Preferred mexiletine block of human sodium channels with IVS4 mutations and its pH-dependence*

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The effects of extracellular pH (6.2, 7.4 and 8.2) and 0.1 mM mexiletine, a channel blocker of the lidocaine type, are studied on two mutations of the fourth voltage sensor of the $Na_v1.4$ sodium channel, R1448H/C. The fast inactivated channel state to which mexiletine preferentially binds is destabilized by the mutations. By contrast to the expected low response of R1448H/C carriers, mexiletine is particularly effective in preventing exercise-induced stiffness and paralysis from which these patients suffer. Our measurements performed in the whole-cell mode on stably transfected HEK cells show for the first time that the mutations strikingly accelerate closed-state inactivation and, as steady-state fast inactivation is shifted to more negative potentials, stabilize the fast inactivated channel state in the potential range around the resting potential. At pH 7.4 and 8.2, the phasic mexiletine block is larger for R1448C (55%) and R1448H (47%) than for wild-type channels (31%) due to slowed recovery from block (τ is approximately 520 ms for R1448C versus 270 ms for wild-type at pH 7.4) although the recovery from inactivation is slightly faster for the mutants (τ is approximately 1.9 ms for R1448C versus 3.8 ms for wild-type at pH 7.4). At pH 6.2, recovery from block is relatively fast (τ is approximately 35 ms for R1448H/C and 14 ms for wild-type) and thus shows no use-dependence. We conclude that enhanced closed-state inactivation expands the concept of a mutation-induced uncoupling of channel inactivation from activation to a new potential range and that the higher mexiletine efficacy in R1448H/C carriers compared to other myotonic patients offers a pharmacogenetic strategy for mutation-specific treatment. *Pharmacogenetics and Genomics* 15:235–244 © 2005 Lippincott Williams & Wilkins.

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Introduction

Gain-of-function mutations of the voltage-gated human sodium channel destabilize channel inactivation and thus lead to an increased sodium inward current that generates additional action potentials. In the heart, this activity results in potentially life-threatening dysrhythmias (long QT syndrome type 3); in skeletal muscle, this repetitive firing of action potentials leads to involuntary muscle contractions (i.e. myotonia as in potassium-aggravated myotonia), sometimes followed by flaccid muscle weakness (hyperkalemic periodic paralysis, paramyotonia congenita); and, in the brain, a persistent sodium current is thought to cause generalized epilepsy with febrile seizures plus other seizure forms ($GEFS^+$) [1]. Inhibitors of voltage-gated sodium channels, such as mexiletine, flecainide and other lidocaine-like drugs, are clinically used in patients with sodium channelopathies caused by gain-of-function mutations [2–4]. These drugs are highly effective as anti-arrhythmics in patients with long QT syndrome type 3 (LQT3) and as antimyotonics in myotonia and paramyotonia congenita patients with Nav1.4 mutations V445, I1160 and R1448 [1,5]. By contrast, patients with hyperkalemic periodic paralysis [6] and paramyotonia patients carrying mutations at positions T1313 or F1473 respond less positively [7,8].

Two types of drug blocks have been described, a low-affinity tonic (or first pulse or resting) block, and a high-affinity phasic block that occurs during repetitive stimulation, and therefore is also called a use-dependent block [9]. Dependence of the block on the channel state appears to be responsible for the different efficacy of the drugs: the low-affinity binding site refers to the resting state whereas the high-affinity binding site refers to the inactivated state. Binding to the fast inactivated state has been consistently reported [9,10]. This binding is strengthened by the hydrophobic domain of drugs such

*This study is dedicated to Dr Kenneth Ricker who identified the particularly beneficial effect of tocainide and mexiletine in paramyotonia congenita patients and who died in 2004.

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as lidocaine (i.e. the aromatic ring which probably interacts with a hydrophobic complex formed by the inactivation particle, IFM, and its receptor). Binding, as well as unbinding from the fast inactivated state, occurs on a slow time scale of several 100 ms [11]. Slow and ultra-slow channel inactivation appear to be prevented by lidocaine by a 'foot in the door' process in the inner vestibule of the pore [12].

The present study aimed to examine the cause of the particularly high mexiletine sensitivity of paramyotonia congenita patients carrying R1448 substitutions. Because all R1448 substitutions (H, C, P, S) reduce the number of IVS4 charges, the IVS4 voltage sensor within the electric field may be displaced and thus alter the fast inactivated channel state [13]. We studied the effects of mexiletine on wild-type and the most frequently occurring R1448 channel mutations, R1448H and R1448C. Because the charges of both R1448H and mexiletine depend on extracellular pH, we determined the mexiletine block at various pH values. Because paramyotonic stiffness followed by weakness occurs during heavy exercise, particularly in a cold environment, low pH is a parameter decisive for the evaluation of antimyotonic treatment. A concentration of 0.1 mM mexiletine was chosen to permit comparison with previous studies [8,14,15].

Methods

Wild-type and mutant α subunit constructs of human skeletal muscle sodium channels were assembled in the mammalian expression vector pRC/CMV and transfected into human embryonic kidney cells (HEK 293) by the Ca^{2+} phosphate precipitation method. Because transient expression was low ($< 10\%$), stable cell lines were obtained by antibiotic selection [16].

The solutions were composed as follows (in mM): the pipette contained 135 CsCl, 5 NaCl, 2 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.4); the control bath solution contained 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 4 dextrose and 5 HEPES. The control solution was prepared at pH values of 6.2, 7.4 and 8.2. Mexiletine (Boehringer Ingelheim, Mannheim, Germany) was added to the control solution at concentration of 0.1 mM. The solutions were freshly prepared and the pH was screened before each experiment.

