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Molecular Determinants of Mexiletine Structure for Potent and Use-Dependent Block of Skeletal Muscle Sodium Channels[1]

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ABSTRACT

On the basis of the information about drug receptor on voltage-gated sodium channels, mexiletine (Mex) analogs with substitutions at either the asymmetric carbon atom or the aromatic ring were synthesized as pure enantiomers. The compounds were tested in vitro for their ability to produce voltage- and use-dependent block of sodium currents (I_{Na}) of frog muscle fibers by the vaseline-gap voltage-clamp method. In all experimental conditions, the drug potency was highly correlated with the lipophilicity of the group on the asymmetric center, the derivative with a benzyl moiety (Me6) having IC_{50} values more than 10 times lower than those of Mex, followed by the phenyl (Me4) and the isopropyl (Me5) derivative. All of the compounds showed a further reduction of IC_{50} values at depolarized membrane potentials and at high frequency of stimulation (10 Hz). Mex and Me5, but not Me4, produced a stereoselective tonic block of I_{Na}, the *R*-(−) isomers being 2-fold more potent than the *S*-(+) ones. The removal of both methyl groups from the aromatic ring of Mex (Me3) caused a 7-fold reduction of the potency, whereas similar substitutions on the phenyl derivative Me4 (Me7 and Me8) produced opposite effects. In fact, the IC_{50} of *R*-(−) Me7 for use-dependent block of I_{Na} was 30 times lower than that of *R*-(−) Mex. Me8 and Me7 were stereoselective during both tonic and use-dependent blockade. All of the compounds left-shifted the steady-state inactivation curves in relation to their potency and to the duration of the inactivating prepulse. Finally, the presence of apolar groups on the asymmetric center of mexiletine is pivotal to reinforce hydrophobic interactions with the proposed aromatic residues at the receptor, and lead to potent and therapeutically interesting inactivated channel blockers.

Voltage-gated sodium channels are the target of clinically important drugs such as antiarrhythmics, anticonvulsants, and local anesthetics (LAs; Catterall, 1987). Recently, sodium channel blockers have been proven beneficial also for neuropathic pain treatment and neuroprotection (Taylor and Meldrum, 1995). Mexiletine (Mex) is an orally effective lidocaine analog belonging to Ib antiarrhythmic drugs, although it retains the wide variety of therapeutic potential described above (Taylor and Meldrum, 1995). Also, Mex is among the few drugs used to symptomatically solve the hyperexcitability of myotonic syndromes, hereditary disorders of skeletal muscle due to genetic alterations of either sodium or chloride channels (Rüdel et al., 1994; Cannon, 1996; Ptacek, 1998). As for most LA-like drugs, the clinical usefulness of Mex resides in the ability to block sodium channels with a stronger potency in situations of excessive burst of action potentials (use-dependent block) and/or prolonged depolarization (voltage-dependent block), as occurring in diseased tissues, rather than on physiological excitability (resting or tonic block). This blocking mechanism relies on the high-affinity drug binding to the receptor on the α subunit of the channel when the latter is open and/or inactivated, and to a slow recovery from inactivation of the drug-bound channels during membrane repolarization (Catterall, 1987; De Luca et al., 1991, 1997a; Sunami et al., 1993). Mutagenesis studies in rat brain type II channel have identified two aromatic amino acids lining the pore, Phe1764 and Tyr1771, in the D4-S6 transmembrane segment of the α subunit that are important for state-dependent block by LA-like drugs (Ragsdale et al., 1994, 1996). Corresponding amino acids in other sodium channel types have a similar role, strongly corroborating that this channel region, highly conserved between tissues and species, forms part of the drug receptor (Marban et al., 1998; Wright et al., 1998; Li et al., 1999). A certain degree of overlapping pharmacology between sodium channels of different tissues is clinically observed; for instance, the use of

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ABBREVIATIONS: Mex, mexiletine; Me4, α-[(2,6-dimethylphenoxy)methyl] benzenemethanamine; Me5, 1-(2,6-dimethylphenoxy)-3-methyl-2-butanamine; Me6, α-[(2,6-dimethylphenoxy)methyl]benzenethanamine; Me3, 1-phenoxy-2-propanamine; Me7, α-[(2-methylphenoxy)methyl]benzenemethanamine; Me8, α-(phenoxymethyl)benzenemethanamine; LA, local anesthetic; I_{Na}, sodium current; h_∞, steady-state availability function; $Vh_{1/2}$, potential for half-maximal inactivation of sodium channels; log P, log of the octanol/water partition coefficient; h.p., holding potential.

