Molecular basis for exaggerated sensitivity to mexiletine in the cardiac isoform of the fast Na channel

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Abstract Cardiac sodium channels have been shown to have a higher sensitivity to local anesthetic agents, such as lidocaine, than the sodium channels of other tissues. To examine if this is also true for mexiletine, we have systematically measured mexiletine sensitivity of the Na channel isoforms, rH1, μ1, and rBII, which were transiently expressed in human embryonic kidney (HEK) 293 cells. We confirmed that the cardiac isoform rH1 exhibited the highest sensitivity among the three tested channel isoforms. In rH1, μ1, and rBII, the respective IC_{50} values were 62, 294, and 308 μM mexiletine, in regard to tonic block, and 18, 54, and 268 μM mexiletine, in relation to use (8 Hz)-dependent block. The relatively high drug sensitivity of rH1 was an invariant finding, irrespective of channel state or whether channels were subjected to infrequent or frequent depolarizing stimuli. Mutating specific amino acids in the skeletal muscle isoform μ1 (namely, μ1-I433V and μ1-S251A) to those of the cardiac isoform at putative binding sites for local anesthetic agents revealed that only one of the point mutations (μ1-S251A) has relevance to the high cardiac drug sensitivity, because mexiletine produced significantly more use-dependent and tonic block in μ1-S251A than wild-type μ1. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mexiletine; Na channel; Block; Whole-cell patch clamp; HEK 293

1. Introduction

Mexiletine is an orally effective lidocaine analog classified as a class Ib antiarrhythmic agent. This drug is potentially useful in various clinical settings, because it is effective as an antiarrhythmic [1], anticonvulsant [2], and local anesthetic agent [3]. The use- and voltage-dependent blocking effects of mexiletine explain the suppressive action of mexiletine on neuropathic pain [4] associated with post-traumatic neuromas, post-amputation pain, and diabetic neuropathy [5]. Recently, a neuroprotective effect of mexiletine has been described [6] at drug concentrations which have the potential also to produce side effects on organs that are not targeted, and must therefore be used with caution. The direct effects of mexiletine on various excitable tissues [7] depend on the membrane potential and stimulation frequency [8–10]; thus the organ-specific mexiletine affinity could be largely due to differences in action potential duration and frequency. There may also be substantial differences in the intrinsic drug affinity of the organ-specific Na channel isoforms. However, a direct comparison has yet to be made between the effects of local anesthetics (LAs) on different Na channel isoforms under uniform experimental conditions. Thus, in the present study, our aim was to determine the mexiletine sensitivity of three Na channel isoforms, rH1, rBII and μ1, when expressed in human embryonic kidney (HEK) 293 cells. By analysis of mutated channel isoforms, we further undertook to explain the distinctly higher affinity of mexiletine in the cardiac isoform of the Na channel.

2. Materials and methods

2.1. Transient transfection and cell culture

The Na^+ channels were constructed using three cDNA clones coding the α-subunits of rBII [11], rH1 [12] and μ1 [13]. For introduction of point mutations in domain 1 segment 6 (D1-S6) and domain 1 segment 4-segment 5 (D1S4-S5) intracellular loop, we used PCR-based and site-directed mutagenesis (Promega Corp.). Resulting point mutants were confirmed with restriction mapping and sequencing using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Each cDNA clone was co-transfected with CD8 cDNA transiently into HEK 293 using SuperFect transfection reagent (Qiagen). The cells were grown to 50% confluence in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (Bio-whittaker), 30 units/ml penicillin G (Invitrogen) and 30 μg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Transfection-positive cells were identified by immunobeads (CD8-Dynabeads, Dynal) before the sodium current recording.

2.2. Electrophysiological recording

Macroscopic sodium currents from the transfected cells were measured using the whole-cell variation of the patch clamp method. Electrode resistances ranged from 1 to 2 MΩ. Voltage clamp command pulses were generated by pCLAMP software (v. 7.0; Axon Instruments, Inc.). An Axopatch 200B patch clamp amplifier was used with series resistance compensation. The standard holding potential in all pulse protocols was −120 mV.

In every cell studied, we determined the blocking effects of mexiletine, at a given concentration, and under infrequent and frequent pulsing, according to the standard experimental protocol shown in Fig. 1.

Data are presented as mean ± S.D. Experiments were carried out at

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Abbreviations: HEK, human embryonic kidney; rBII, α-subunit of a rat brain type II Na^+ channel; rH1, α-subunit of a rat cardiac-specific Na^+ channel; μ1, α-subunit of rat skeletal muscle Na^+ channel; LA, local anesthetic

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a bath temperature of 19.0°C rather than at room temperature, thus allowing improved resolution of I_{Na} and greater viability of the cells subjected to whole-cell patch clamp.

2.3. Drugs and solutions

The bath solution contained 70 mM NaCl, 67 mM *N*-methyl-D-glucamine, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose, and 5 mM *N*-2-hydroxyethylpiperazine- *N*′-2-ethanesulfonic acid (HEPES) (pH 7.4). The pipette solution contained 70 mM CsF, 60 mM CsCl, 12 mM NaF, 5 mM ethylene-bis(oxyethylenenitrilo)-tetraacetic acid (EGTA), and 5 mM HEPES (pH 7.4). Mexiletine-hydrochloride was obtained as a gift from Boehringer Ingelheim (Tokyo, Japan).

