Two human paramyotonia congenita mutations have opposite effects on lidocaine block of Na^+ channels expressed in a mammalian cell line

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1. Two mutant human skeletal muscle voltage-gated Na^+ channel α-subunits (hSkM1), with mutations found in patients with hereditary paramyotonia congenita (T1313M on the III–IV linker and R1448C on the outside of S4 of repeat IV), and wild-type hSkM1 channels were expressed in a human embryonic kidney cell line (tsA201) using recombinant cDNA.

2. Compared with wild-type, both mutants exhibited altered inactivation phenotypes. Current decay was slowed for both, but voltage-dependent availability from inactivation was shifted in the negative direction for R1448C and in the positive direction for T1313M.

3. The hypothesis that a local anaesthetic, lidocaine (lignocaine), binds primarily to the inactivated state to block the channel was reassessed by testing lidocaine block of these two mutants and the wild-type channel.

4. T1313M showed reduced phasic block, but R1448C showed increased phasic block for trains of depolarizations.

5. Rest block (from −120 mV) was increased for R1448C ($IC_{50} \approx 0{\cdot}2$ mM) and decreased for T1313M ($IC_{50} \approx 1{\cdot}3$ mM) compared with wild-type ($IC_{50} \approx 0{\cdot}5$ mM), but these differences were diminished at a holding potential of −150 mV, suggesting that the differences were caused by binding to the inactivated state rather than a different affinity of lidocaine for the resting state.

6. Inactivated state affinity measured from lidocaine-induced shifts in voltage-dependent availability was reduced for T1313M ($K_d = 63$ μM) but little changed for R1448C ($K_d = 14$ μM) compared with wild-type ($K_d = 11$ μM). Two pulse recovery protocols showed faster recovery from lidocaine block for T1313M and slower recovery for R1448C. Together these accounted for the opposite effects on lidocaine phasic block observed for the mutant channels.

7. Neither mutation is located at a putative lidocaine binding site in domain 4 S6, yet both affected lidocaine block. The data suggest that R1448C altered phasic lidocaine block mainly through altered kinetics, but T1313M altered block through a change in affinity for the inactivated state. These findings have implications for drug therapy of paramyotonia congenita, and also provide an insight into structural requirements for drug affinity.

Sodium channel opening causes an inward current resulting in the rapid depolarization of the cell membrane in many excitable tissues, including skeletal muscle. Reduction or block of Na^+ current (I_{Na}) by drugs such as lidocaine (lignocaine) decreases the excitability of these cells. Lidocaine is used clinically on peripheral nerves as a local anaesthetic and also on heart as an antiarrhythmic agent. Lidocaine analogues have also been used to treat skeletal muscle sodium channel disorders such as paramyotonia congenita (for review see Rüdel, Ricker & Lehmann-Horn, 1993). Lidocaine, like many Na^+ channel blocking drugs, causes both a resting, or tonic, block and a phasic block, also called use-dependent or frequency-dependent block. Phasic block is additional block caused by single or repetitive depolarizations. The mechanism for this block can be explained in the context of the modulated receptor model

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(Hondeghem & Katzung, 1977; Hille, 1977) as the channel having an increased affinity for the drug when it enters different kinetic states such as the open state or the inactivated state during the depolarization cycle. In particular, phasic block of I_{Na} by lidocaine has been attributed to a greater affinity for the inactivated state of the channel.

Investigations on cloned voltage-dependent Na^+ channel α-subunits, the pore-forming unit of the channel, have provided insight into the structures responsible for various channel functions such as permeation and gating (for review see Goldin, 1994). The mechanisms for local anaesthetic interactions with the channel are being investigated using designed mutations at various sites. For example, mutations on the transmembrane-spanning segment in S6 in the fourth homologous domain (D4) of the rat brain sodium channel (Ragsdale, McPhee, Scheuer & Catterall, 1994) affected etidocaine block, leading the authors to suggest that S6 is the binding site for local anaesthetic drugs (Fig. 1). A mutation in human heart sodium channels (IFM→QQQ) on the cytoplasmic linker between D3 and D4, a structure associated with inactivation, resulted in non-inactivating channels and also a greatly reduced lidocaine block (Bennett, Valenzuela, Chen & Kallen, 1995).

Recently, efforts to trace autosomal dominant hereditary diseases characterized by painless stiffness (myotonia) of voluntary muscles led to the discovery of mis-sense mutations in the human skeletal muscle sodium channel α-subunit (hSkM1) gene *SCN4A* (George, Komisarof, Kallen & Barchi, 1992). Biophysical measurements of the expressed mutant channel I_{Na} showed a defect in inactivation: I_{Na} decayed more slowly and/or resulted in a persistent current, and these electrophysiological phenotypes have been implicated in the pathogenesis of the clinical phenotype (for review see Rüdel *et al.* 1993). These naturally occurring mutations of the hSkM1 provide a channel model with altered inactivation with which to assess the drug–channel interaction and the role of inactivation in drug block. An intriguing and potentially useful aspect of this model is that the mutation sites, although clustered mostly on D4 and the

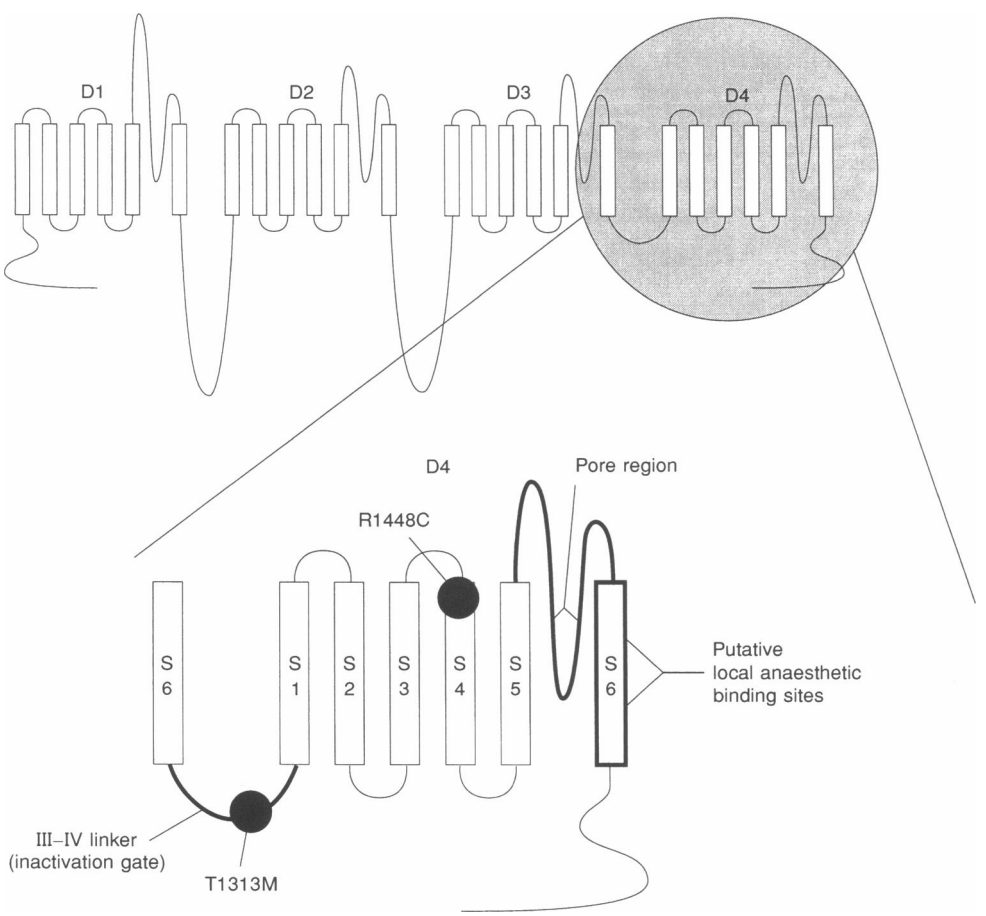

Figure 1. Diagram of the α-subunit for the hSkM1 Na^+ channel

Four homologous domains, D1, D2, D3 and D4 each consist of 6 transmembrane segments termed S1–S6. The expanded diagram of the carboxyl end of the channel shows S6 of D3, the D3–D4 linker and D4. The inactivation gate, pore region and putative local anaesthetic binding sites are labelled along with approximate locations of the mutations T1313M and R1448C.

