investigation of the wild-type cells with the algorithm presented by Crumb and Clarkson, the unexpected increase in peak currents when exposed to very low propafenone concentrations (0.03 μM) made the evaluation of the steady-state affinity of propafenone to the resting state challenging because the baseline peak current is unclear. The reason for this peak variation for low propafenone concentrations is not clear. However, the peak current was found to remain elevated at an unchanging level until a threshold propafenone concentration of 0.3 μM had been reached. In addition, the propafenone concentration used for the algorithm (3 μM) was 10-fold higher than the threshold of 0.3 μM yielding a resting block of approximately 10%. Therefore, the initial peak current was redefined to occur not during perfusion with the control solution, but during perfusion with 0.03 μM propafenone. Of course, this may have introduced an error because the algorithm of Crumb and Clarkson requires the measurement of the resting block. The change in rate constants resulting from an error in the resting state can be calculated. Table 3 lists the rate constants that would have resulted had the algorithm of Crumb and

Table 3. Error analysis to determine the sensitivity of the rate constants $k_{r,o,i}$ (resting, open, and inactivated) and $l_{r,o,i}$ to errors in the estimation of the resting block by propafenone

	Rate coefficients assuming different resting blocks		
	Resting block = 5%	Resting block = 10%	Resting block = 15%
k_r	0.00071	0.0014	0.0021
l_r	0.04	0.038	0.036
k_o	54.0	51.1	48.3
l_o	12.2	20.7	29.3
k_i	Same as 10%	0.0758	Same as 10%
l_i	Same as 10%	0.0299	Same as 10%

The algorithm of Crumb and Clarkson was repeated for assumed resting blocks of 5, 10 and 15%. Our measured value was 10%. $k_{r,o,i}$ are in s^{-1} μM^{-1} and $l_{r,o,i}$ are in s^{-1}

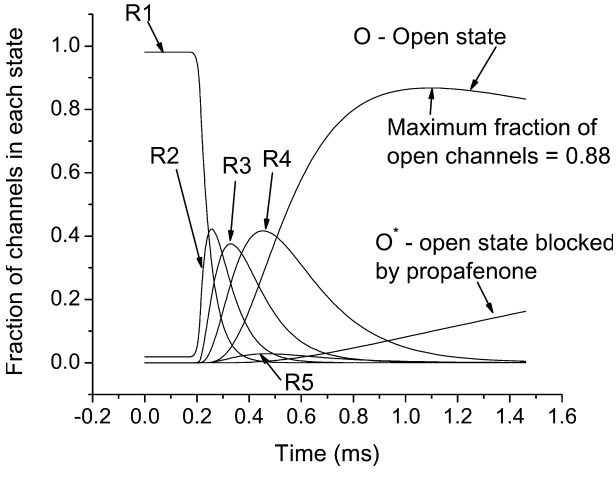

Fig. 10. Analysis of the transition between states in the kinetic model (*see* Fig. 3) during application of a voltage pulse while applying 3 μM propafenone. There is overlap between the initial resting state *R*1 and the subsequent resting states *R*2–*R*5. The open state (*O*), which is permeable to sodium ions, overlaps with both the resting states and the blocked state (*O**). Thus, the maximum fraction of open channels is reduced by 12%. Note that the effect of slow inactivation is not visible yet (time constant $\tau_{O \rightarrow SI}$ = 24.4 ms).

Clarkson been calculated with a smaller (5%) or larger (15%) resting block than the value measured (10%).

Thus, the rate constants l_o and k_r would be the most sensitive to errors in the assumed resting block. However, the open-channel rate constants, k_o and l_o, remain approximately 1000-fold higher than the resting rate coefficients, k_r and l_r. The constants k_i and l_i are not affected by errors in measurement of the resting block because they are calculated from the double-pulse experiment alone.

A "gating" model of the sodium channel that was presented previously (Kuo & Bean, 1994) was modified to represent the inactivation-deficient channels by excluding the original inactive states but including one inactivated state that is reached by "slow inactivation", as shown in Fig. 3. The rate constants, k_o, l_o, k_{si}, and l_{si}, were derived from the experiments on the inactivation-deficient channels; the other parameters were initially adopted from Kuo and Bean (1994) and then optimized by repetitive approximation. The model was found to compare well to the actual behavior of the sodium channels.

The rising phase and peak of the simulated current traces could be modified by changing the parameters α, β, γ, and δ. One set of parameters was found that minimized the MSE between actual and simulated current traces, and that matched the rising phase (slope) of the currents. Interestingly, the model displayed a reduction in peak current as the propafenone concentration was increased, even though there was no formal mechanism for a "resting block" because the inactivated states coming from the resting states had been removed from the model, as shown in Fig. 3. The model enables a detailed inspection of each simulated state and revealed that a portion of the open channels become blocked by the drug before all the channels in the resting states reach the open state (*see* Fig. 10). This effect lends the channel the appearance of having a so-called "resting" or "tonic" block even though the reduction in peak current stems from significant binding of propafenone early during the depolarization. This finding has also been made in measurements of sodium channels of toad axons exposed to lidocaine (Chernoff, 1990). Further investigation, perhaps with modified voltage driving pulses, is needed to further elucidate this phenomenon before assuming that this is the actual cause of the reduced peak current when propafenone concentrations increase.

This model differs significantly from a recently presented quantitative model also describing the interaction of propafenone with sodium channels (Pasek & Simurda, 2004). These authors had developed their model based on the premise that open-channel blockade is very small or absent, which conflicts with our findings.

In our model, a discrepancy between the actual and simulated tail currents was noted with a much faster response in the model than the experimental

data. Accurate modeling of the tail currents may require addition of sequential open states or interspersed inactive states between two or more open states, as suggested from experiments using squid axons (Correa & Bezanilla, 1994).

It should be noted that in the experiments above, a depolarizing voltage of + 50 mV was used. Therefore, a possible voltage dependency of the calculated rate constants for a range of driving voltages has not been established. Whether the model is applicable at other depolarizing voltages will require further studies.

Also, although the structure of the model in Fig. 3 can be used to explain many of the experimental findings of this study, it does not exclude the possibility that propafenone induces other inactivated states in addition to the above postulated drug-bound and slow-inactivation states.

Conclusion

Investigation of the normally very short open state of cardiac sodium channels is important to evaluate medications for treatment of diseases characterized by persistent currents, such as Brugada Syndrome. An ideal medication should block pathologically persistent currents but not prevent the normal opening of the channel. In this in-vitro setting, propafenone attenuates the persistent current at clinically relevant concentrations, while causing a small reduction in the peak current (10% reduction at 3 μM).

The prolonged open state of inactivation-deficient channels made direct kinetic analysis possible. However, even in wild-type channels that have very brief open times before inactivation (approx. 1 ms), a previously described indirect algorithm yielded similar results (Crumb & Clarkson, 1990). This supports the claim that the algorithm should be applicable in other experimental settings when there is only a very short time period available for kinetic analysis.

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