3. Results

3.1. Voltage-dependent activation and inactivation of wild-type rBII, rH1 and μ1 Na^+ channels

Fig. 2 displays sodium currents through rBII (a), rH1 (b) and μ1 (c) channels expressed in HEK 293 cells, and their current–voltage relationships. Currents through all three Na channel isoforms had reversal potentials of approximately +45 mV, which is in agreement with the theoretical value for E_{Na} (i.e. the reversal potential for Na^+) calculated from the Na concentration of the internal and external solutions used (see Section 2). Compared to both rBII and μ1 isoforms, rH1 Na^+ channels were activated at less depolarized voltages and had a more negative half-activation voltage (Fig. 2d). Na^+ currents through rH1 channels had longer inactivation time constants than rBII and μ1 (see the figure legend for the numerical data). Steady-state inactivation curves were obtained with a standard double-pulse protocol using a 500-ms prepulse duration. The rH1 sodium channel isoform was inactivated at more negative transmembrane potentials than the other two isoforms. The half-inactivation potentials ($V_{0.5i}$) and slope factors (k) were -94.0 ± 0.2 mV and -6.7 ± 0.2 for rH1 ($n = 7$), -72.1 ± 0.1 mV and -5.4 ± 0.1 for rBII ($n = 7$), and -82.5 ± 0.1 mV and -6.0 ± 0.1 for μ1 ($n = 7$), respectively.

3.2. Use-dependent block

Use-dependent block is a key feature of LAs such as lidocaine and mexiletine, i.e. their blocking effect is enhanced as the frequency of depolarizing pulses is increased. We compared the three Na channel isoforms with respect to the use-dependent block produced by application of mexiletine at depolarization frequencies of 1, 2, 4, or 8 Hz. Fig. 3 depicts the use-dependent block caused by mexiletine at a frequency of 8 Hz. Mexiletine blocked all three sodium channel isoforms in a concentration-dependent manner. The concentration dependence of the block, at each of the stimulus frequencies employed, has been summarized in Fig. 4 for all three isoforms. IC_{50} values, obtained by fitting the equation $1/(1+([\text{mexiletine}]/IC_{50}))$ to these data, are listed in Table 1. At any given pulsing frequency, the order of drug sensitivity for the three isoforms was (from highest to lowest) rH1 > μ1 > rBII.

3.3. Tonic block

Use-dependent block of LAs has been explained by the modulated receptor hypothesis [9,10,14], according to which

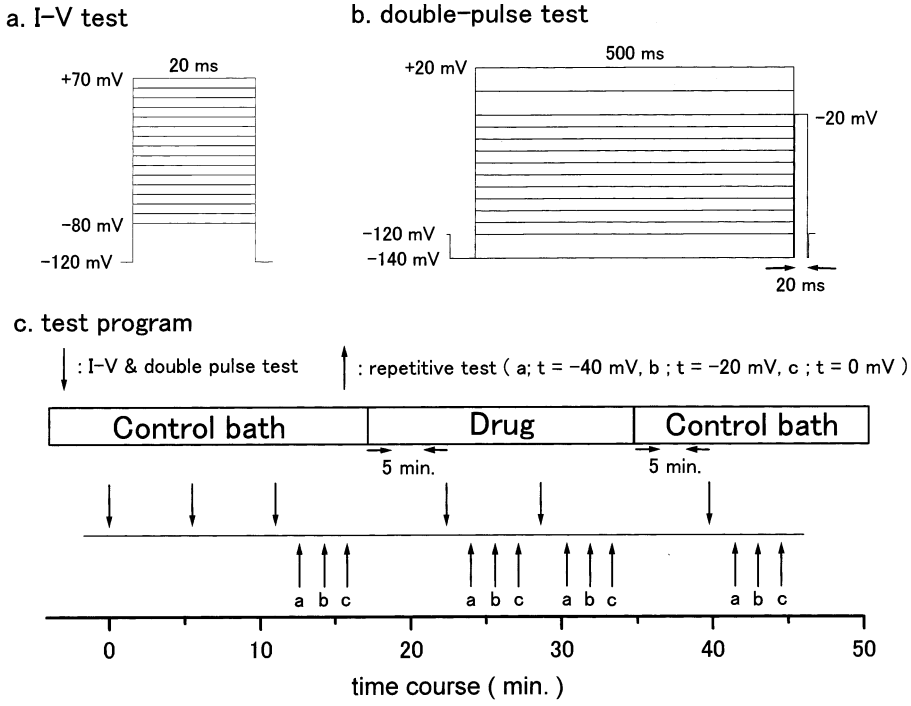

Fig. 1. Experimental protocols used throughout this study. The pulse protocols used are shown in a and b. a: Current–voltage (*I–V*) relationship for I_{Na} was obtained by delivering, at 1 Hz, 20-ms depolarizing pulses to membrane potentials between −80 mV and +70 mV, from a holding potential of −120 mV. In the repetitive pulsing mode, we stimulated cells at 1, 2, 4, or 8 Hz with a depolarizing pulse train of 5 s to one of three fixed potentials (−40, −20 and 0 mV). b: The voltage dependence of steady-state Na inactivation was assessed by the double-pulse protocol. 500-ms conditioning prepulses of various amplitudes were applied from a potential of −140 mV followed by a 20-ms test pulse to −20 mV. Pulse pairs were applied every 5 s; holding potential was −120 mV. c: Diagram showing the overall experimental procedure. The *I–V* and double-pulse protocols were given sequentially where indicated (downward arrows). Trains of repetitive pulses to different test potentials (a = −40 mV, b = −20 mV, and c = 0 mV) were given, as shown (upward arrows). Drugs were administered between 17 and 35 min from start of experiments. The first *I–V* protocol was carried out 5 min after the beginning of drug perfusion.