D3–D4 linker of hSkM1, are located on diverse structures within these regions. We chose two paramyotonia congenita mutants (T1313M on the cytoplasmic III–IV linker and R1448C on the outside of S4 repeat IV, see Fig. 1) because they both affected characteristics of sodium current inactivation (Yang *et al.* 1994), but the mutated sites were predicted to be far from each other by consensus molecular models for the channel (Durell & Guy, 1992; Catterall, 1995). In addition, these two mutations are among the most common in patients with paramyotonia congenita (15% for T1313M and 12% for R1448C; R. Rüdel, personal communication). We characterized lidocaine interaction for the wild-type hSkM1 and these two mutants. Although, based upon a similar inactivation phenotype a concordant alteration in lidocaine block of the channel might be expected, we found that the T1313M mutant channel had decreased affinity, whereas R1448C had increased affinity for lidocaine. This dichotomy has implications for drug treatment of the clinical disease, and also provides insight into the structure–function relationships for inactivation and local anaesthetic binding to the channel.

METHODS

Construction and expression of hSkM1 mutants

The construction and expression of hSkM1 and its mutants have been described previously (Yang *et al.* 1994; Chahine *et al.* 1994). Full-length mutant hSkM1 constructs were assembled in the mammalian expression vector pRc/CMV and introduced into tsA201 cells (a derivative of human embryonic kidney cells) by transient transfection (calcium phosphate co-precipitation method). After 24–60 h the transfected cells were used for electrophysiological recording, or were frozen (−80 °C freezer) for at least 2 days, but not longer than a week, and then transferred to storage in liquid nitrogen. These cells were later thawed and used the same day for electrophysiology recording. Electrophysiological properties of cells that had been frozen were identical to those not frozen.

Electrophysiological recordings

An Axon-1D patch clamp with a series resistance compensation circuit (Axon Instruments) was used for recording currents at 20–22 °C in a flowing bath solution consisting of (mM): 140 NaCl, 4·0 KCl, 1·8 $CaCl_2$, 0·75 $MgCl_2$ and 5 Hepes, pH 7·4. The electrode pipettes were filled with a solution consisting of (mM): 120 CsF, 20 CsCl, 2 EGTA and 5 Hepes. Lidocaine in appropriate amounts was added from a 100 mM stock solution in distilled water. Data were acquired using pCLAMP 6 software (Axon Instruments) onto an Intel 486-based computer. Data were digitized at 42 kHz and were low-pass filtered at 10 kHz (−3 dB).

Experimental protocols and analysis

Details of the voltage protocols are provided with the data. The holding potential was −120 mV unless otherwise noted. The frequency of stimulation within a protocol was carefully considered and tested to ensure that no effects of a previous stimulation would be present in a subsequent test. For recovery protocols, we inverted the order of the recovery times to ensure that the order did not influence the recovery time course. Peak currents were measured as the difference between maximum negative current and the current at the end of the depolarization. Data were fitted to model equations using non-linear regression (procedure NLIN) using SAS statistical software (SAS Institute, Inc., Cary, NC, USA) running on a SUN IPX workstation. Parameter estimates for fits are given with the standard error. For the exponential fits for recovery and decay, the data were fitted to both one- and two-exponential component models and the two-component model is reported only if the F ratio showed the fit to be statistically significantly better ($P < 0.05$) given the increase in the degrees of freedom. Mean data are reported with their standard deviation. All determinations of statistical significance of mean data were performed by using Student's t test for comparisons of two means, or by one-way analysis of variance (ANOVA) followed by the Bonferroni t test for comparison of more than two means.

RESULTS

T1313M and R1448C slow I_{Na} decay

Na^+ channel current (I_{Na}) was recorded by whole-cell voltage clamp from tsA201 cells transiently transfected with α-subunits of the wild-type human skeletal muscle Na^+ channels (hSkM1) or the paramyotonia congenita mutant hSkM1 channels T1313M or R1448C (Fig. 2). A series of depolarizing voltage clamp steps from a holding potential of −120 mV showed that I_{Na} for the mutant channels decayed more slowly than for the wild-type channel (Fig. 2*A*), in agreement with earlier reports (Yang *et al.* 1994). The I_{Na} decay time course for the wild-type and mutant channels was assessed by fitting the decay with a single-exponential function to obtain a decay time constant (τ_{decay}), and these were then plotted against the magnitude of the depolarizing step (Fig. 2*B*). I_{Na} decay times for both mutants were slower than for the wild-type, but R1448C was even slower than T1313M, being about 4-fold slower than the wild-type. Activation kinetics, evaluated by a conductance transform of the peak current–voltage relationship and by a plot of the time to peak, were similar for the three channel types (data not shown). Steady-state availability from inactivation (500 ms conditioning pulse) was shifted by 10 mV in the hyperpolarizing (negative) direction for R1448C (Table 1 and Fig. 5*A*). These control data confirm I_{Na} kinetics previously described for these channels (Yang *et al.* 1994).

Measurement and classification of lidocaine block of I_{Na} in hSkM1

Drug block of I_{Na} is generally classified as phasic or non-phasic block. Non-phasic block, also called rest block, is block occurring either in the absence of prior depolarizations (first-pulse block) or at very low pulsing frequencies (tonic block). Phasic block, also called use-dependent, frequency-dependent or extra block, is the additional block occurring after prolonged or repetitive depolarizations. I_{Na} was recorded in response to a train of 10 ms long depolarizing pulses from a holding potential of −120 mV to a test potential of −10 mV. Wild-type channel recordings of I_{Na} for the 1st and 20th pulses in a 10 Hz train in control solutions and after addition of 1 mM lidocaine are shown in Fig. 3*A*. Peak inward currents were measured and plotted against the pulse number in the train (Fig. 3*B*). First-pulse block, defined as the relative decrease in I_{Na} for the first depolarization in lidocaine compared with the current measured without lidocaine, was used as a measure of non-

Table 1. Slope factors and $V_{½}$ of inactivation

	Control			Lidocaine (100 μM)		
	Wild-type	T1313M	R1448C	Wild-type	T1313M	R1448C
n	25	16	24	9	5	7
Slope factor (k)	6·6 ± 0·3	6·8 ± 0·5	8·8 ± 0·2*	7·0 ± 0·7	6·9 ± 0·6	9·2 ± 0·2*
$V_{½}$ (mV)	−78·5 ± 1·9**†	−75·5 ± 2·5**††	−88·3 ± 2·7**†††	−87·2 ± 2·9***†	−83·9 ± 3·7***††	−104·0 ± 1·1***†††

The slope factor (k) and $V_{½}$ are parameters of the Boltzmann fit to the steady-state availability protocol as in Fig. 5. n represents the number of experiments. * ANOVA test detected the significant difference between these values and other members in the same row of the table, both $P < 0·0001$. **, *** ANOVA test detected the significant difference among these values. †, ††, ††† Bonferroni t test detected the significant difference between the two values.