Standard whole-cell patch-clamp recordings were performed at 20°C (temperature controller II, Luigs & Neumann, Ratingen, Germany) using an EPC-9 patch-clamp amplifier with 'Pulse' software (HEKA, Lambrecht, Germany). Cell dishes were placed between two temperature control elements and a temperature sensor was placed within the solution in the cell dish. The voltage error due to series resistance was below 3 mV. Leakage and capacitive currents were subtracted automatically by a prepulse protocol (−P/4). All data were low pass filtered at 5 kHz and sampled at 20 kHz. The amplitude of sodium currents in non-transfected cells was always below 0.3 nA ($n = 8$). A maximum peak current in transfected cells was up to 20 nA. To minimize both serial resistance and contribution of endogenous sodium channels, data were only recorded from cells with currents of 2–5 nA.

Analysis was based on HEKA and Excel (Microsoft Corporation, Redmond, Washington, USA) software. Student's *t*-test was applied for statistical analysis using SPSS software (SPSS Inc., Chicago, Illinois, USA). $P < 0.01$ was considered statistically significant. Data are provided as mean ± SEM, unless indicated otherwise. Correlation coefficients were calculated according to the Pearson test.

Results

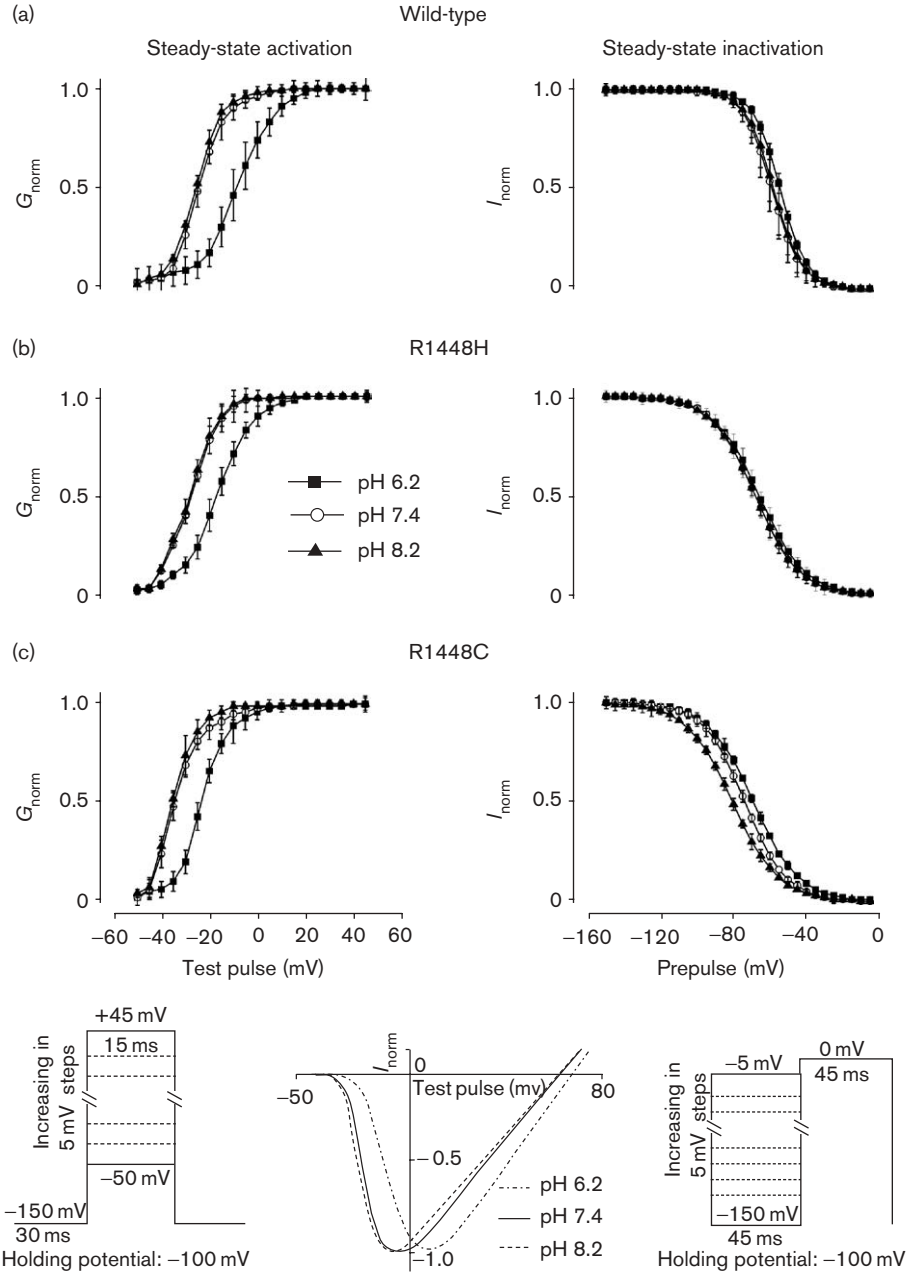
Activation

At all three pH values, the midpoint potentials $V_{m,0.5}$ of the steady-state activation curves were more negative for the mutants (R1448C > R1448H) than for wild-type channels (Fig. 1, left; significant differences marked by asterisks in Table 1). For the three channel types, a pH reduction from 7.4 to 6.2 caused a right-shift to less negative potentials of the activation curve (Fig. 1, left; significant differences marked by open circles in Table 1). An example of the underlying shift of the voltage–current relationship is given in Fig. 1 (bottom): the panel also shows a shift of the reversal potential pointing to an altered ion selectivity of the channels (Fig. 1, bottom) which is in accordance with a decrease of the current amplitude by some 50% (not shown). By contrast, an increase from 7.4 to 8.2, corresponding to a much smaller proton concentration change, caused no significant left-shift of the curve.