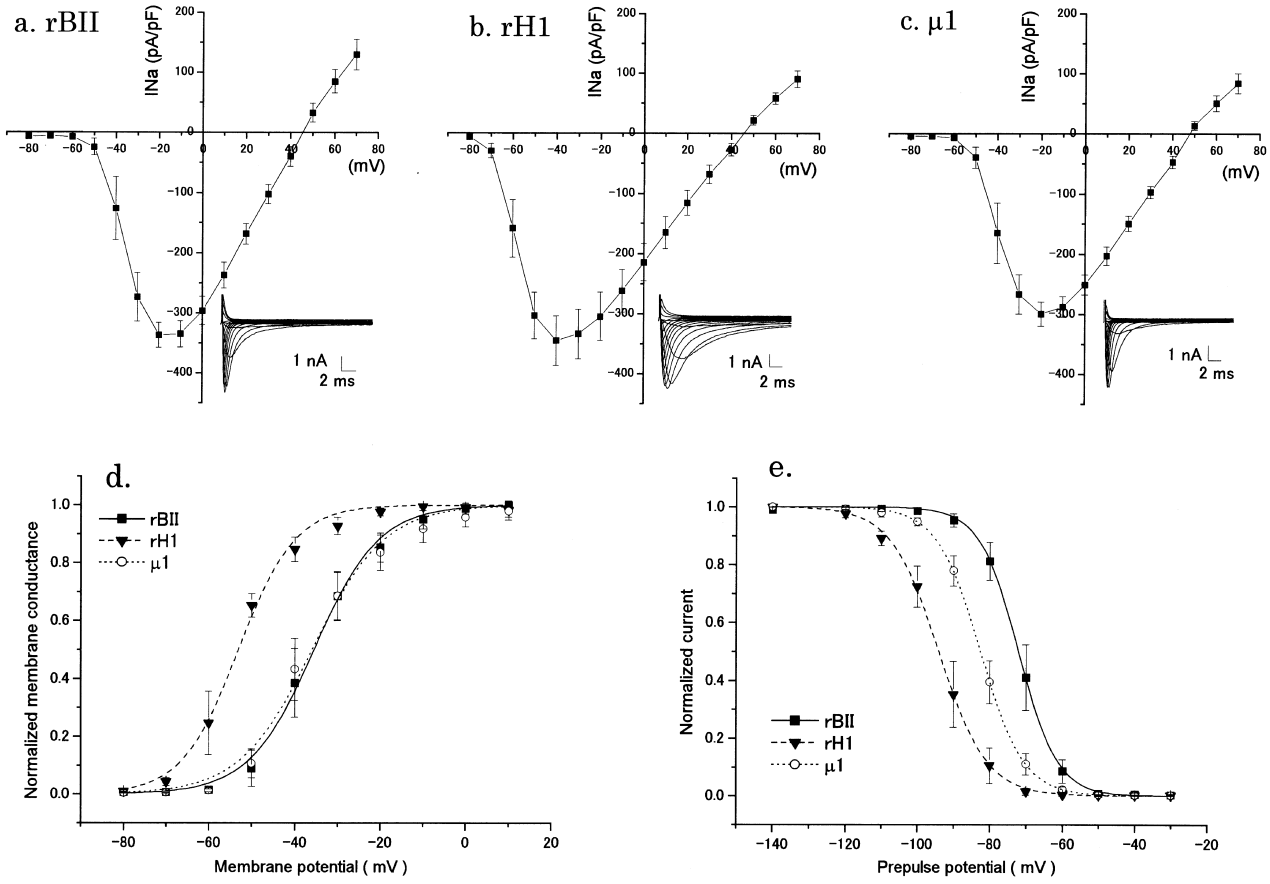

Fig. 2. Voltage-dependent properties of the three Na channel isoforms, rBII, rH1, and μ1. The *I–V* relationships for currents through rBII, rH1, and μ1 channels are shown in a, b, and c, respectively. Data are expressed as mean (■) ± S.D. (error bars). Current records for each Na^+ channel isoform are shown in the insets. The inactivation phase of the currents are composed of two time constants, namely τ_{fast} and τ_{slow} (at −20 mV, $\tau_{fast} = 0.63 \pm 0.086$ ms and $\tau_{slow} = 2.4 \pm 1.1$ ms for rBII, $n = 16$; $\tau_{fast} = 0.81 \pm 0.18$ ms and $\tau_{slow} = 3.4 \pm 0.83$ ms for rH1, $n = 12$; $\tau_{fast} = 0.58 \pm 0.070$ ms and $\tau_{slow} = 2.2 \pm 0.93$ ms for μ1, $n = 15$). d: Normalized activation curves are shown for each isoform. The data points (mean ± S.D.) have been fitted with a Boltzmann function $(1/[1+\exp((V_{0.5a}-V)/k)])$, where $V_{0.5a}$ is the half-activation voltage, and k is the slope factor. $V_{0.5a}$ and k were −35.6 mV and 8.0, −36.0 mV and 8.4, and −55.6 mV and 6.8 for rBII ($n = 7$), μ1 ($n = 7$), and rH1 ($n = 7$), respectively. e: Steady-state inactivation (h_∞) curves for rBII, rH1, and μ1 obtained by the double-pulse protocol. Normalized peak Na^+ currents during the test pulse are plotted against the potential of the conditioning prepulse. The data points (mean ± S.D.) have been fitted with a Boltzmann function $(1/[1+\exp((V-V_{0.5i})/k)])$, where $V_{0.5i}$ is the half-inactivation voltage, and k the slope factor. $V_{0.5i}$ and k were −72.7 mV and 6.0, −83.3 mV and 5.6, and −99.7 mV and 7.1 for rBII ($n = 7$), μ1 ($n = 7$), and rH1 ($n = 7$), respectively.