Mex as antimyotonic is hindered by its ability, at the clinically effective doses, to affect excitability in the nervous system and heart (Rüdel et al., 1994). However, cardiac sodium channel is intrinsically more sensitive to lidocaine-like drugs versus skeletal muscle isoform, suggesting possible subtle structural differences in drug binding site between channel types; in addition, the long-lasting inactivated states of the cardiac channels can favor inactivated-state block by the drug (Wang et al., 1996; Wright et al., 1997). The available biochemical and biophysical information encourages the rational design of more potent and/or tissue-selective drugs. The recognized pharmacophore moieties of sodium channel blockers are an ionizable amino group and an aromatic ring at a distance matching that found between the two aromatic amino acids in D4-S6, suggesting specific π-cation and hydrophobic interactions, respectively (Liu et al., 1994; Ragsdale et al., 1994, 1996; Li et al., 1999). Our previous studies suggest that the stereogenic carbon atom bearing the amino group also contributes to Mex activity. An increased potency in exerting a tonic block of skeletal muscle sodium currents but a concomitant decrease of use-dependent behavior is obtained by increasing the lipophilicity of the molecule with modifications at both the asymmetric carbon atom and the aromatic ring (Me1), whereas the opposite is observed by increasing the distance between the chiral center and the amino terminal group (Me2) (De Luca et al., 1997a). Both of these compounds are more effective than Mex in reducing in vitro the hyperexcitability of intercostal muscle of myotonic adr/adr mouse (De Luca et al., 1997b). The pivotal role of the asymmetric carbon atom is also corroborated by the stereoselective effects of the enantiomers of LA-like drugs on sodium channels of various tissues (Yeh, 1980; Hill et al., 1988; Tricarico et al., 1991; De Luca et al., 1995, 1997a), suggesting that the groups present at this position contribute to a proper binding with the receptor by influencing a third point interaction in the channel protein. In this study we evaluated in more detail the structure-activity relationship for chiral Mex derivatives, synthesized as pure enantiomers, on sodium currents of native frog skeletal muscle fibers. By introducing apolar groups of increasing molecular weight and lipophilicity on the asymmetric carbon atom, we tested the hypothesis that this part of the molecule directly interacts with a proposed hydrophobic pocket in the binding site; structural modifications at the aryloxy moiety, able to modify the electronic cloud and the orientation of the aromatic ring, should modulate the strength of a π-π interaction with the other aromatic amino acids, possibly contributing to the receptor (Fig. 1). The results showed that substitutions at the level of the asymmetric center greatly enhance the drug potency and can minimize or change the effects brought about by the structural modifications at the aromatic ring. In line with the proposed residues accounting for drug receptor (Ragsdale et al., 1996; Li et al., 1999), the lipophilicity of the groups on the asymmetric center may allow high-affinity hydrophobic interactions and lead to potent and therapeutically interesting compounds.