Steady-state, closed-state and open-state inactivation and pH effects

For all pH values, the steepness of the steady-state inactivation curves was significantly reduced for the mutant channels compared to wild-type, and $V_{h,0.5}$ was shifted to the left (e.g. −12 mV for R1448H and −17 mV for R1448C relative to wild-type) (Table 1, Fig. 1, right). To test whether this voltage-shift of the steady-state fast inactivation of mutant channels resulted from a kinetic change of closed-state inactivation or from recovery or both, we determined the time constants for the mutant which showed the largest shift (i.e. R1448C at voltages around the resting membrane potential; negative to −65 mV). In addition, open-state inactivation was determined for comparison with earlier reports and with closed-state inactivation. The latter was determined at pH 7.4 in a potential range at which no activation occurred (i.e. negative to −70 mV) and open-state inactivation in the potential range of −65 to +20 mV.

Fig. 1



Steady-state activation and inactivation of wild-type and mutant channels at various pH. The voltage dependences of steady-state activation and inactivation are shown for wild-type, R1448H and R1448C channels at various pH values (6.2, 7.4 and 8.2). The left panels show the activation curves which indicate the fraction of channels that is activated by depolarization from a certain resting potential to various test potentials. The right panels show the inactivation curves that reflect the voltage dependence of the maximal fraction of channels available for activation. Conductance G and current I were normalized to the maximal amplitude of the respective experiment and fit to the Boltzmann equation G/G_{max} or $I/I_{max} = 1/(1 + \exp[(V - V_{0.5})/k])$ to estimate the number of equivalent gating charges $Q = -RT/k$ transferred during gating. The relationships are characterized by $V_{0.5}$ and the slope factor k which is inverse to the steepness of the curve (for exact values, see Table 1). Bottom: the left panel shows the pulse protocol of activation, the right panel that of inactivation and, in the middle, fit curves of the wild-type channel voltage–current relationship are given for the three pH values. The reversal potentials correspond to the highly positive voltages at which the currents are zero.

Closed-state inactivation was found to be strikingly accelerated compared to wild-type ($\tau = 6.9 \pm 0.5$ ms for R1448C and 23.0 ± 1.4 ms for wild-type at -90 mV), suggesting facilitated closed-to-closed channel state transition in this negative potential range (Fig. 2a, left part of the curve). As in earlier studies, open-state inactivation was markedly slowed compared to wild-type ($\tau = 2.11 \pm 0.14$ ms for R1448C and 0.40 ± 0.03 ms for

Table 1 Steady-state activation and inactivation parameters of sodium channels at various pH

Channel type	$V_{0.5}$ k	pH control solution			pH mexiletine solution		
		6.2	7.4	8.2	6.2	7.4	8.2
Activation							
Wild-type	$V_{m,0.5}$	−9.3 ± 2†	−18.6 ± 2	−19.7 ± 3	−9.6 ± 1†	−17.8 ± 2	−20.3 ± 3
	k	−6.9 ± 1	−6.9 ± 1	−7.6 ± 2	−6.2 ± 1	−7.2 ± 1	−5.2 ± 1
R1448H	$V_{m,0.5}$*	−16.5 ± 3†	−24.1 ± 3	−26.8 ± 4	−14.3 ± 3†	−25.6 ± 2	−26.7 ± 3
	k	−7.3 ± 2	−7.8 ± 1	−8.7 ± 1	−6.5 ± 1	−8.2 ± 1	−7.9 ± 1
R1448C	$V_{m,0.5}$*	−22.9 ± 4†	−31.1 ± 3	−33.8 ± 5	−21.3 ± 2†	−32.6 ± 2	−33.7 ± 4
	k	−8.1 ± 1	−9.6 ± 1	−8.5 ± 1	−8.6 ± 1	−10.2 ± 1	−9.4 ± 1
Inactivation							
Wild-type	$V_{h,0.5}$	−54.2 ± 1	−55.6 ± 5	−55.7 ± 4	−54.6 ± 2	−60.5 ± 1	−67.3 ± 4
	k	5.2 ± 0.3	4.9 ± 0.5	5.3 ± 0.4	3.6 ± 0.3	3.2 ± 0.7	3.9 ± 0.8
R1448H	$V_{h,0.5}$*	−65.8 ± 2	−67.9 ± 3	−68.1 ± 2	−67.3 ± 2	−74.2 ± 2	−75.9 ± 2
	k	7.8 ± 0.5	7.5 ± 0.4	8.2 ± 0.7	5.2 ± 0.7	4.9 ± 0.6	6.4 ± 0.4
R1448C	$V_{h,0.5}$*	−70.5 ± 1	−73.1 ± 1	−80.5 ± 3	−74.1 ± 2	−79.4 ± 1	−86.3 ± 1
	k	8.5 ± 1.2	9.5 ± 2	8.6 ± 1	6.8 ± 0.9	6.9 ± 1	7.5 ± 1.2

For each channel type and each pH, at least six cells were analysed. $V_{0.5}$ = midpoint potentials (mV) of (in)activation curves, k = slope factor (mV) inverse to steepness.
†Values at pH 6.2 significantly different from those for pH 7.4 and 8.2.
*Mutation values significantly different from wild-type.

wild-type at 0 mV), suggesting impaired open-to-closed channel state transition in this less negative potential range (Fig. 2a, right part of the curve). Recovery from closed-state inactivation was the same as that from open-state inactivation (i.e. slightly accelerated), suggesting a destabilized inactivated state ($\tau = 1.9$ ms for R1448C and $\tau = 3.7$ ms for wild-type at −100 mV; see also Table 2). Because the transition from the resting closed to the inactivated closed state was much more facilitated than the backward transition destabilized, it is reasonable to assume that closed-state inactivation is stabilized in the mutant channels. The left-shift of the steady-state inactivation curve is in agreement with this assumption. By contrast, slowed open-state inactivation and accelerated recovery from inactivation lead to the well known destabilization of the fast inactivated channel state in the potential range at which channel activation occurs. In addition, the time course of open-state inactivation was determined for all three channel types at pH 6.2 and 8.2 (Fig. 2b). R1448H revealed a distinct pH dependence: at pH 6.2, it behaved somewhat like wild-type but, at pH 8.2, more like R1448C. For R1448C, a flattening of the voltage-dependence curve was observed (Fig. 2b) which was previously interpreted as uncoupling of inactivation from activation [17].