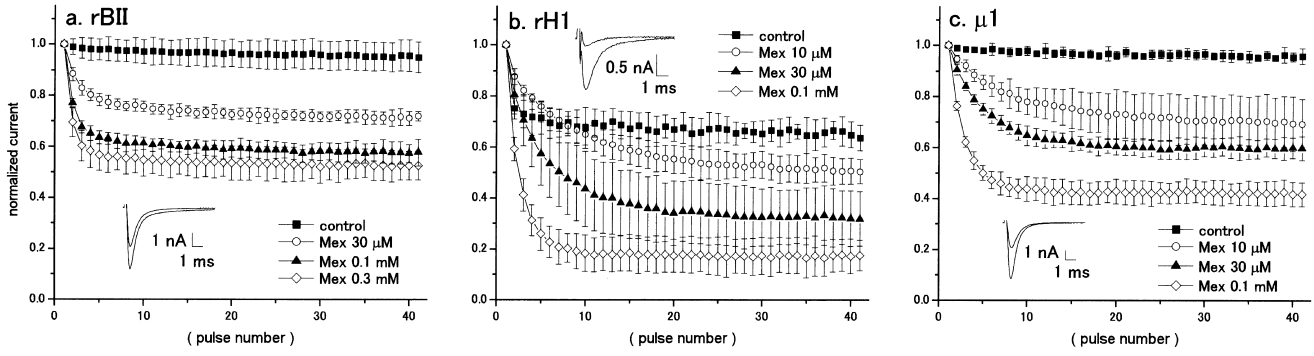

Fig. 3. Time course of use-dependent block caused by mexiletine in the three Na channel isoforms, rBII, rH1 and μ1. Cells were depolarized to −20 mV with a 5-s train of frequent (8 Hz) pulses in the absence or presence of mexiletine at the indicated concentrations. Data were expressed as mean ± S.D. and plotted against time for rBII (a), rH1 (b), and μ1 (c) isoforms of Na channel α-subunits. Mexiletine concentration-dependent block was observed in all the isoforms. Similar data were obtained with −40 and 0 mV test pulses, showing that block was not voltage dependent (data not shown).

Table 1
IC_{50} values for use-dependent and tonic block produced by mexiletine

	n	Use-dependent block				Tonic block
		1 Hz	2 Hz	4 Hz	8 Hz	
rBII	3	2576	1068	522	268	308
rH1	3	192	93	40	18	62
μ1	3	390	186	96	54	294
μ1-I433V	3	585	214	92	44	278
μ1-S251A	3	391	159	76	41	174

IC_{50} values are expressed in μM

LAs possess a higher affinity for open and inactivated channels than for resting channels. However, mexiletine was capable of suppressing I_{Na} without conditioning prepulses, indicating that resting block could be occurring. For this reason, we measured the blocking effect of mexiletine (tonic block) on I_{Na} under conditions in which fast Na inactivation was fully removed by holding the membrane at −140 mV for 500 ms (according to the double-pulse protocol used to study steady-state inactivation of I_{Na}). As shown in Fig. 5a, mexiletine suppressed I_{Na}, elicited following a large hyperpolarizing prepulse, in a dose-dependent manner. This effect was compared among the three Na channel isoforms (Fig. 5b). IC_{50} values for tonic block are summarized in Table 1. Again, rH1 was the most sensitive of the three isoforms to tonic block induced by mexiletine. The two remaining isoforms (rBII and μ1) exhibited equivalent sensitivity to the tonic block that mexiletine caused.

3.4. An amino acid residue of rH1 potentially conferring high sensitivity to mexiletine block

Lipid-soluble toxin binding sites have been considered to be potential binding sites for LAs that could influence Na channel sensitivity to LAs [15,16]. There are five known binding sites for lipid-soluble toxins in domain 1 segment 6 (D1-S6) and D4-S6 of the Na channel α-subunit (μ1-N434, μ1-L437, μ1-I1575, μ1-F1579 and μ1-Y1586) [15–18]. The indicated amino acids are all homologous to those at corresponding rH1 sites. In addition to these sites, we have recently found grayanotoxin (GTX)-specific binding sites in D1-S6 and the S4–5 linker of D1 [19] that contain heterologous amino acid residues in the rH1 and μ1 isoforms (rH1-V406/μ1-I433 and rH1-A252/μ1-S251). Among the seven known lipid-soluble toxin binding sites, these are the only two toxin sites in rH1 and μ1 that might be responsible for the higher sensitivity of rH1 to the blocking effects of mexiletine. Thus, we made four