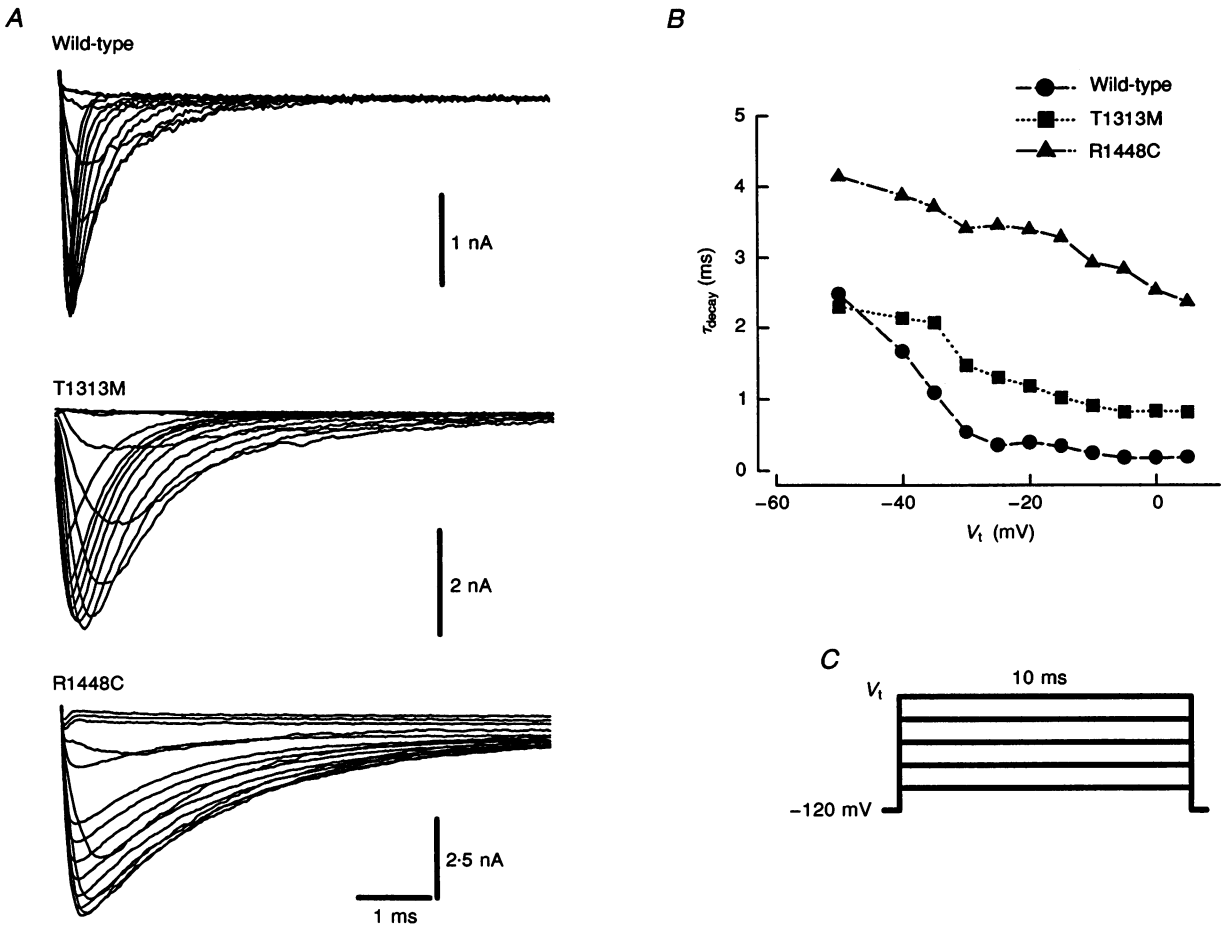

Figure 2. Slow I_{Na} decay for wild-type and two mutant paramyotonia hSkM1 Na^+ channels

A, currents recorded for wild-type, T1313M and R1448C mutated hSkM1 Na^+ channels expressed in tsA201 cells. The currents were elicited by step depolarizations from a holding potential of −120 mV to various test potentials (V_t). A diagram of the protocol is shown in *C*, and particular V_t values can be read from the abscissa in *B*. *B*, plots of the I_{Na} decay time constants (τ_{decay}) at the different V_t. Current decays were fitted by fitting the trace after peak I_{Na} with $I_{Na} = I_{Na,max} \exp(-t/\tau_{decay})$, where I_{Na} is the current magnitude, $I_{Na,max}$ is the peak I_{Na} and t is the time (ms).

phasic block. Phasic block was then defined as the additional I_{Na} decrease for the last pulse relative to the first pulse in a test train in the presence of lidocaine. Care was taken that pulse trains had a sufficient number of pulses such that I_{Na} achieved a steady-state pulse-to-pulse value by the end of the train.

First-pulse block by lidocaine

First-pulse block was measured at different lidocaine concentrations for wild-type, T1313M and R1448C, and concentration–response relationships were plotted and fitted with a single site binding equation (Fig. 3*C* and *D*). First-pulse lidocaine block of wild-type hSkM1 Na^+ channels had a half-concentration for block (IC_{50}) of 0·46 ± 0·08 mM. The mutant channel T1313M exhibited lesser sensitivity to lidocaine (IC_{50} = 1·30 ± 0·13 mM), but the mutant channel R1448C exhibited greater sensitivity (IC_{50} = 0·19 ± 0·05 mM) compared with both wild-type and T1313M channels (P = 0·0001 by 3-way ANOVA). First-pulse block is often assumed to reflect drug binding to the resting state of the channel. The first-pulse block at −120 mV, however, may not represent solely binding to the resting state because at this potential a small fraction of the channels may be in the inactivated state. An even greater

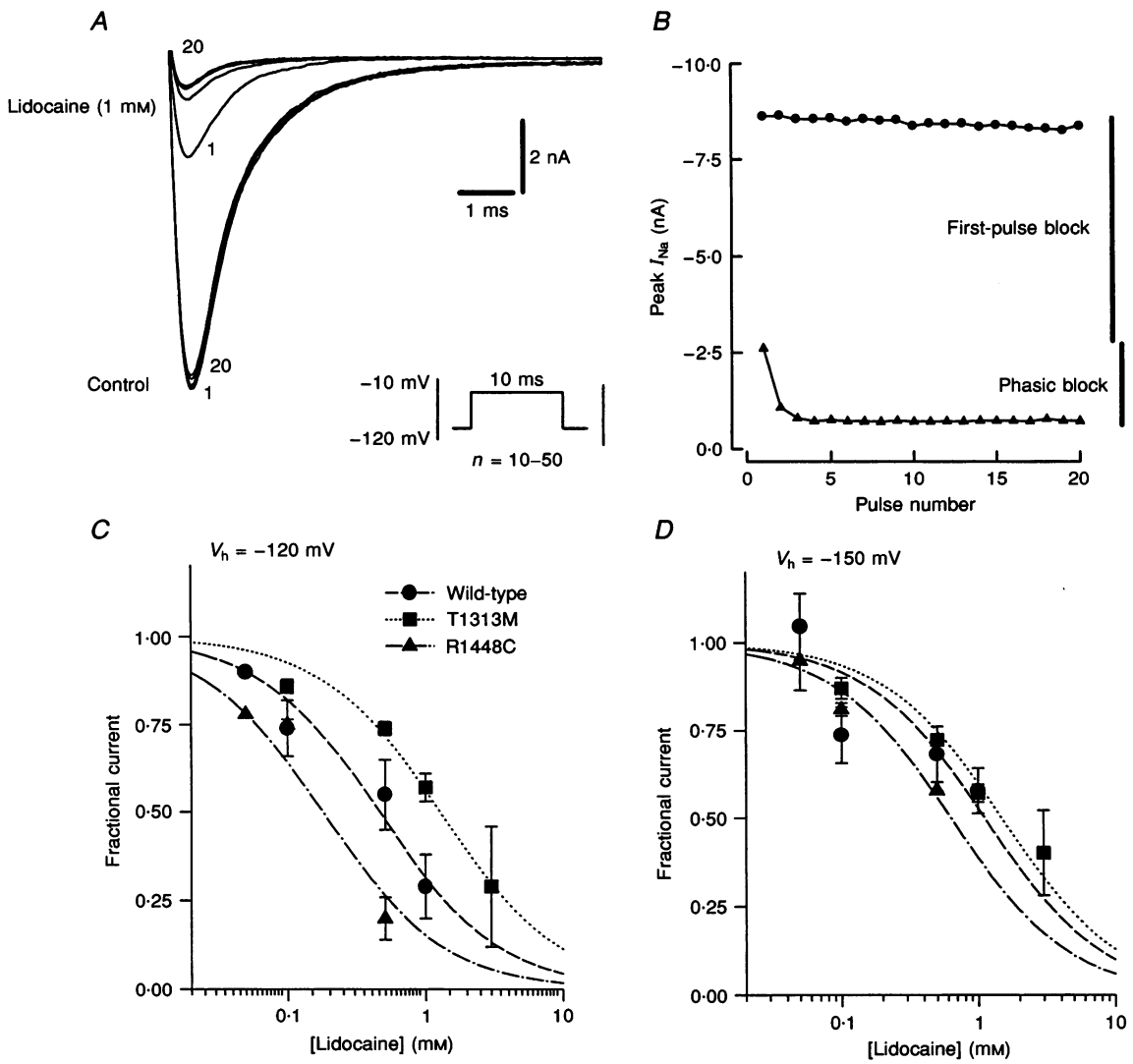

Figure 3. First-pulse lidocaine block of wild-type, T1313M and R1448C hSkM1 I_{Na}