Mexiletine effects on steady-state activation and inactivation

Mexiletine had no effect on the voltage dependence of steady-state activation (Table 1). By contrast, it significantly reduced the steepness of the steady-state inactivation curves of all three channel types and shifted $V_{h,0.5}$ to the left, at least at pH 7.4 (Table 1, Fig. 3). Whereas the effect of mexiletine on steady-state inactivation was not significant for any of the channels at pH 6.2, the left-shift at pH 8.2 depended on the channel ($n = 7$ cells for each type): −5.8 mV for R1448C, −7.8 mV for R1448H and −11.6 mV for wild-type. Mexiletine had no effect on the kinetics of inactivation (data not shown).

Mexiletine-induced first pulse block on wild-type and mutant channels and its pH dependence

For the determination of the first pulse or resting block, we had to choose a holding potential at which no channels were in the inactivated state, otherwise the resulting block could have been attributed to the binding of mexiletine to the inactivated state. According to the results shown in Table 1 (Fig. 3), we decided to hold the membrane potential at −150 mV, a value at which also all R1448C channels are in the resting state. At this holding potential, mexiletine reduced the peak sodium current elicited by the first stimulus. The effect was minimal at pH 6.2 for all three channel types. It was larger at pH 7.4 and further increased at pH 8.2 (Figs 4a–c and 5). At a holding potential of −100 mV, the resulting block was over twice as high under all conditions indicating that a portion of the channels underwent closed-state inactivation (Figs 4d and 5).

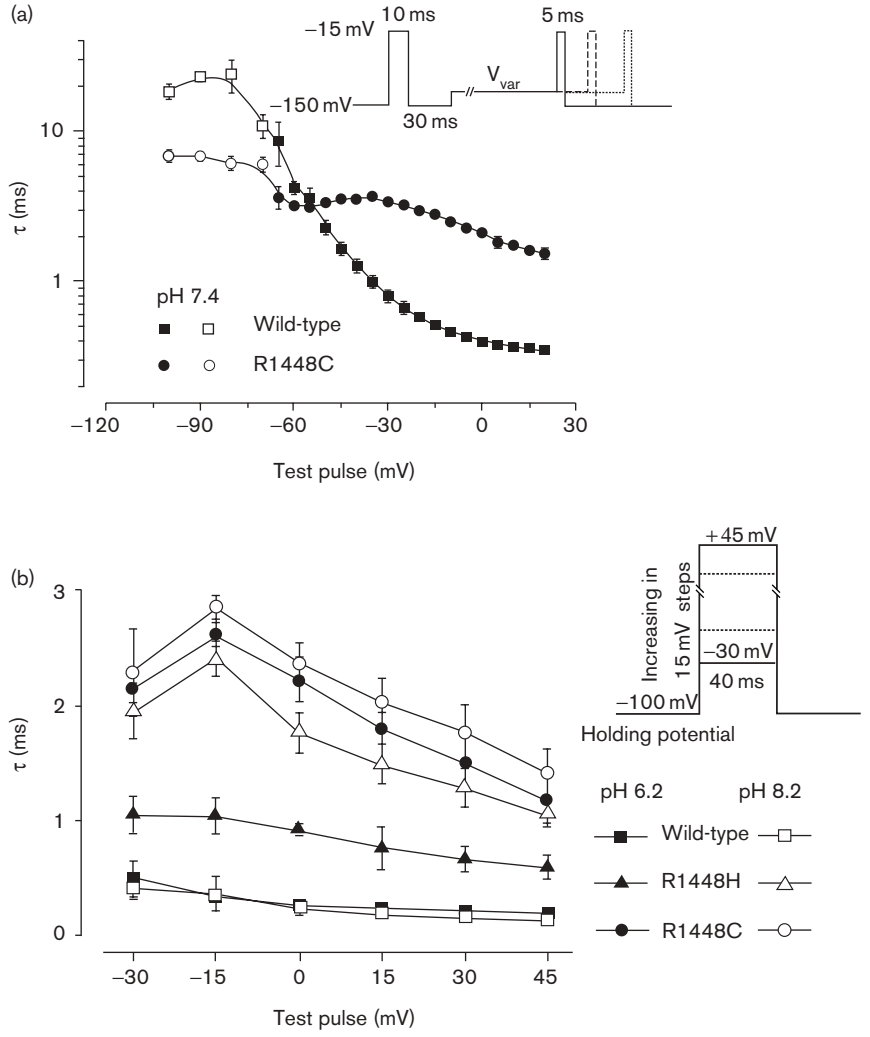
Mexiletine-induced phasic block on wild-type and mutant channels and its pH dependence

In the presence of mexiletine, current amplitudes within a train always decreased from pulse to pulse (Fig. 6), whereas this was not observed without mexiletine (data not shown). For all three channel types, the decrease of the current amplitude was more prominent at pH 7.4 and 8.2 compared to 6.2. A quantitative analysis of the phasic block, induced at a holding potential of −100 mV, confirmed the significant difference between pH 6.2 and 7.4 for all channel types (Table 3). A further increase in pH to 8.2 had no significant effect. As already shown for the first pulse block, the phasic block was most effective for R1448C channels (i.e. channels with pronounced closed-state inactivation).