Materials and Methods

Fiber Preparation and Voltage-Clamp Apparatus. Segments of undamaged single muscle fibers (about 1 cm in length) were obtained by microsurgery (plucking procedure) from the ventral branch of the semitendinosus muscle of *Rana Esculenta* bathed in normal physiological solution at room temperature. The cut-end fiber was then superfused with an internal solution and mounted across three chamber partitions, which delineated the four pools. Three strips of vaseline were applied over the fiber and carefully sealed to the fiber to reduce leakage. The width of the gaps of the central pools (A and B) had been previously set to 70 to 100 and to 200 μm, respectively. Four KCl/agar bridges electrodes connected the recording chamber to the voltage-clamp amplifier based on methods described by Hille and Campbell (1976) and detailed elsewhere (De Luca et al., 1995, 1997a). When the solution level was lowered below the vaseline strips, the four pools were physically and electrically independent from each other; this was confirmed by verifying that no leak current was flowing when increasing the amplifier gain. Then the solution in pool A was replaced with the external solution and after about 10 min of equilibration the recordings were performed at 10°C. Unless changed for particular protocols, the usual holding potential (h.p.) was −100 mV. Sodium currents were recorded using an amplifier connected via a A/D and D/A Digidata 1200 Interface (Axon Instruments, Inc., Foster City, CA) to a 486 DX2/66 personal computer and stored on the hard disk. The stimulation protocols and data acquisition were driven by the Clampex program (pClamp software package; Axon Instruments, Inc.). The currents flowing in response to depolarizing command voltages were low pass filtered at 10 kHz (Frequency Devices, Inc., Haverhill, MA), visualized on an oscilloscope, and sampled at 20 kHz. When necessary, leak and capacities currents were subtracted by the P/4 method. The acquired traces were analyzed later using the Clampfit program (pClamp software package; Axon Instruments, Inc.).

Drug and Solutions. The following solutions were used: Normal physiological solution: 115 mM NaCl, 1.8 mM $CaCl_2$, 2.15 mM Na_2HPO_4, 0.85 NaH_2PO_4; External solution: 77 mM NaCl, 38 mM choline-Cl, 1.8 mM $CaCl_2$, 2.15 Na_2HPO_4, 0.85 mM NaH_2PO_4; Inter-

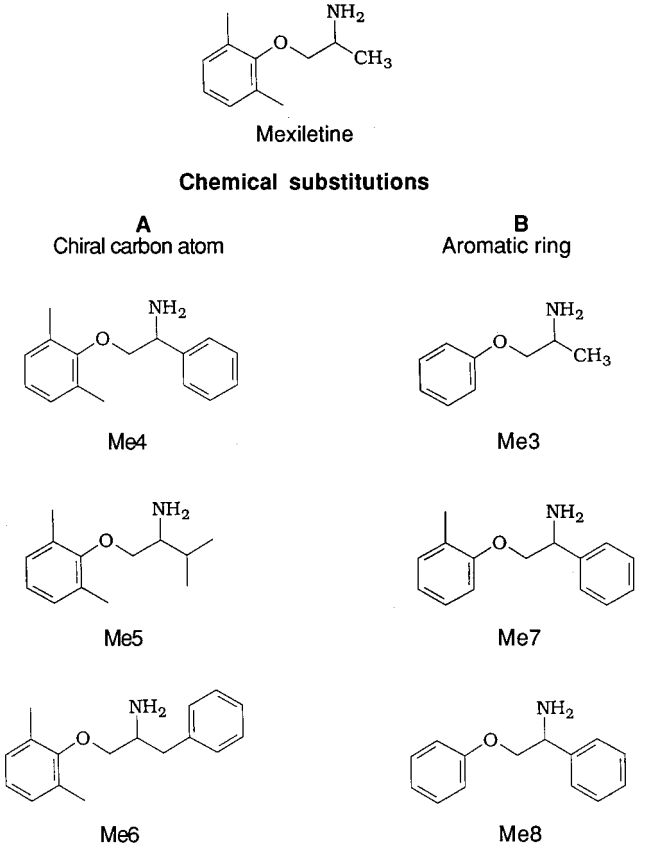

Fig. 1. Chemical structure of Mex and its newly synthesized chiral analogs.

nal solution: 105 mM CsF, 5 mM MOPS, 2 mM $MgSO_4$, 5 mM EGTA, 0.55 mM Na_2ATP. The pH was adjusted at 7.2 with a standard NaOH-concentrated solution.