A, superimposed I_{Na} records from wild-type hSkM1 before (control) and after exposure to 1 mM lidocaine. A train of 20 depolarizing pulses of 10 ms duration (see protocol inset) was applied from a holding potential of −120 mV to −10 mV at 10 Hz. The first (1) and last (20) depolarizations in control and in lidocaine are labelled. *B*, plot of peak I_{Na} against pulse number in a train of depolarizations. A representation of the definitions for first-pulse block and phasic block amplitude is displayed (see text for details). *C*, concentration dependence of the first-pulse block measured at a holding potential of −120 mV. *D*, first-pulse block measured at a holding potential of −150 mV. Symbols represent the means and the bars represent the S.Ds for between n = 2–7 experiments; bars were omitted when smaller than the symbols. Lines in the figures are fittings of first-order binding function: fractional current = $1/(1 + [\text{lidocaine}]/K_d)$ where K_d is the apparent dissociation constant for lidocaine.

proportion of channels may be in the inactivated state for R1448C at −120 mV because the steady-state inactivation relationship is more negative for this channel (Table 1). The inactivated state of the Na^+ channel has a greater affinity for lidocaine, so the affinity for the resting state could be overestimated. We measured rest block at the more hyperpolarized holding potential of −150 mV where the proportion of channels in the inactivated state would be lower than at −120 mV. At −150 mV, rest block IC_{50} values were 1·12 ± 0·39, 1·49 ± 0·23 and 0·63 ± 0·09 mM for wild-type, T1313M and R1448C, respectively. Although these sensitivities tended to differ in the same direction as at −120 mV, the differences were much smaller and no longer statistically significant ($P = 0·19$ by ANOVA), and appeared to be converging towards a common value at about 1·5 mM. These results suggest that, although first-pulse block is different for the three channels, the actual affinity of lidocaine for the resting state in the three channels may not be different, but rather differences in first-pulse block at a particular potential may be 'contaminated' by inactivated state binding.

Phasic block of I_{Na} by lidocaine

Phasic block in response to pulse trains was reduced by the T1313M mutation and enhanced by the R1448C mutation. Typical examples of phasic block in response to a train of 10 ms-long depolarizing pulses from a holding potential of −120 mV to a test potential of −10 mV at 10 Hz are shown in Fig. 4*A*. In control solutions, I_{Na} did not decline in response to the pulse train. The control data shown in Fig. 4*B* are for the wild-type channel; the mutant channel data are not shown for clarity of illustration, but they also

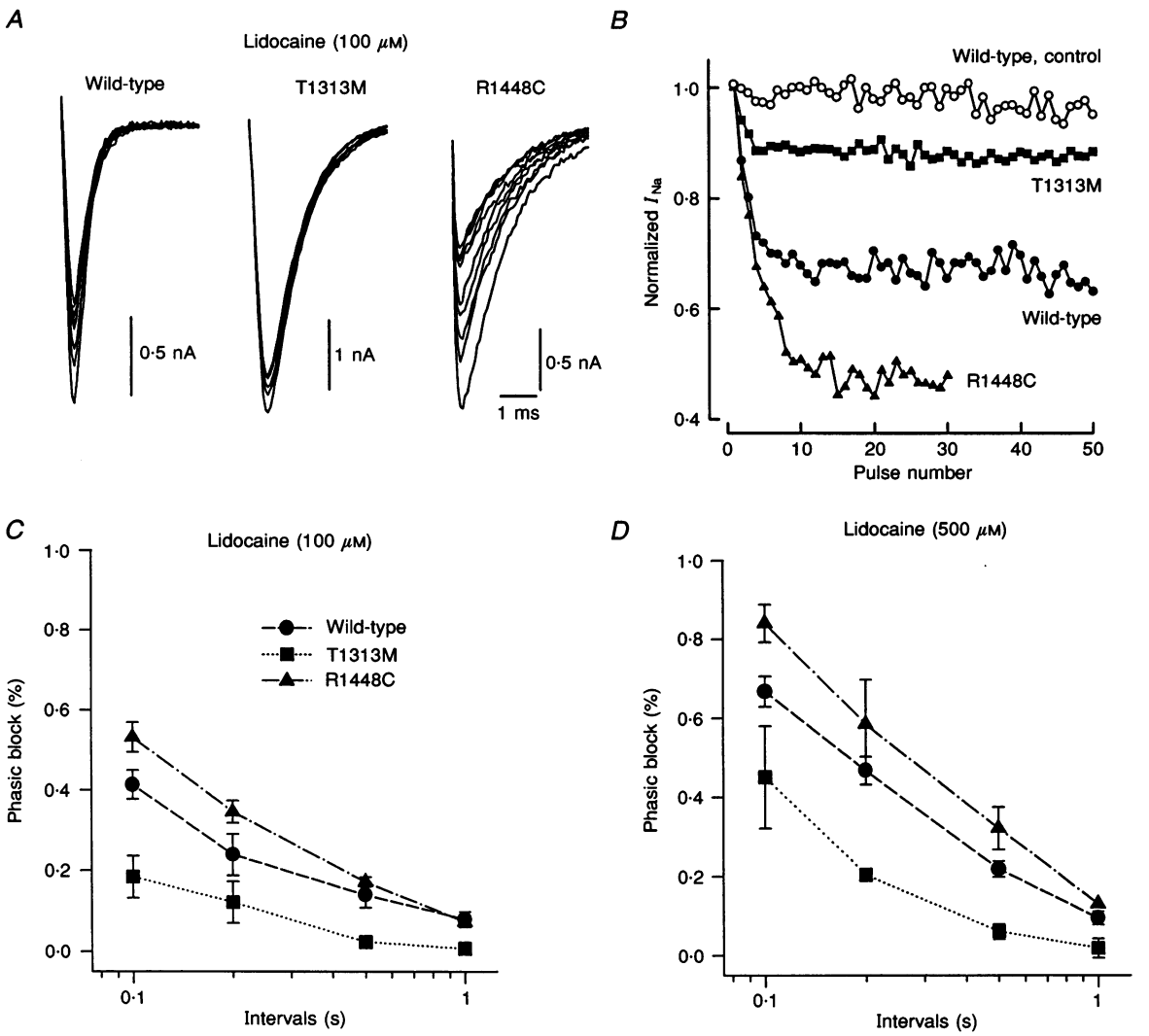

Figure 4. Phasic lidocaine block of wild-type, T1313M and R1448C hSkM1 I_{Na}

A, superimposed I_{Na} records from wild-type, T1313M and R1448C hSkM1 in the presence of 100 μM lidocaine. The pulse train clamp protocols are described in the legend to Fig. 3. *B*, peak I_{Na} plotted against pulse number in the train for wild-type in control solutions, and wild-type, T1313M and R1448C in 100 μM lidocaine. *C*, fractional current blocked at the end of a pulse train for 100 μM lidocaine. Symbols represent the means and the bars represent S.D. for $n = 4$–10 experiments; bars were omitted when smaller than the symbols. *D*, same as *C* but for 500 μM lidocaine, $n = 3$–6 experiments.