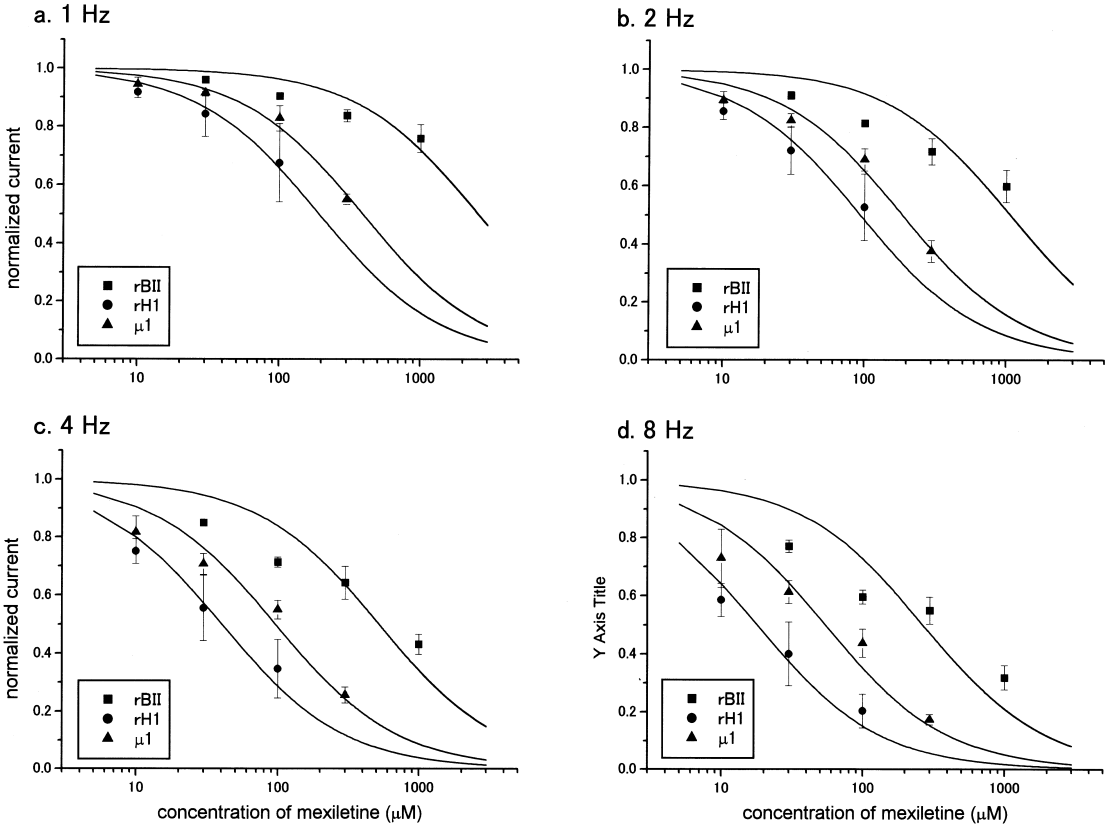

Fig. 4. Concentration dependency of use-dependent block caused by mexiletine. After treatment with mexiletine, use-dependent block of I_{Na} through all the three Na channel isoforms (rBII, rH1 and μ1) was elicited by pulsing at four different stimulation frequencies (i.e. 1, 2, 4, or 8 Hz, as shown in a, b, c, and d, respectively). Lines through the data points were drawn according to the expression, $1/(1+([\text{mexiletine}]/IC_{50}))$, where [mexiletine] indicates concentration of mexiletine, and IC_{50}, the concentration at which I_{Na} was decreased to half of its basal value (in the absence of mexiletine). We estimated IC_{50} values for mexiletine at each pulse frequency used. Values of IC_{50} are summarized in Table 1.

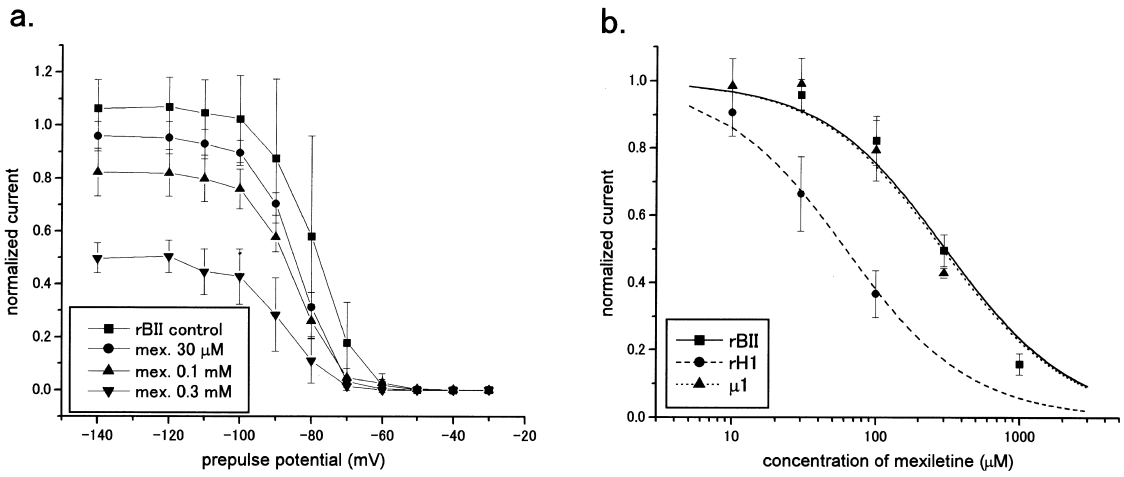

Fig. 5. Concentration dependency of tonic block among the Na channel isoforms. Tonic block of I_{Na} induced by mexiletine was measured under maximal relief of inactivation. a: Test I_{Na} was elicited, following the pulse protocol for steady-state inactivation, and measured at different concentrations of mexiletine. b: Test I_{Na}, elicited following a hyperpolarizing prepulse to −140 mV, was used as the index of tonic block. Lines through symbols were drawn as in Fig. 4. IC_{50} values for tonic block are listed in Table 1.