The compounds tested and shown in Fig. 1 were Mex, α-[(2,6-dimethylphenoxy)methyl]benzenemethanamine (Me4), 1-(2,6-dimethylphenoxy)-3-methyl-2-butanamine (Me5), α-[(2,6-dimethylphenoxy)methyl]benzenethanamine (Me6), 1-phenoxy-2-propanamine (Me3), α-[(2-methylphenoxy)methyl]benzenemethanamine (Me7), and α-(phenoxymethyl)benzenemethanamine (Me8). All of the compounds were prepared in our laboratories, as pure or highly enriched enantiomeric forms (enantiomeric purity ≥93%), as hydrochloride, or as hydroiodide salts according to procedures described in detail elsewhere (Franchini et al., 1994; Loughhead et al., 1999; Franchini et al., submitted). Briefly, hydrochloride salts of Mex, Me3, Me4, Me7, and Me8 stereoisomers were prepared through a three-step route starting from optically active propylene (Mex and Me3) or styrene oxide (Me4, Me7, and Me8). Me5 and Me6 isomers were obtained by condensing N-protected aminoalcohol with 2,6-xylenol via standard Mitsunoby oxidoreductive substitution; the free bases, obtained by removing the N-protecting group, were then converted to corresponding hydro halides. The abbreviated numbered nomenclature used throughout the text was assigned at the time the compounds were synthesized and is, therefore, arbitrary. All compounds were fully characterized by routine spectroscopic analyses; analytical results for C, H, and N were within ±0.4% of the theoretical values. The enantiomers were stable under the conditions used in this study. Stock solutions of Mex, Me5, and Me3 were prepared by dissolving the compounds in external solution, whereas stock solutions in dimethyl sulfoxide (50 μl/mg) were used for Me4, Me6, Me7, and Me8. Dimethyl sulfoxide, at the highest concentration used for dilution (0.2%), was without effect on the parameters recorded. All other chemicals used were of analytical grade and obtained from Sigma Chemical Company (St. Louis, MO).

Pulse Protocols and Statistical Analysis. The curves describing the voltage dependence of sodium current (I-V curve; not shown) were constructed with a cycle of 10-ms test pulses from the h.p. of −100 mV to increasing potentials (from −60 to +60 mV). The intervals between each test pulse were long enough (~3 s) to allow complete recovery of sodium channel from inactivation. The exact value of membrane potential, at which peak sodium current (I_{Na}) reached its maximum ($I_{Na\ max}$) calculated from the I-V curves, was −38.2 ± 1.5 mV (n = 35). Thus, the test pulse used for evaluating drug block (see below) was −20 mV because this membrane potential value is in the linear part of the I-V curve, when the dynamic process of activation is complete. No differences were found in the calculated half-maximal concentration values of each drug evaluated either on maximal sodium current during the construction of the I-V curve or on the nearly maximal I_{Na} elicited by single test pulses from the h.p. to −20 mV. Sodium channels rapidly transit between resting, open, and inactivated states in relation to threshold membrane depolarization; however, at each membrane potential a steady-state distribution of channels between resting and inactivated states also exists, the more depolarized the voltage, the higher the fraction of channels that enters the inactivated state (Marban et al., 1998). Such an equilibrium already exists at membrane potentials close to the physiological ones for striated fibers (−90/−70 mV). A different affinity of the drug in relation to the channel state, as well as the ability to influence the above equilibrium, (Catterall, 1987) can therefore strongly affect drug effect on slight modifications of membrane potential, allowing the prediction of the drug potency in pathological conditions in which membrane depolarization is responsible for abnormal excitation pattern in skeletal muscle and other excitable tissues. To evaluate the voltage-dependent effect of each drug that would reflect the steady-state binding to the channel in the resting and/or inactivated state, we used a pulse protocol of infrequent depolarizing stimulation to −20 mV for 10 ms from three different h.p. values: very negative (−140 mV), close to the physiological resting membrane potential of frog striated fibers (−100 mV), and depolarized, resembling a pathological condition (−75 mV). At −140 mV, all of the channels are virtually in the resting activatable state, and the I_{Na} reaches its maximal value. No difference in the peak I_{Na} was observed when the h.p. was −100 mV, suggesting that at this potential the channels are mostly in the resting state. Thus the drug-induced reduction of peak I_{Na} at −140 and −100 mV was considered tonic block (block of the channel in the resting state on a physiologically polarized membrane). On the contrary, at −75 mV, a membrane potential depolarized with respect to the normal physiological one and rather resembling a pathological state, I_{Na} was reduced to 64.6 ± 4.8% (n = 39), and, therefore, more than 30% of the channels were in the inactivated nonconducting state. The reduction I_{Na} produced by an inactivated channel blocker at this h.p. reflects the combination of binding to both resting and inactivated channels. However, if the affinity for the inactivated state is much higher than that for the resting state, the former would be the main determinant for the remarkable shift of the channel population toward the adsorbing nonconductive inactivated state. Taking into account the above considerations, and in agreement with similar assumptions in the literature, we conventionally considered the potency of the compounds at a depolarized potential of −75 mV as a rough estimate of drug affinity for the inactivated state (Wright et al., 1997, 1998; Li et al., 1999; Nau et al., 1999). The use-dependent behavior of each compound was evaluated with 30-s trains of test pulses from the h.p. of −100 to −20 mV at the frequency of 2 and 10 Hz. With this protocol in the presence, but not in the absence, of use-dependent compounds, a further reduction in peak I_{Na} over the tonic block was observed, which progressively accumulated until a new equilibrium was reached. The value of the current at the equilibrium normalized with respect to the current in the absence of drug was used to calculate the potency of the drug for blocking the channels under conditions of excessive stimulation (e.g., high-frequency firing).