The pH effects on the recovery from inactivation and block from mexiletine

In the absence of mexiletine, the time course of recovery from inactivation was best fit to a mono-exponential

Fig. 2



Gating kinetics of the three channel types at various pH values. The time constants τ for various transitions were plotted against the corresponding membrane potentials. (a) Closed-state and open-state inactivation were determined for wild-type and R1448C channels in control solution at pH 7.4. The time constants of closed-state inactivation (−100 mV to −70 mV, left part of the diagram) were obtained by the two-step protocol as shown in the inset in (a). The time constants of open-state inactivation (−65 mV to +20 mV, right part of the diagram) were studied by the same standard protocol as shown in the inset in (b) and determined by a mono-exponential fit to the decay of the normalized current. (b) Open-state inactivation was measured for wild-type, R1448H and R1448C channels in control solution at two pH values (6.2 and 8.2). Note that open-state inactivation of the mutant channels is slower but closed-state inactivation is faster compared to wild-type channels.

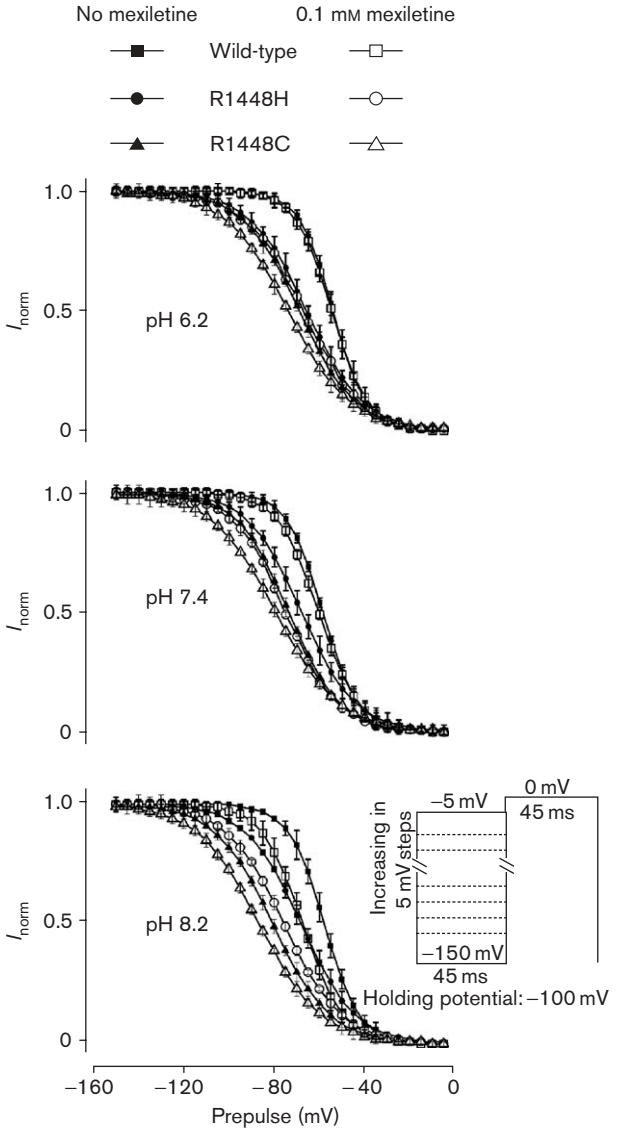
Table 2 Time constants τ (ms) of recovery from inactivation at −100 mV and various pH with and without mexiletine

Channel type	τ_{rec}			$\tau_{rec,fast}$			$\tau_{rec,slow}$		
	pH of control solution			pH of mexiletine solution					
	6.2	7.4	8.2	6.2	7.4	8.2	6.2	7.4	8.2
Wild-type	2.86 ± 0.5	3.74 ± 0.7	5.20 ± 0.4	2.9 ± 0.5	3.81 ± 0.5	5.32 ± 0.4	14 ± 2	268 ± 31	317 ± 35
R1448H	2.68 ± 0.5	2.86 ± 0.1	3.41 ± 0.3	2.7 ± 0.8	2.89 ± 0.2	3.51 ± 0.2	36 ± 4	431 ± 30	418 ± 24
R1448C	1.29 ± 0.2	1.91 ± 0.3	2.71 ± 0.2	1.4 ± 0.4	1.88 ± 0.4	2.51 ± 0.3	34 ± 3	522 ± 43	439 ± 57

Except for the difference between wild-type and R1448H at pH 6.2, all others were statistically significant. For each channel type and each pH, at least six cells were analysed.

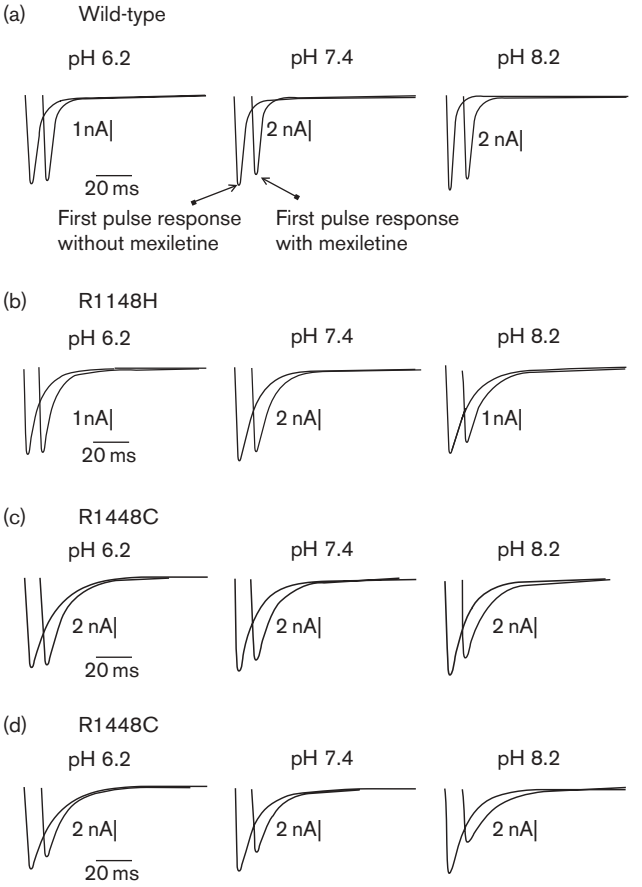
function (Fig. 7, Table 2). Its time constant τ_{rec}, significantly increased with higher pH values for all three channel types. At pH 7.4, recovery from inactivation was slightly but significantly faster for R1448C (τ_{rec} = 1.9 ms) and R1448H (τ_{rec} = 2.9 ms) than for wild-type (τ_{rec} = 3.7 ms). The same was true for pH 8.2; however, at pH 6.2, only recovery of R1448C channels was significantly faster than that of the wild-type.