mutant Na channels, including rH1-V406I, μ1-I433V, rH1-A252S and μ1-S251A, and examined effects of mexiletine on these mutants. We could not obtain an IC_{50} value with respect to tonic block from two of the mutants (rH1-A252S and rH1-V406I), because a large hyperpolarizing shift of the steady-state inactivation curve hampered evaluation of the tonic blocking effect of mexiletine. (As Na inactivation could not be fully removed, drug-bound inactivated channels might tend to accumulate during the course of an experiment, thus obscuring the tonic blocking effect of mexiletine.) Unexpectedly,

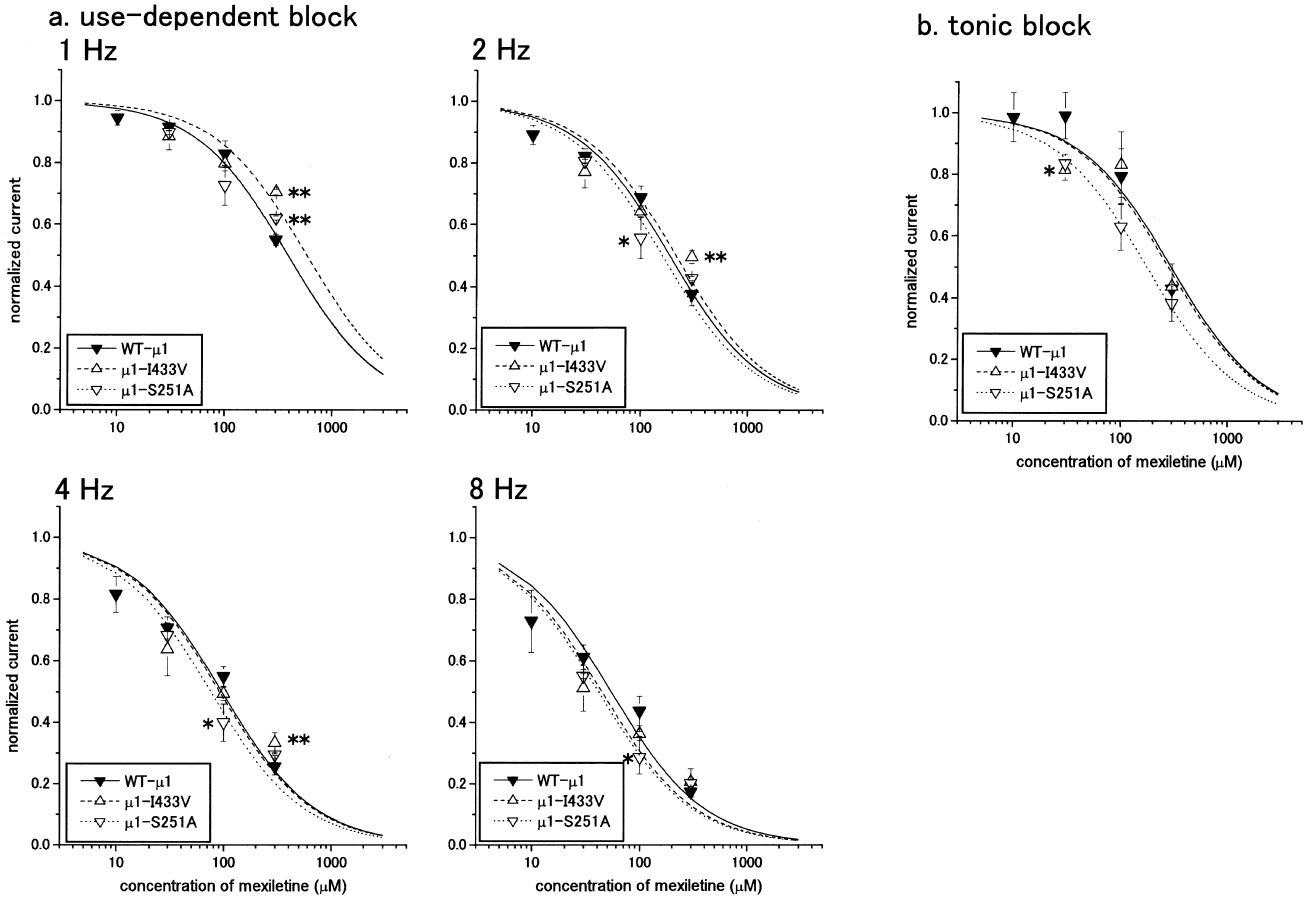

Fig. 6. Concentration dependency of tonic and use-dependent block produced by mexiletine in WT-μ1 and its mutants. Lines drawn through symbols are fitted as in Fig. 4. a,b: Use-dependent (a) and tonic (b) blocking effect of mexiletine in WT-μ1, μ1-I433V and μ1-S251A. Significant increase in sensitivity to mexiletine compared with WT-μ1 is indicated by *, and significant decrease in sensitivity compared with WT-μ1 is indicated by ** ($P < 0.05$).