Steady-state inactivation curves were determined by cyclic protocol of pulse sequences. Each sequence consisted of a conditioning pulse to −140 mV for 500 ms (to have most of the sodium channels in the "activatable" state), a prepulse of variable potential of either 50 or 1000 ms duration, and the 10-ms test pulse to −20 mV; after a pause of 1 s the sequence was cyclically repeated 18 to 20 times with the prepulse potential value increased each time by 5-mV steps (De Luca et al., 1995, 1997a).

The data were expressed as mean ± S.E. The estimates of S.E.M. of normalized I_{Na} values have been obtained as described previously (De Luca et al., 1995). Molar concentrations of the drugs tested producing a 50% block of I_{Na} (IC_{50}) were determined by using a nonlinear least-squares fit of the concentration-response curves to the following logistic equation: $\text{Effect} = -100/\{1+(K/[\text{drug}])^n\}$, where Effect = percent change of I_{Na}; −100 = maximal percent block of I_{Na}; K = IC_{50}; n = logistic slope factor; [drug] = molar concentration of the compound. The steady-state availability function (h_∞) curves have been fitted with a single Boltzmann distribution and the potential for half-maximal inactivation of sodium channels ($Vh_{1/2}$) was calculated at the inflection point of the curves (De Luca et al., 1991). Correlation between K_d values and log of the octanol/water partition coefficient (log P) was calculated by fitting the experimental data points with classical single exponential decay functions. Nonlinear equation fitting and processing for data graphics were done by Fig. P Software (Biosoft, Cambridge, UK). Theoretical log P values of Mex derivatives were calculated by using C Log Software v. 3.0 (Biobyte Corp., Clermont, CA). Statistical significance of differences between mean values has been estimated by the unpaired Student's t test.

Results

1. Voltage- and Use-Dependent Block of Na^+ Channels by Chiral Derivatives of Mex

Substitutions on Asymmetric Carbon Atom. An increased hindrance on the asymmetric carbon atom obtained

by replacing the methyl group of Mex with either a phenyl (Me4), an isopropyl (Me5), or a benzyl (Me6) group caused a marked increase in the potency for producing a tonic block of sodium current. A sample of typical recordings showing the effect produced on I_{Na} (elicited with single test pulses from −100 to −20 mV) by the *R*-(−) enantiomers of these analogs is shown in Fig. 2A. As it can be seen, Me4 and Me6 at 10 μM and Me5 at 30 μM produced a comparable or even larger tonic block than that observed with 50 μM *R*-(−) Mex. The concentration-response curves of the three derivatives, constructed by calculating the percent reduction of I_{Na} versus the current value in the absence of drug, were clearly shifted to the left with respect to that of *R*-(−) Mex with the following order of potency: Me6 > Me4 > Me5 (Fig. 3A). The calculated concentration for having the half-maximal block of I_{Na} (IC_{50}) showed that *R* enantiomers of Me6, Me4, and Me5 were 8, 4.5, and 3 times more potent than *R*-(−) Mex, respectively (Table 1). The IC_{50} values of each drug were clearly voltage dependent; however, at any membrane potential used, the compounds showed the same scale of potency described above (Fig. 3B). It is worthwhile to notice that although the absolute I_{Na} amplitude did not change between −140 and −100 mV in the absence of drug, all of the compounds showed a decreased potency at −140 mV, suggesting that −100 mV, close to the physiological resting potential of frog striated fibers, is a borderline voltage at which the channels, although mostly present in the resting state as assumed by the I_{Na} amplitude, have an easier tendency to enter the inactivated state, equilibrium that is further shifted in the presence of the drugs. Accordingly, all of the compounds were much more potent at −75 mV. As shown in Fig. 3, A and D, Me4 was up to 16 times more potent at this potential than at −140 mV, followed by Me5, Me6, and Mex.