Fig. 3



Steady-state inactivation at various pH in the absence and presence of mexiletine. The voltage dependences of inactivation are shown for wild-type, R1448H and R1448C channels in the absence (filled symbols) and presence of 0.1 mM mexiletine (open symbols) at pH 6.2 (upper panel), 7.4 (middle panel) and 8.2 (lower panel). The steady-state inactivation was determined by the two-pulse protocol shown in the inset: the prepulse depolarized the cell membrane for 45 ms from a holding potential of −150 mV to up to −5 mV in steps of 5 mV. The following test pulse always depolarized the membrane to zero. The currents were normalized to the maximal amplitude of the respective experiment and fit to the Boltzmann equation.

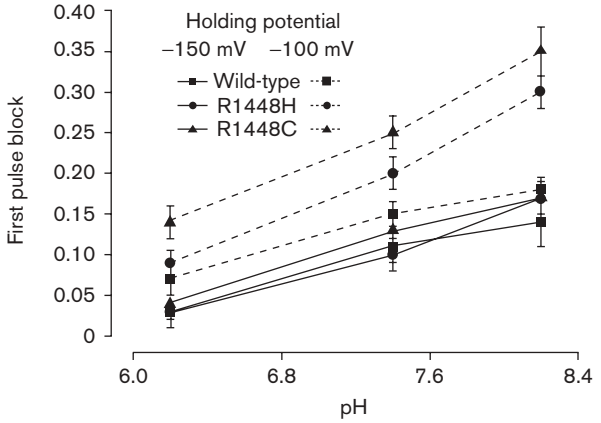
Fig. 4



Sodium currents at various extracellular pH with and without mexiletine. Representative whole-cell current traces of (a) wild-type, (b) R1448H and (c) R1448C sodium channels at pH values of 6.2, 7.4 and 8.2 of the extracellular solution. The first trace of each current twin is measured in control solution and the second trace under application of 0.1 mM mexiletine. The currents are activated by depolarizing test pulses from −150 mV to 0 mV, each lasting 40 ms. By contrast, currents which are elicited by depolarizing test pulses from −100 mV to 0 mV are much more reduced by mexiletine as shown for R1448C (d). The values for the relative reduction of the current peaks by mexiletine are given in Table 3.

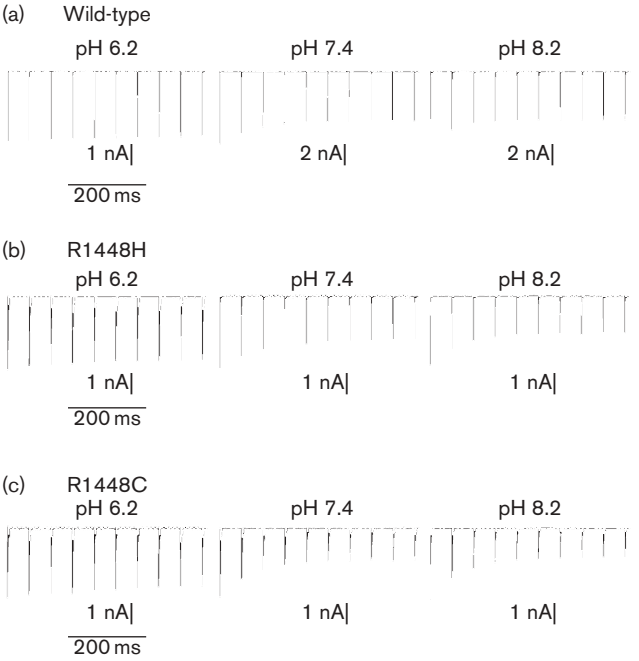
In the presence of mexiletine, the time course of recovery of all three channel types from inactivation and block showed the best fit to a double-exponential function at all three pH values (Fig. 7, Table 2). The fast component $\tau_{rec,fast}$ was not different from τ_{rec} determined in the absence of the drug. The second time constant $\tau_{rec,slow}$, which corresponded to the recovery from mexiletine block, was more than 100-fold greater than $\tau_{rec,fast}$ at pH 7.4 and 8.2 and 10-fold greater than $\tau_{rec,fast}$ at pH 6.2. The relative number of channels which were blocked and unblocked depended on the pH and the channel type (Table 3). At pH 7.4, only the minority of wild-type channels was blocked by mexiletine and recovered from block in the presence of mexiletine. By contrast, the majority of mutant channels was blocked. At pH 8.2, the portion of blocked and recovered channels was slightly greater than at 7.4 but the differences were not statistically significant. At pH 6.2, the slight delay of the recovery curves suggests some efficacy of mexiletine being in agreement with the phasic block in Fig. 6, but the much faster recovery compared to that at higher pH points to a different type of block.