Table 2
Half-inactivation voltages under mexiletine

		N	$V_{0.5(C)}$	$V_{0.5(D)}$	$\Delta V_{0.5(D-C)}$	$V_{0.5(W)}$	$\Delta V_{0.5(W-D)}$
rBII	(30 μM)	3	−74.7 ± 0.5	−76.9 ± 1.4	−2.2 ± 0.9	−76.1 ± 1.6	0.8 ± 0.9
	(0.1 mM)	3	−67.7 ± 5.3	−77.4 ± 0.6	−9.7 ± 5.3	−74.7 ± 2.8	2.7 ± 3.3
	(0.3 mM)	3	−75.1 ± 4.6	−81.8 ± 6.1*	−6.7 ± 1.7	−74.8 ± 5.1	7.0 ± 1.1
rH1	(10 μM)	3	−90.1 ± 4.1	−89.7 ± 3.7	0.4 ± 0.5	−88.3 ± 5.0	1.4 ± 1.3
	(30 μM)	3	−91.0 ± 0.2	−89.2 ± 2.2	1.7 ± 2.3	−85.3 ± 1.2	3.9 ± 1.2
	(0.1 mM)	3	−94.9 ± 7.0	−96.0 ± 1.9	−1.1 ± 5.1	−89.7 ± 1.9	6.3 ± 3.0
μ1	(10 μM)	3	−83.9 ± 1.1	−85.4 ± 4.1	−1.5 ± 4.7	−84.8 ± 6.2	0.6 ± 2.2
	(30 μM)	3	−80.2 ± 0.2	−82.4 ± 1.1	−2.2 ± 1.0	−80.2 ± 0.6	2.2 ± 1.7
	(0.1 mM)	3	−78.4 ± 1.6	−80.5 ± 3.1	−2.2 ± 2.3	−74.0 ± 4.1#	6.6 ± 1.2

Data are corrected for spontaneous voltage shifts that occurred in the absence of mexiletine (see text). $V_{0.5(C)}$ were measured at 11 min in the absence of mexiletine, $V_{0.5(D)}$, at 29 min during application of mexiletine. Washout of drug was begun after 23 min of mexiletine application, and $V_{0.5(W)}$ was determined 5 min after the start of washout. Thus, $\Delta V_{0.5(D-C)} = V_{0.5(D)} - V_{0.5(C)}$ and $\Delta V_{0.5(W-D)} = V_{0.5(W)} - V_{0.5(D)}$, indicating the true shifts in $V_{0.5}$ that occurred during the various treatments. Significant difference ($P < 0.05$) from $V_{0.5(C)}$ or $V_{0.5(D)}$ is indicated by * or by #, respectively.

there was no clear difference in sensitivity to mexiletine between WT- (wild-type) μ1 and μ1-I433V, and μ1-I433V even showed reduced sensitivity at some concentrations of mexiletine (Δ indicated with ** in Fig. 6). By contrast, μ1-S251A exhibited higher sensitivity to mexiletine (Δ indicated with * in Fig. 6) than WT-μ1 with respect to both use-dependent block at 2, 4, and 8 Hz and tonic block (Fig. 6 and Table 1). These results thus revealed a novel site in the S4–5 linker of D1 (μ1-S251) of the Na channel that alters the channel's affinity to mexiletine.

3.5. Mexiletine-induced shift of voltage dependency of activation and inactivation curves

It has been reported that LAs affect the voltage dependency of activation and inactivation in addition to their blocking effects [20,21]. However, in the present study, we noticed that these curves were spontaneously shifted in the negative direction (without drug application) during the course of whole-cell voltage clamp experiments. Thus, we measured the size of spontaneous negative shifts in separate control experiments using our standard experimental protocol (see Fig. 1), but without mexiletine application. Between the 13th and 30th min of these control experiments, $V_{0.5i}$ was shifted in the negative direction by 7.4 ± 1.3 mV ($n = 3$) for rBII, by 11.5 ± 2.3 mV ($n = 3$) for rH1, and by 7.4 ± 3.1 mV ($n = 3$) for μ1. The corrected half-inactivation voltages ($V_{0.5i}$) at 30, 100, or 300 μM mexiletine (applied between min 17 and 35), which we estimated by taking into account the values obtained in the control experiments above, are tabulated in Table 2. There was a tendency for mexiletine to cause a negative shift in $V_{0.5i}$, which was reversible on drug washout in rBII and μ1. For rH1, there was no negative shift in $V_{0.5i}$ on application of mexiletine. Perhaps the effect of mexiletine causing a negative shift in $V_{0.5i}$ was also present in rH1 experiments, but a correction of large spontaneous shift of $V_{0.5i}$ in rH1 could have obscured a shift produced by mexiletine.