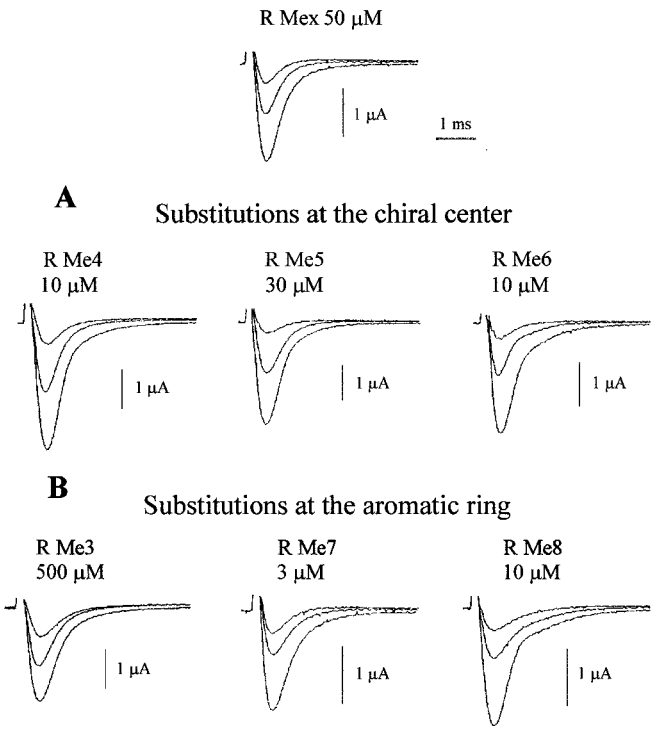

Fig. 2. Na^+ current transients recorded by the three vaseline gap voltage-clamp method from single fibers of frog semitendinosus muscle in the absence and presence of *R*-(−) enantiomers of Mex, and of its derivatives with a phenyl (Me4), an isopropyl (Me5) or a benzyl (Me6) group on the chiral carbon atom (A) and with substitutions on the aromatic ring i.e., Me3, without methyl groups, and Me7 and Me8, that are Me4 analogs without one or both methyl groups from the aromatic ring, respectively (B). In particular, the effects produced by 50 μM Mex are compared with those produced in (A) by 10 μM Me4 and Me6 and by 30 μM Me5 and in (B) by 500 μM Me3, 3 μM Me7, and 10 μM Me8. In each group of traces, the control current (in absence of drug) is the greatest one obtained with single depolarizing pulses from −100 to −20 mV for 10 ms. Ten minutes after application of each drug, a similar depolarization allowed us to estimate the tonic block exerted by the drug. Afterward the depolarizing stimulus has been repetitively applied at a 10-Hz frequency for 30 s and a cumulative block developed over the tonic block due to the use-dependent behavior, until a new equilibrium is reached. The smallest traces show the residual current at the end of the 10-Hz stimulation protocol.