Table 2. Parameters of a two-exponential fit to the recovery time course

	Control, $t_c = 50$ ms			Control, $t_c = 1$ s			Lidocaine (100 μM), $t_c = 50$ ms		
	Wild-type	T1313M	R1448C	Wild-type	T1313M	R1448C	Wild-type	T1313M	R1448C
n	14	5	10	15	6	10	5	5	6
τ_f (ms)	1·7 ± 0·1	1·7 ± 0·1	1·7 ± 0·1	2·9 ± 0·4	2·5 ± 0·3	1·7 ± 0·3	2·6 ± 0·3	2·2 ± 0·2	3·5 ± 0·4
a_s (%)	13 ± 2	13 ± 2	13 ± 2	57 ± 2	55 ± 2	61 ± 2	48 ± 1	43 ± 1	43 ± 2
τ_s (ms)	25·1 ± 6·1	25·7 ± 8·0	27·6 ± 7·8	168 ± 18	170 ± 19	245 ± 37	392 ± 42*	124 ± 10*	564 ± 99*

τ_f is fast and τ_s is slow time constant of recovery, and a_s is the relative contribution of the slow component to overall recovery from the two-exponential fit described in Fig. 6. t_c is the conditioning potential duration for the recovery protocol as shown in Fig. 6. n represents the number of experiments. * Significant difference within the group by ANOVA with $P < 0.0001$.

did not decline. After fifty pulses, 100 μM lidocaine caused a 43% decline in I_{Na} for wild-type, a 14% decline for T1313M, and a 69% decline for R1448C. Summary data for the three channel types at two lidocaine concentrations and for four different frequencies (interpulse intervals) confirm that R1448C exhibits greater phasic block and T1313M less phasic block than the wild-type (Fig. 4*C* and *D*).

Inactivated state affinity estimated by steady-state availability shifts

The voltage dependence of steady-state availability was assessed by a classical 'h_∞ type' protocol where the cell membrane was first conditioned by a 500 ms-long step to various potentials then stepped to −10 mV, where peak I_{Na} in response to this step was measured and normalized to assess the fraction of channels that were available for opening (see protocol inset of Fig. 5*A*). The normalized peak currents were then plotted against the conditioning potential (V_c) and the data were fitted to a Boltzmann relationship to yield a mid-point ($V_{½}$) and a slope factor (k). Summary data for steady-state availability for the three channel types in control solutions (Fig. 5*A*) show that the mid-point for R1448C is negative by about 10 mV to those for wild-type and T1313M, consistent with the data reported previously (Yang *et al.* 1994). The T1313M $V_{½}$ was 3 mV positive to that of the wild-type. Lidocaine shifted the $V_{½}$ in the negative direction for all three channel types; results for 100 μM lidocaine are summarized in Fig. 5*B*. This shift can be explained in terms of the diagram inset to Fig. 5*C* (Hondeghem & Katzung, 1977). In control solutions, the steady-state availability curve represents the voltage-dependent distribution between the resting state (R) and the inactivated state (I). When drug is added, it binds to and blocks the inactivated state (I_{block}). By 'mass action' more channels are drawn off from R into the blocked state, resulting in reduced availability at any given conditioning potential. In this simplified scheme, binding to the resting state is ignored. Summary data for the $V_{½}$ in lidocaine (Fig. 5*C*) show that, as predicted by this model, the $V_{½}$ values are more negative with increasing lidocaine concentration. In order to estimate the affinity of lidocaine for the inactivated state of the Na^+ channel, we adapted the three-state model and the equation of Bean, Cohen & Tsien (1983) that describes the concentration dependence of $V_{½}$ in lidocaine:

$$V_{½} = k \ln(1/(1 + C/K_d)) + V_{½,c},$$

where $V_{½}$ is the mid-point in lidocaine, $V_{½,c}$ is the $V_{½}$ in control solutions (no lidocaine), k is the Boltzmann slope factor for the availability curve, C is the lidocaine concentration, and K_d is the dissociation constant for lidocaine from the inactivated state. The continuous lines in Fig. 5*C* represent non-linear regression fits of this equation to the data. For these fits, k was assigned the value found in the control (Fig. 5*A* legend) and the equation was fitted over the mid-points for the different lidocaine concentrations to yield a dissociation constant (K_d) for the inactivated state. We also tested a model that included resting state binding, but the results were not different, probably because resting state affinity is negligible compared with inactivated state binding. We therefore report only the results of fitting to the simpler model. The estimated K_d for the wild-type, T1313M and R1448C were 11, 63 and 14 μM, respectively. Although mid-points were very negative for R1448C in lidocaine, this analysis suggests that this could be wholly accounted for by the negative shift in availability in control, and does not reflect a greater affinity of lidocaine for the R1448C inactivated state.

Effects of T1313M and R1448C on the recovery from lidocaine block

Recovery from inactivation and lidocaine block was assessed by a two-pulse recovery protocol (see inset to Fig. 6*A*). In this protocol a conditioning pulse of either 50 ms or 1 s was used to inactivate the channel and, in the case of lidocaine, to allow for drug binding to inactivated states. This was followed by a variable recovery interval at −120 mV after which a test depolarization to −10 mV was used to elicit a peak current to assess the fraction of channels that had recovered (or unblocked) during the preceding recovery interval. The normalized peak currents were plotted *versus* the recovery interval and the data were fitted to a one- or two-exponential recovery model (see legend to Fig. 6) to

yield either a single rapid time constant (τ_f), or in the case of a two-exponential fit, an additional slow time constant (τ_s) and a percentage amplitude of the recovery attributable to the slow component (a_s). At a holding potential of −120 mV, a short 10 ms conditioning pulse step to −10 mV produced a rapid ($\tau_f < 5$ ms) single-exponential recovery time course from inactivation (data not shown). Prolonging the conditioning pulse duration to 50 ms induced a second, slower ($\tau_s \approx 25$ ms) exponential component comprising about 13% of the recovery amplitude. These data are shown as a log(time) plot in Fig. 6*A* with the single-component fit shown as a dotted line, and the two-component fit shown as a continuous line. Summary data for the fitted values (τ_f, τ_s and a_s) are shown in Table 2. When the conditioning potential duration (t_c) was increased to 1 s (Fig. 6*B*), the slower component became slower ($\tau_s > 150$ ms) and comprised more than 50% of the recovery amplitude. Except for a trend towards a larger and slower slow recovery component for R1448C, no significant differences (ANOVA) were noted for recovery in control for the two mutant channels and the wild-type channel.

In the presence of lidocaine at a conditioning duration of 50 ms (Fig. 6*C*), the slow component of block was slower ($\tau_s > 100$ ms) compared with control ($\tau_s < 30$ ms) at the same conditioning duration and it was larger in magnitude (a_s, ~43% in lidocaine compared with ~13% in control).