4. Discussion

We have shown that rH1 was more sensitive to mexiletine block than two other Na channel isoforms (rBII and μ1) under uniform experimental conditions in which the α-subunit of the respective Na channels was transfected into HEK 293 cells. This demonstrates that the site responsible for the organ-specific differences in sensitivity to mexiletine block resides in the α-subunit of the Na channel. Although mutation of μ1-I433V did not reveal a clear-cut site responsible for the higher sensitivity of the cardiac isoform, the μ1-S251A mutant exhibited a smaller IC_{50} than WT-μ1 with respect to both use-dependent block at 2, 4, and 8 Hz and tonic block. Thus, this site, located in the S4–5 linker of D1, has an influence on the sensitivity of the channel to mexiletine.

4.1. Physiological relevance of sensitivity differences of Na channel isoforms to mexiletine

The higher LA sensitivity of cardiac Na channels in comparison to channel isoforms in other organs has not been systemically explored, although the clinical use of LAs as antiarrhythmic agents without significant side effects may suggest that this is the case. Recently, comparisons of lidocaine effects between hH1 and hSkM1 [22], and R-mexiletine effects between rH1 and rBIIA [23] have been made. These reports show a higher LA sensitivity of Na channel isoforms of cardiac muscle than isoforms of other tissues. In this study, we have now clearly shown the order of sensitivity to mexiletine both in terms of use-dependent and tonic block, i.e. rH1 > μ1 > rBII. This is important; because the higher sensitivity of the heart compared to other tissues emphasizes that any clinical application of mexiletine to skeletal muscle or the central nervous system requires careful monitoring of cardiac rhythms.

4.2. Possible sites responsible for sensitivity differences among the Na channel isoforms

It has not been clearly shown what sites within the α-subunit are responsible for the higher LA sensitivity of the cardiac isoform of Na channels. Thus, it is important to compare the effects of mexiletine on Na channel α-subunit isoforms without co-transfection with β-subunit, although recent report indicates that the presence of the β-subunit may affect the affinity on LAs to sodium channels [24].

Weiser et al. indicated that mutation of the homologous phenylalanine residue of rBIIF1764/rH1F1762 to alanine reduced block to a similar extent but with different mechanisms, one by slowing the rate of drug binding (rBIIF1764A) and the other by increasing the rate of drug dissociation (rH1F1762A). These investigators also showed that mutation of Y1771A in rBII exhibited the strongest reduction of block by R-mexiletine. These sites in D4-S6 coincide with the receptor sites for lipid-soluble toxins, such as batrachotoxin (BTX)

[16] or grayanotoxin (GTX) [18]. Thus, investigating other lipid-soluble toxin binding sites may lead to the discovery of sites required for the high cardiac sensitivity of mexiletine. Indeed, we recently reported that mutation of μ1-I433 and μ1-S251 to the corresponding amino acid residues of rH1-V406 and rH1-A252 reduced the sensitivity to GTX [19]. Therefore, we presumed that mutation of μ1-I433V and μ1-S251A should enhance, and that of rH1-V406I and rH1-A252S should reduce mexiletine sensitivity. However, our experimental results differed from expectation. Unfortunately, we could not obtain useful data from the mutants of rH1 due to a severe hyperpolarizing shift of the steady-state inactivation curve. Unexpectedly, μ1-I433V did not show a significant difference in sensitivity from that of WT-μ1. μ1-S251A did increase the sensitivity. Thus, we could at least point out one possible site responsible for the high cardiac sensitivity to mexiletine. At the same time, we should give some explanation for the increased sensitivity of μ1-S251A. Does this mutation cause a direct modulation of mexiletine binding affinity? We cannot give a clear answer to this question, because this mutant exhibited changes in inactivation properties. $V_{0.5i}$ of μ1-S251A (-86.3 ± 0.8 mV, $n = 9$) was significantly shifted in the hyperpolarizing direction compared with that of wild-type μ1 (-81.4 ± 0.7 mV, $n = 12$), and the fast time constant for current decay ($\tau_{fast} = 0.52 \pm 0.02$ ms, $n = 9$) of μ1-S251A was significantly briefer than that of μ1 (0.58 ± 0.07 ms, $n = 15$). However, τ_{slow} was not significantly different (2.2 ± 0.2 ms, $n = 15$ for μ1 vs. 2.5 ± 0.7 ms, $n = 9$ for μ1-S251A). The change in inactivation properties may well imply increased numbers of inactivated Na channels under the same experimental conditions, leading to a higher sensitivity of these mutants to mexiletine block, because LAs are thought to bind more tightly to the inactivated channel state [9].

4.3. Voltage shift of steady-state inactivation curve

Depolarization-enhanced effects of LAs were explained by the modulated receptor hypothesis [9] according to which drug binds more tightly to inactivated states of the channel than to the resting state. If that is so, and given that mexiletine is a known blocker of inactivated channels [25–27], the steady-state inactivation curve in the presence of mexiletine should shift to more negative membrane potentials. In fact, very small negative shifts (~ 5 mV) of the steady-state inactivation curve of hSkm1 Na channels expressed in HEK 293 cells have been reported to occur in the presence of 100–500 μM mexiletine [28,29], and a large shift was observed only at a very high dose (1 mM) [29]. The shift is more obvious (~ 20 mV) in Na channels expressed in *Xenopus* oocytes, where a spontaneous negative was not observed [30]. In our study, mexiletine-specific negative shifts were variable among the Na channel isoforms (2–9 mV): rBII exhibited most marked shift, while rH1 did not show any significant shift after correction for spontaneous shifts. The shift could be masked by two properties of our experimental system utilized in this study: an intrinsically small negative shift due to LAs in mammalian expression systems, and a large spontaneous shift in rH1.

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