The potency of the compounds was enhanced by increasing the frequency of depolarizing stimulation (Table 1). The protocol at 10 Hz showed a remarkable use-dependent behavior of the three compounds because after 30 s of such a stimulation in the presence of the drugs the current amplitude was markedly reduced with respect to the tonic block (Fig. 2A). As shown by the concentration-response curves in Fig. 3E, the order of potency was the same of that found for tonic block. The calculated IC_{50} values of Me4, Me5, and Me6 for use-dependent block at 10 Hz were reduced by about 3-fold with respect to those for tonic block versus the 2-fold decrease observed with Mex (Table 1). Me 5 showed a slightly higher use-dependent behavior with respect to Me4 and Me6, whereas the latter showed an IC_{50} value that was 10 times lower than that of Mex (Table 1). Assuming that IC_{50} value is a good measure of drug affinity constant (K_d), we found that K_d for both voltage- and use-dependent block decreased exponentially and with a very high correlation with the increase in the log P of the molecule, which in this case is entirely due to the lipophilicity of the groups linked to the asymmetric carbon atom (Fig. 3F). Substitution at this position may also influence the steric disposition of the molecule at the receptor and, therefore, its stereoselectivity (De Luca et al., 1997a). This property was evaluated for Me4 and Me5 for which the two enantiomers were available. Me4 action was almost devoid of stereoselectivity, the two enantiomers being equieffective in producing both the tonic and the use-dependent block of I_{Na} (Table 1). Similar to the results obtained previously (De Luca et al., 1997a), both Mex and Me5 produced a stereoselective tonic block, the *R*-(−) enantiomers being twice as potent than the *S*-(+) ones (Table 1). However, when increasing the stimulation frequency, the stereoselectivity of Me5, as well as that of Mex, attenuated. The eudismic ratio [IC_{50} Distomer/IC_{50} Eutomer] for Me5 decreased from 2, found for tonic block, to 1.02 at 10 Hz (Table 1). The attenuation of stereoselectivity during use-dependent blockade has been observed already with other chiral analogs of Mex, and has been attributed to a slow time course of recovery from inactivation of the drug-blocked channels that can be influenced by the unblocking kinetic of the drug (De Luca et al., 1997a). In contrast to Mex and Me5, whose effects were rapidly abolished on washout, the effects of Me4 and Me6 were slowly reversible.

Substitutions on Aromatic Ring. The Mex derivative Me3, from which the two *ortho*-methyl groups on the aromatic ring were removed, showed a dramatic reduction of potency with respect to the parent compound in producing both a tonic and a use-dependent block, as shown in Fig. 2B in which the effects produced by 500 μM *R*-(−)Me3 and 50 μM *R*-(−) Mex are compared. Consequently, the concentra-