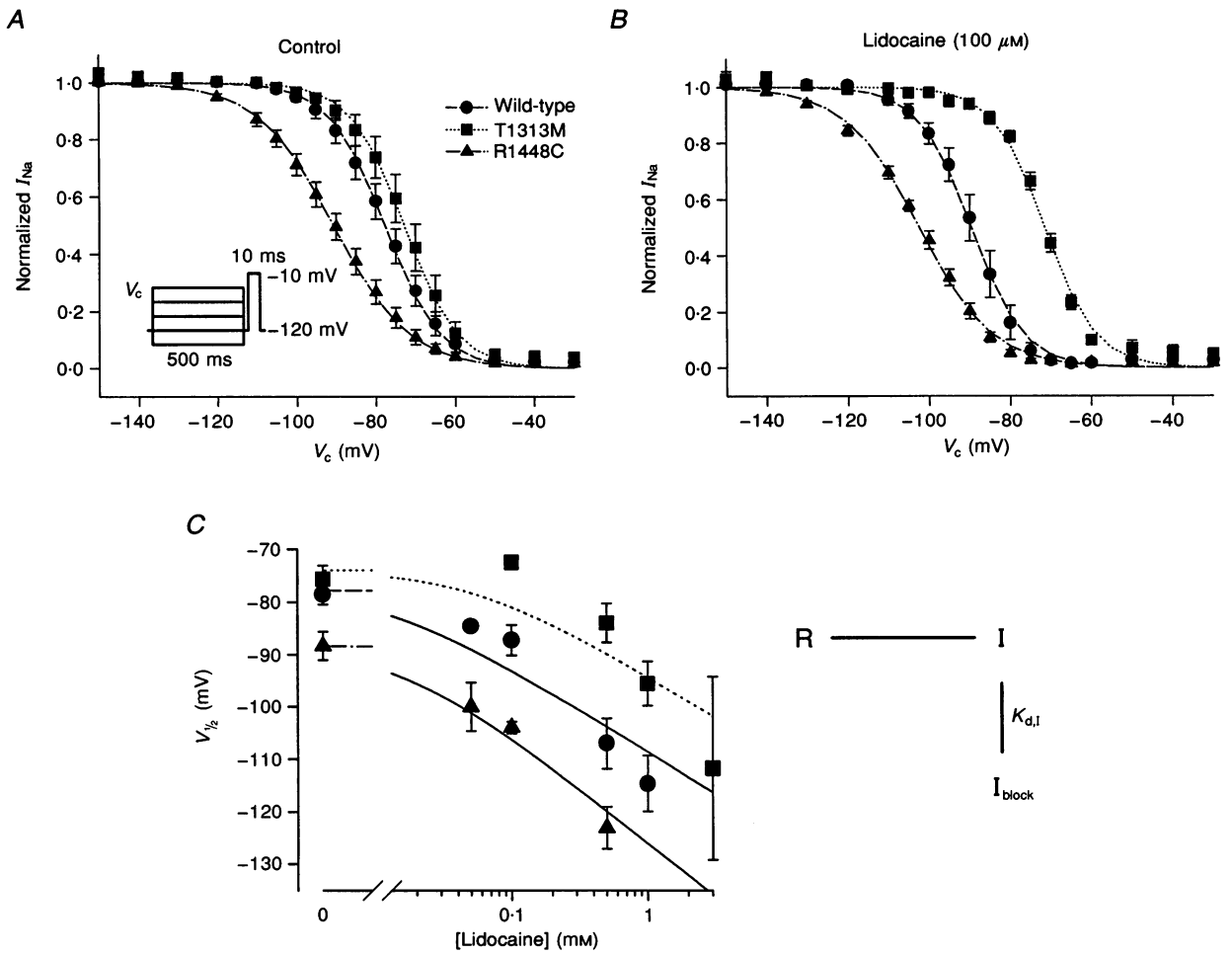

Figure 5. Effect of T1313M and R1448C mutations on the voltage-dependent availability of I_{Na} in the absence and presence of lidocaine

A, symbols represent means and bars represent S.D. for measurements of peak I_{Na} measured for a test depolarization to −10 mV after a 500 ms clamp step to various conditioning potentials, V_c (see protocol inset). A 1 ms stepback to −120 mV between the conditioning step and the test step allowed for normalization of the capacity transients. The data were normalized to the peak I_{Na} at −140 mV and plotted against V_c. The lines represent fits to a Boltzmann relationship: normalized $I_{Na} = [1 + \exp(V_c - V_{½})/k]^{-1}$ where $V_{½}$ and k are the mid-point and slope, respectively, of the Boltzmann relationship. The slopes, mid-points and n numbers are reported in Table 1. *B*, same as *A* but in the presence of 100 μM lidocaine. *C*, lidocaine-induced shifts in $V_{½}$ of inactivation in wild-type, T1313M and R1448C mutated hSkM1 Na^+ channels. Symbols represent the means and the bars represent the S.D. for up to 19 experiments. Lines are fittings of the model shown in the inset. The equation used is listed and explained in the text. $K_{d,I}$ is the calculated apparent dissociation constant for the inactivated state and values are reported in the text.

Presumably this new slow component represented the recovery from drug-bound channels. Although recovery from lidocaine block was slower than the slow recovery component in control, this measure of lidocaine recovery may have been contaminated by slow recovery from inactivation. We were unable to discern the third exponential component attributable to slow recovery from inactivation when lidocaine was present. This could have been because this component had a very similar time constant, or because the amplitude may have been small and had been reduced proportionately or eliminated preferentially in the presence of lidocaine. Not knowing which of these possibilities pertained, we chose to present the recovery time constants for a conditioning duration of 50 ms, where we obtained a strong lidocaine recovery amplitude (nearly 50%), whilst the control experiments with the identical protocol showed a small (~13%) and relatively rapidly recovering ($\tau_s < 30$ ms) slow component. We concluded that the time constants of recovery for conditioning durations of 50 ms were very likely to reflect recovery from lidocaine block, and these demonstrated important and statistically significant (ANOVA $P < 0{\cdot}0001$) differences for lidocaine recovery for the three channel types (asterisked values in Table 2). T1313M recovered more quickly from lidocaine block and R1448C recovered more slowly.

DISCUSSION

For wild-type hSkM1, lidocaine was estimated to have a 100-fold higher affinity for the inactivated state ($K_d = 11$ μM) compared with the resting state ($IC_{50} = 1{\cdot}12$ mM at −150 mV). Measuring the affinity of lidocaine for different kinetic states of the Na^+ channel is difficult because it depends upon underlying assumptions regarding gating kinetic models for the channel, the model of drug block, the specific protocols used to measure block, how they are interpreted in terms of the models of kinetics and block and finally, upon a range of experimental variables (such as temperature, pH and ions such as Na^+ and Ca^{2+} (for review see Hille, 1992)). In this analysis we kept the protocols and the underlying assumptions as simple as possible. We assumed a single resting state, open state and inactivated state for the underlying kinetic model, and we assumed lidocaine binding produced a blocked channel and that it occurred predominantly to two states: the resting state and the inactivated state. This is consistent with the original formulation of the modulated receptor models (Hondeghem & Katzung, 1977; Hille, 1977). As such it does not account for more complex possibilities such as pre-open (Starmer, Nesterenko, Undrovinas, Grant & Rosenshtraukh, 1991) or post-repolarization (Makielski, Satin & Fan, 1993) block,

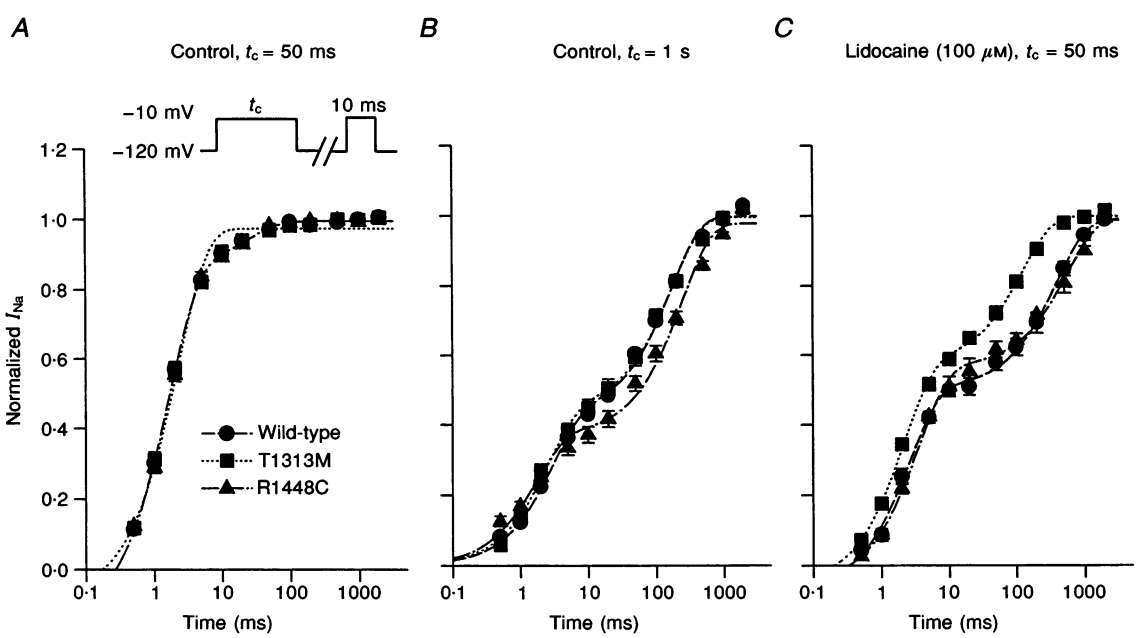

Figure 6 . Effect of T1313M and R1448C mutations on the recovery from lidocaine block