Fig. 5



Mexiletine-induced blocks at various pH and two holding potentials. The channel block induced by 0.1 mM mexiletine is plotted against the extracellular pH. According to the steady-state inactivation curves shown in Fig. 1, all channels remain in the resting state at a holding potential of −150 mV. The resulting first pulse block (solid lines) (i.e. a true resting block) showed statistically significant differences for the three pH values, but no differences between the three channel types at a given pH. By contrast, at a holding potential of −100 mV, some channels were in the inactivated state as shown in Fig. 1. The resulting first pulse block (dotted line) is much more pronounced at this more physiological membrane potential, indicating that much more mexiletine is bound during closed-state inactivation than in the resting state [see also Fig. 4(d) versus 4(c)]. The block showed statistically significant increases with pH elevation for all three channel types, except the step from 7.4 to 8.4 for the wild-type channel, which behaves as if in the resting state at this holding potential.

Fig. 6



Currents trains of normal and mutant channels exposed to mexiletine at various pH. Whole-cell currents were activated by a train of 10 brief depolarization steps from −100 mV to 0 mV applied at a frequency of 10 Hz. Original current traces of (a) wild-type, (b) R1448H and (c) R1448C channels are shown at various pH values (6.2, 7.4 and 8.2) in a solution containing 0.1 mM mexiletine.

Discussion

Effects of histidine and cysteine 1448 on channel gating

By contrast to several reports on a destabilization of the fast inactivated state of R1448 substitutions [17–20], we report an enhanced inactivation of R1448H/C channels in a potential range which has not been studied before. This potential range concerns potentials between −100 and −60 mV and is of particular physiological importance for the inactivation from the resting closed to the inactivated closed state. This novel result fits to the concept of a mutation-induced uncoupling of channel inactivation from activation and expands it over an additional potential range (Fig. 2). The previously published results concerning open-state inactivation, and recovery from it, are confirmed in this study: Compared to the wild-type channel: (i) the R1448 mutants slow fast inactivation from the open state and reduce the voltage dependence of inactivation kinetics; (ii) the R1448 mutants accelerate recovery from the inactivated state; and (iii) the R1448 mutants reduce steepness of the steady-state fast inactivation curve of fast inactivation. For these three changes, R1448C exerts stronger effects than R1448H. The reduced voltage dependence of the inactivation kinetics and the steady-state fast inactivation may result from the reduction of the fourth voltage sensor charge which, in contrast to all other sensors, is important for the transition along the activation pathway [21] and/or for the transitions to fast inactivated states.

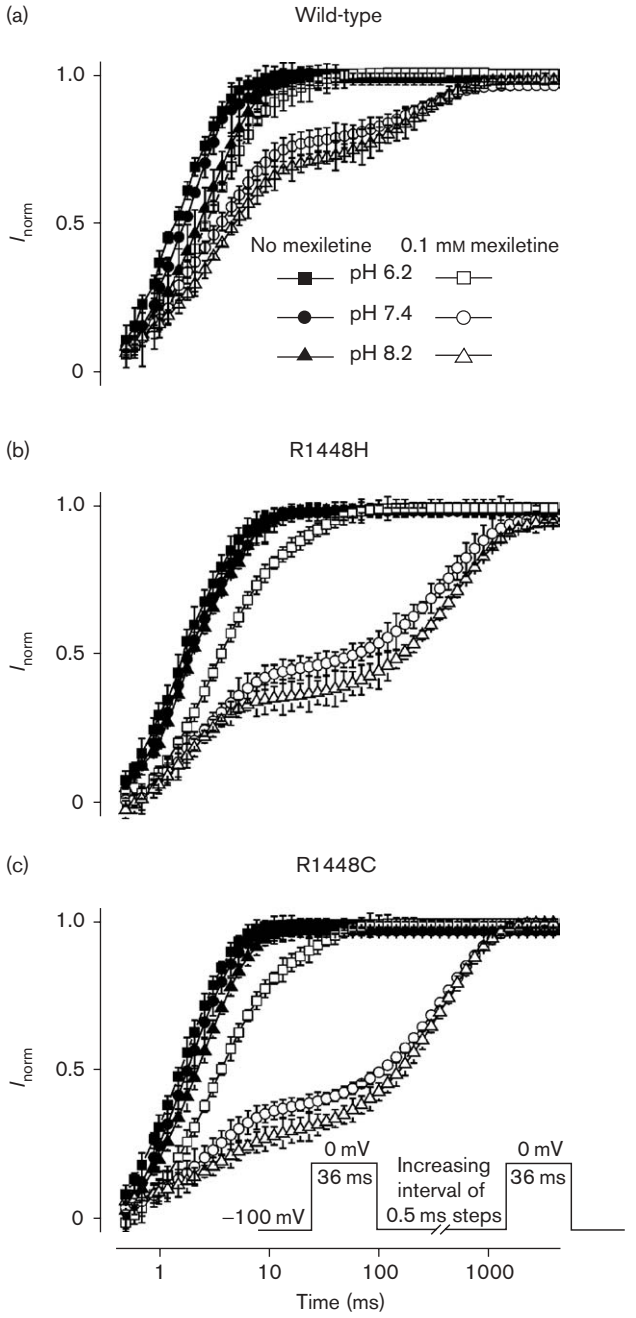
Table 3 Comparison of various mexiletine blocks at various pH values

Channel type	Holding potential	pH of solution containing mexiletine		
		6.2	7.4	8.2
Reduction of first current peak amplitude (%)				
Wild-type	−150 mV	3 ± 2	11 ± 2	14 ± 3
R1448H		3 ± 1	10 ± 2	17 ± 2
R1448C	−100 mV	4 ± 1	13 ± 2	17 ± 2
		14 ± 2	25 ± 2	35 ± 3
Reduction of current peak amplitude due to phasic block (%)				
Wild-type	−100 mV	6 ± 2	32 ± 4	31 ± 5
R1448H		11 ± 2	46 ± 2	48 ± 2
R1448C		17 ± 2	54 ± 3	56 ± 3
Percentage of channels with slow recovery from inactivation (i.e. from block)				
Wild-type	−100 mV	3 ± 1	33 ± 6	34 ± 4
R1448H		6 ± 3	57 ± 5	63 ± 3
R1448C		8 ± 2	58 ± 4	64 ± 4