A, peak I_{Na} was measured in response to a second test depolarization which was preceded by a conditioning depolarization to −10 mV lasting 50 ms ($t_c = 50$ ms) followed by a variable recovery interval, as shown by the diagram in the inset. Peak I_{Na} at each time was divided by peak I_{Na} recorded in the absence of a conditioning step to give normalized I_{Na}, which was plotted against the recovery time. Lines are fits to a two-exponential equation:

$$\text{normalized } I_{Na} = (a_f + a_s) - \{a_f \exp[-(t - D)/\tau_f] + a_s \exp[-(t - D)/\tau_s]\},$$

where t is the recovery time, the fitted parameters are τ_f (fast recovery time constant) and τ_s (slow recovery time constant), a_f and a_s are the amplitudes of the fits, and D is a delay factor that ranged from 0·2 to 0·33 ms for $t_c = 50$ ms to 0 for $t_c = 1$ s. Parameters of the fit are reported in Table 2. *B*, I_{Na} recovery for $t_c = 1$ s in the absence of lidocaine. *C*, I_{Na} recovery for $t_c = 50$ ms in the presence of 100 μM lidocaine.

multiple inactivated or resting states, gating of drug-bound channels (Hanck, Makielski & Sheets, 1994), or even drug-bound conducting channels (McDonald, Courtney & Clusin, 1989). With these caveats noted, the affinities we obtained are comparable to those obtained for rat skeletal muscle sodium channels expressed in oocytes (Nuss, Tomaselli & Marban, 1995).

Inactivated state binding affects rest block

The seemingly straightforward measurement of rest block as an indication of resting state affinity was complicated by a voltage dependence. Rest block decreased at −150 mV compared with −120 mV (Fig. 3*C* and *D*), most probably because a higher proportion of the block at −120 mV represented binding to the inactivated state (per Fig. 5*C* inset) rather than binding to the resting state. The mutant channel R1448C has a steady-state availability relationship 10 mV negative to that for wild-type, and therefore would have more rest block caused by binding to the inactivated state at any given potential than the wild-type channel. This could be misinterpreted as the R1448C mutation having altered the affinity for the resting state of the channel, whereas the apparent differences in rest block should be attributed to the altered kinetics of distribution between resting and inactivated states. Hyperpolarizing to −150 mV decreased the apparent affinity of lidocaine for the resting state, and also narrowed the differences in apparent affinity for the three channels (Fig. 3*C* and *D*). Cells did not survive well at more hyperpolarized potentials, but a few experiments at −180 mV suggested that rest block was saturating as predicted by the simple model (Fig. 5*C* inset) at a common value of between 1·5 and 2·0 mM for all three channels. The differences in voltage-dependent availability from inactivation are not, however, the only factor to affect tonic block at a given potential. The IC_{50} for T1313M at −120 mV was 1·3 mM compared with 0·5 mM for wild-type, but the $V_{½}$ for T1313M was only 3 mV positive to that of wild-type. This difference in $V_{½}$ alone cannot account for the much greater IC_{50}. The explanation lies with the lower affinity of T1313M for the inactivated state. Because of this lower affinity, T1313M tonic block is less 'contaminated' by binding to the inactivated state at this potential than wild-type, and thus has an affinity lower than expected from $V_{½}$ differences alone. We conclude, therefore, that whereas the mutations may have affected rest block at a given potential, the mechanism for this difference can be accounted for by both the differences in the voltage dependence of availability from inactivation and differences in affinity for the inactivated state, rather than an intrinsic change in lidocaine affinity for the resting state of the channel.

Paramyotonia congenita mutations altered phasic block by different mechanisms

Measuring blocking affinity for the inactivated state of the channel is less straightforward than measuring resting affinity, and depends even more upon assumptions about the underlying kinetic models used to make the estimates. The method using the shift in $V_{½}$ is based on a simple three state model (Fig. 5*C* inset) adapted from the original descriptions of the modulated receptor model (Hondeghem & Katzung, 1977; Hille, 1977). This model has the advantage of having been used frequently in the past to estimate inactivated affinity (e.g. Bean, Cohen & Tsien, 1983; Chahine, Chen, Barchi, Kallen & Horn, 1992) and it also accounts for the voltage dependence of steady-state availability from inactivation of the channel (the $R \rightleftharpoons I$ distribution), but it has the disadvantage of ignoring contributions of transitions of the drug-bound channel between a drug-bound resting state and a drug-bound inactivated state. We had confirmed previously, however, in experiments with cloned human heart channels, that the $V_{½}$ method produced estimates consistent with those obtained from a two-pulse development of block protocol, an independent and more direct measure of block (Makielski, Limberis, Chang, Fan & Kyle, 1996). This two-pulse protocol could not be used with hSkM1 because a relatively large component (compared with heart) of slow recovery from inactivation under control conditions overlapped the time course of recovery from lidocaine block (Table 2). We tried many different conditioning and recovery conditions, but we were unable to separate these components. Therefore, we have not reported our results with two-pulse development protocols. Another method to estimate inactivated state affinity is the guarded receptor model formalism for block in response to pulse trains (Starmer & Grant, 1985), but this method did not yield the linear results predicted and required to use the model. We therefore used the $V_{½}$ estimate to measure lidocaine affinity for the inactivated state of the channel. Despite having similar clinical and electrophysiological phenotypes (Yang *et al.* 1994), the two mutations produced opposite results for phasic block in response to pulse trains (Fig. 4), with less block for T1313M and more block for R1448C. T1313M was shown to have a reduced affinity for the inactivated state (K_d, 63 μM) compared with wild-type (K_d, 11 μM) which would, at least in part, account for the reduced block in pulse trains because of reduced binding to the inactivated state. The inactivated state affinity for R1448C (K_d, 14 μM), however, was not greater and indeed slightly less than the wild-type. Thus a difference in affinity for the inactivated state cannot by itself account for the greater phasic block for R1448C. This effect, however, can be explained by the fact that this mutation significantly slowed recovery from lidocaine block (Table 2). Slower recovery in the interpulse interval would allow for a greater accumulation of phasic block in response to the pulse trains

Structural implications for lidocaine interactions with the sodium channel

The sodium channel α-subunit (Catterall, 1995) consists of four homologous repeats (D1–D4) each with six transmembrane spanning segments (S1–S6; see Fig. 1). Myotonia-associated mutations of the human skeletal muscle α-subunit described to date are distributed over D2, D3 and D4, and over segments S3, S4, S5, S6 and on the D3–D4 linker; those mutations studied have abnormal inactivation (Rüdel *et al.*

1993). One of the mutations we studied, T1313M, is on the cytoplasmic D3–D4 linker associated with inactivation, and the other, R1448C, is located on D4 S4, a structure near the extracellular space and associated with activation (Fig. 1). Classically, the lidocaine binding site has been postulated to be on the inner pore (Hille, 1992). By analogy with K^+ channels, and by molecular modelling (Durell & Guy, 1992), S6 is a candidate for the inner pore. Ragsdale *et al.* (1994) made mutations on S6 of D4 that affected block by the local anaesthetic etidocaine and suggested that S6 was the local anaesthetic binding site. Neither of the mutations we studied are on S6, yet both affected lidocaine block. Present concepts of Na^+ channel structure predict T1313M and R1448C to be located on opposite sides of the membrane (Fig. 1). Clearly if present models are correct, both mutations cannot affect block by being part of or by being close to a single binding site. State-dependent binding provides an alternative explanation. Local anaesthetic block is dependent upon the kinetic state of the channel, therefore block might be altered as a result of a mutation-induced change in kinetics. Indeed, Bennett and colleagues (Bennett *et al.* 1995) have recently shown that the inactivation-defective mutation IFM→QQQ in the D3–D4 linker of the human heart sodium channel has greatly reduced affinity for lidocaine. Both R1448C and T1313M caused abnormalities in the rate of current decay, but T1313M decreased block, whereas R1448C increased block. We have provided evidence that the changes in lidocaine affinity induced by R1448C can be explained by changes in kinetics induced by the mutation. For T1313M, however, the actual affinity for the inactivated state is decreased. This suggests that the inactivation gate interacts actively and specifically with the local anaesthetic molecule in the pore. Such a view is consistent with the 'trapping' hypothesis (Yeh, 1978), with the added concept that the molecule is not merely passively trapped by the gate, but rather provides a 'glue' for the gate to the channel pore. We conclude that structures on the D3–D4 linker are also likely to make up part of the local anaesthetic binding site. The S6 mutations purported to be at the local anaesthetic binding site also affected inactivation kinetics (McPhee, Ragsdale, Scheuer & Catterall, 1995); it will therefore be important to determine how much of the block altered by S6 mutations can be explained by altered kinetics before concluding that the altered residues are located at the binding site itself. In addition, this analysis assumes a single binding site for local anaesthetics with access from the inside. Additional binding sites or alternative access to the inside site (Alpert, Fozzard, Hanck & Makielski, 1989; Ragsdale, McPhee, Scheuer & Catterall, 1994; Qu, Rogers, Tanada, Scheuer & Catterall, 1995) have also been suggested, which would provide additional mechanisms by which mutations can affect local anaesthetic block.

Clinical implications

Lidocaine analogues such as mexiletine and tocainide have been reported to have been effective in treating symptoms in some cases (reviewed by Rüdel *et al.* 1993). Our data predict that such drugs might be more effective for patients with the underlying defect at R1448 than for those with the defect at T1313. Although absolute affinities of drugs for the kinetic states of the Na^+ channel may have some clinical relevance, they are primarily of biophysical interest. Resting potential of muscle is much less hyperpolarized than the holding potentials used in our experiments. Thus interactions with the inactivated state, and the shifts in inactivation induced by the mutants, are likely to be important. Detailed evaluation of the other (>10) mutations in hSkM1 that cause muscle diseases will undoubtedly show similar heterogeneity in response to these drugs, accounting for the clinical response, and may also provide insight into the molecular mechanisms for the interaction of local anaesthetics with the sodium channel.

ALPERT, L. A., FOZZARD, H. A., HANCK, D. A. & MAKIELSKI, J. C. (1989). Is there a second external lidocaine binding site on mammalian cardiac cells? *American Journal of Physiology* **257**, H79–84.

BEAN, B. P., COHEN, C. J. & TSIEN, R. W. (1983). Lidocaine block of cardiac sodium channels. *Journal of General Physiology* **81**, 613–642.

BENNETT, P. B., VALENZUELA, C., CHEN, L. Q. & KALLEN, R. G. (1995). On the molecular nature of the lidocaine receptor of cardiac Na^+ channels – Modification of block by alterations in the Alpha-subunit III-IV interdomain. *Circulation Research* **77**, 584–592.

CATTERALL, W. A. (1995). Structure and function of voltage-gated ion channels. *Annual Review of Biochemistry* **64**, 493–531.

CHAHINE, M., CHEN, L., BARCHI, R., KALLEN, R. & HORN, R. (1992). Lidocaine block of human heart sodium channels expressed in *Xenopus* oocytes. *Journal of Molecular and Cellular Cardiology* **24**, 1231–1236.

CHAHINE, M., GEORGE, A. L. JR, ZHOU, M., JI, S., SUN, W., BARCHI, R. L. & HORN, R. (1994). Sodium channel mutations in paramyotonia congenita uncouple inactivation from activation. *Neuron* **12**, 281–294.

DURELL, S. R. & GUY, H. R. (1992). Atomic scale structure and functional models of voltage-gated potassium channels. *Biophysical Journal* **62**, 238–250.

GEORGE, A. L., KOMISAROF, J., KALLEN, R. G. & BARCHI, R. L. (1992). Primary structure of the adult human skeletal muscle voltage-dependent sodium channel. *Annals of Neurology* **31**, 131–137.

GOLDIN, A. L. (1994). Voltage-gated sodium channels. In *CRC Handbook of Receptors and Channels*, vol. II, *Ligand- and voltage-gated ion channels*, ed. NORTH, R. A., pp. 73–112. CRC Press, Boca Raton, FL, USA.

HANCK, D. A., MAKIELSKI, J. C. & SHEETS, M. F. (1994). Kinetic effects of quaternary lidocaine block of cardiac sodium channels: A gating current study. *Journal of General Physiology* **103**, 19–43.

HILLE, B. (1977). Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor reaction. *Journal of General Physiology* **69**, 497–515.

HILLE, B. (1992). *Ionic Channels of Excitable Membranes*, 2nd edn. Sinauer Associates, Sunderland, MA, USA.

HONDEGHEM, L. M. & KATZUNG, B. G. (1977). Time- and voltage-dependent interaction of antiarrhythmic drugs with cardiac sodium channels. *Biochimica et Biophysica Acta* **472**, 373–398.

McDonald, T. V., Courtney, K. R. & Clusin, W. T. (1989). Use-dependent block of single sodium channels by lidocaine in guinea pig ventricular myocytes. *Biophysical Journal* **55**, 1261–1266.

McPhee, J. C., Ragsdale, D. S., Scheuer, T. & Catterall, W. A. (1995). A critical role for transmembrane segment IVS6 of the sodium channel Alpha subunit in fast inactivation. *Journal of Biological Chemistry* **270**, 12025–12034.

Makielski, J. C., Limberis, J. T., Chang, S. Y., Fan, Z. & Kyle, J. N. (1996). Coexpression of beta-1 with cardiac sodium channel alpha subunits in oocytes decreases lidocaine block. *Molecular Pharmacology* **49**, 30–39.

Makielski, J. C., Satin, J. & Fan, Z. (1993). Post-repolarization block of cardiac sodium channels by saxitoxin. *Biophysical Journal* **65**, 790–798.

Nuss, H. B., Tomaselli, G. F. & Marban, E. (1995). Cardiac sodium channels (hH1) are intrinsically more sensitive to block by lidocaine than are skeletal muscle (Mu1) channels. *Journal of General Physiology* **106**, 1193–1209.

Qu, Y. S., Rogers, J., Tanada, T., Scheuer, T. & Catterall, W. A. (1995). Molecular determinants of drug access to the receptor site for antiarrhythmic drugs in the cardiac Na^+ channel. *Proceedings of the National Academy of Sciences of the USA* **92**, 11839–11843.

Ragsdale, D. S., McPhee, J. C., Scheuer, T. & Catterall, W. A. (1994). Molecular determinants of state-dependent block of Na^+ channel by local anesthetics. *Science* **265**, 1724–1728.

Rüdel, R., Ricker, K. & Lehmann-Horn, F. (1993). Genotype-phenotype correlations in human skeletal muscle sodium channel diseases. *Archives of Neurology* **50**, 1241–1248.

Starmer, C. F. & Grant, A. O. (1985). Phasic ion channel blockade: a kinetic model and parameter estimation procedure. *Molecular Pharmacology* **28**, 348–356.

Starmer, C. F., Nesterenko, V. V., Undrovinas, A. I., Grant, A. O. & Rosenshtraukh, L. V. (1991). Lidocaine blockade of continuously and transiently accessible sites in cardiac sodium channels. *Journal of Molecular and Cellular Cardiology* **23**, 73–83.

Yang, N., Ji, S., Zhou, M., Ptacek, L. J., Barchi, R. L., Horn, R. & George, A. L. Jr (1994). Sodium channel mutations in paramyotonia congenita exhibit similar biophysical phenotypes *in vitro*. *Proceedings of the National Academy of Sciences of the USA* **91**, 12785–12789.

Yeh, J. Z. (1978). Sodium inactivation mechanism modulates QX-314 block of sodium channels in squid axons. *Biophysical Journal* **24**, 569–574.

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