Effects of extracellular pH on channel gating

The time constant of open-state inactivation of R1448H resembled, at low pH, the protonated wild-type and, at high pH, the unprotonated R1448C. Thus, the histidine

Fig. 7



Recovery from inactivation at various pH with and without mexiletine. The current amplitudes were measured in double pulse experiments in which the interval between the two depolarization pulses was increased stepwise. The recovery is shown for (a) wild-type, (b) R1448H and (c) R1448C channels in control solution and under application of 0.1 mM mexiletine at various pH values (6.2, 7.4 and 8.2). The amplitudes were normalized to the largest current of the corresponding test. The time course of recovery from inactivation and block from mexiletine was fit to a double-exponential function with time constants $\tau_{rec,fast}$ and $\tau_{rec,slow}$. Whereas $\tau_{rec,fast}$ was not significantly different from τ_{rec} obtained in the absence of mexiletine, $\tau_{rec,slow}$ was approximately 10-fold (at pH 6.2) or 100-fold higher (at pH 7.4 and 8.2) than $\tau_{rec,fast}$ (Table 2). In addition, the relative amplitude of $\tau_{rec,slow}$ on the total current was significantly greater for mutant than for wild-type channels (Table 3).

residue at position 1448 appears to be in contact with the extracellular fluid and to be protonated at reduced extracellular pH [17]. Our finding that extracellular pH reduction from 7.4 to 6.2 largely shifted the voltage dependence of gating to more positive potentials could be attributed to an altered surface potential due to protonization [22,23]. However, the much less pronounced shift of the steady-state inactivation curve points to a specific alteration of the activation process at pH 6.2. This view is supported by a sodium to calcium selectivity change of the channel and by a striking decrease of the current amplitude by approximately 50%.

The effects of mexiletine on channel gating and the effects of pH on mexiletine-binding

At pH 6.2, 99.9% of the NH_2 groups of mexiletine are protonated (pKa 9.2, Boehringer Ingelheim). At this low pH, the passage through the lipid membrane is almost completely prevented. The degree of the first pulse block correlates well with the portion of inactivated channels (no block at −150 mV, greatest but still slight block at −100 mV for R1448C) indicating a binding of mexiletine to the inactivated state. Its rapid dissociation from the inactivated state might explain why no use-dependent block can occur.

At pH 8.2, 10% of the molecules are uncharged and gain access to the pore receptor site via the lipid bilayer. Again, the degree of the first pulse block correlates well with the portion of inactivated channels. This channel block by mexiletine binding to the inactivated state is in agreement with previous studies [7–9,14,15,24–26]. The long time period required for the dissociation of the drug from the inactivated state delays the recovery from inactivation and causes the striking use-dependence of mexiletine. This biphasic process consists of a fast exponential component that corresponds to the recovery from inactivation of mexiletine-free channels and a second exponential component of channels from which mexiletine dissociates [7,15,24]. The level of the plateau between the phases depends on the fraction of channels in the bound and unbound state. In agreement with the shifts of the steady-state inactivation curves, the fraction of channels in the bound state was in the order R1448C > R1448H > wild-type and also 8.2 > 7.4 > 6.2.

Why do the R1448 mutations respond so well to mexiletine?

Based on the examination of closed-state inactivation, we show that the shift of the steady-state fast inactivation curve to more negative values results from entry into fast inactivation in this potential range being much more accelerated than recovery from inactivation. This pattern leads to an enhanced fast inactivation and predisposes to mexiletine binding [11]. This left-shift is not only seen for all R1448 substitutions such as H, C, S and P, but also

for two other myotonia and paramyotonia mutations, V445 M and I1160 V [26,27]. For all of these sodium channel mutations, the carriers respond very well to tocainide or mexiletine [1,2,5,6,28]. Furthermore, the mexiletine block highly correlates with the extent of the left-shift (correlation coefficients are 0.932 for wild-type, 0.965 for R1448H; and 0.978 for R1448C in the Pearson test, including values from all three pH values 6.2, 7.4 and 8.2). Vice versa, all other paramyotonia (T1313 M, T1313A, F1473S, L1433R) and myotonia mutations (V1589 M), which shift the steady-state fast inactivation curve in the opposite direction [16,18,27,29], show little responsiveness to mexiletine [7,8,30]. Carriers of these mutations derive more benefit from flecainide [31,32].

An alternative hypothesis for why some mutations respond so well to mexiletine and similar drugs is the rapid binding to an open state. The high affinity was reported especially for those channels showing flickery transitions between the open and the closed states (i.e. for channels modified either by gain-of-function mutations similar to R1448H/C). Furthermore, channels activated by certain toxins such as batrachotoxin, and channels exposed to low external sodium concentration or those composed of a specific subunit pattern can show this high affinity [33–37]. These re-opening channels cause a persistent sodium current I_{SS}. However, its extent does not correlate well with the clinical mexiletine effect. I_{SS} is small in the drug-responsive R1448H/C mutations [17,18] but large in the less responsive V1589 M mutation [16]. Moreover, I_{SS} is large in all mutations causing hyperkalemic periodic paralysis [1], a disease known not to respond to mexiletine [6].

Therefore, the effect of mexiletine depends on the probability of a channel assuming a certain state or undergoing a certain interstate transition. The type of mutation, the extracellular pH and the membrane potential all determine this probability and thus the effectiveness of treatment even when the binding site remains unaltered. Apparently, sodium channel mutations that reduce a positive charge of the fourth voltage sensor predipose the channel to closed-state inactivation and thereby increase the effect of mexiletine in the mutation carriers. This pattern explains why mexiletine is so effective even though open-state inactivation of the mutant channels is destabilized.

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