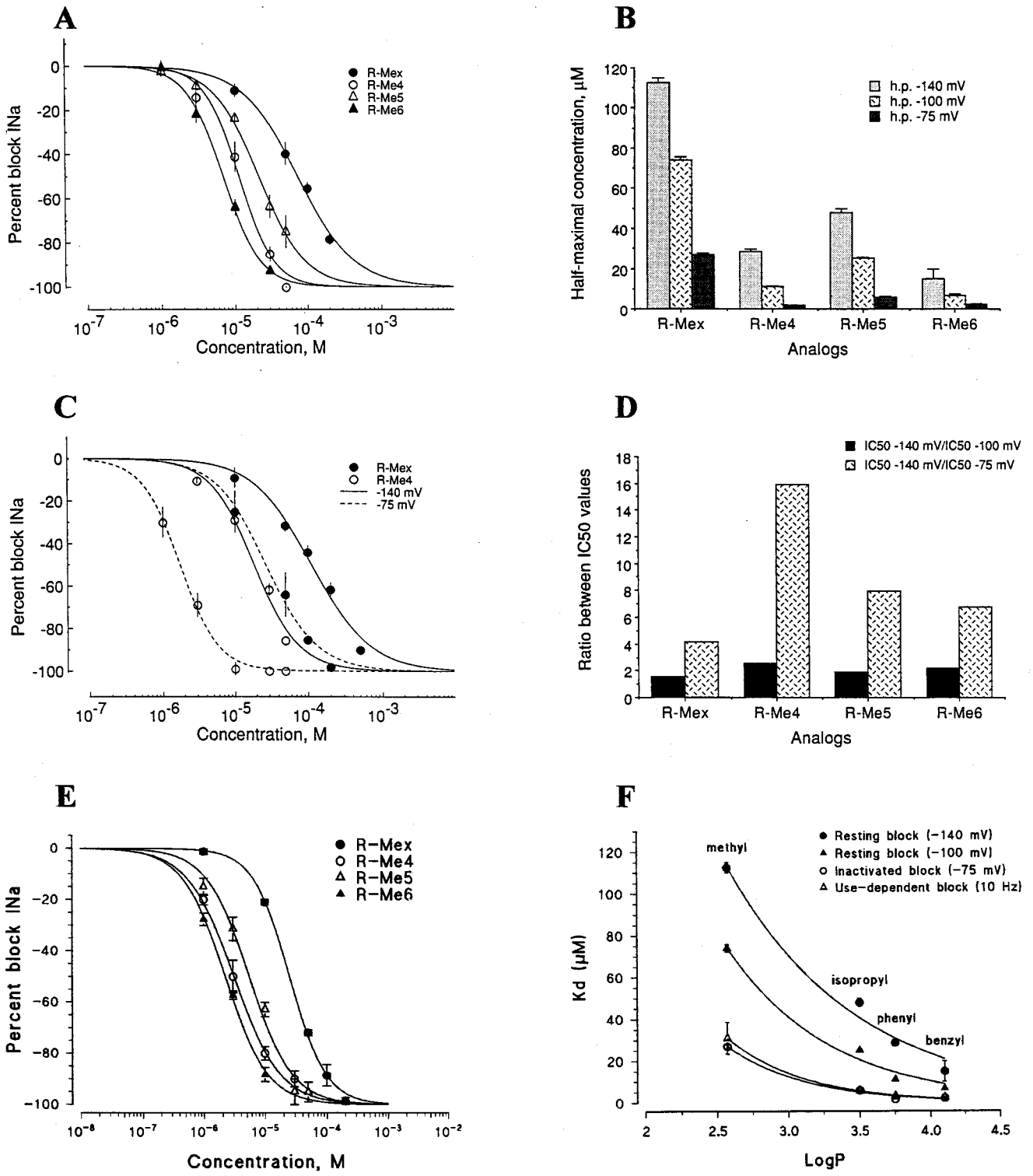

Fig. 3. Voltage- and frequency-dependent drug effects and relative potency of Mex derivatives obtained with substitutions on the chiral carbon atom. A, concentration-response curves of *R*-(−) enantiomers of Mex, Me4, Me5, and Me6 constructed with single depolarizing 10-ms pulses to −20 from the h.p. −100 mV. Each point shows the percent reduction of the current and is the mean ± S.E. from three to eight fibers. B, half-maximal concentrations of *R*-(−) enantiomers of Mex, Me4, Me5, and Me6 obtained with the single pulse protocol from different h.p. values and, in particular, from −140, −100, and −75 mV. Each bar is the value of IC_{50} ± S.E. obtained from fitting the experimental points to the logistic function described in *Materials and Methods*. C, concentration-response curves of *R*-(−) Mex and *R*-(−) Me4 constructed at the h.p. values of −140 mV (continuous lines) and at −75 mV (dotted lines). Each point is the mean ± S.E. from three to eight fibers. D, ratios between IC_{50} values at various h.p. values that show for each compound the gain in potency at more depolarized potentials, as indicative of a high-affinity to inactivated state. E, concentration-response curves for the use-dependent block by *R*-(−) Mex and its derivatives. The amount of use-dependent block is calculated on the residual current at the end of a 10-Hz train of pulses from −100 to −20 mV versus the current in the absence of drug. Each point is the mean ± S.E. from three to eight fibers. F, relation between the apparent affinity (K_d = calculated IC_{50}) and the lipophilicity (log P) of the groups linked to the chiral carbon atom. For both voltage- and frequency-dependent block a high correlation was found between the two parameters ($r > 0.9$).

tion-response curves of Me3 for both tonic and use-dependent block were shifted to the right with respect to those of Mex (Fig. 4, A and B). The calculated IC_{50} values for Me3 were about 10-fold higher than those of Mex in any experimental condition (Table 1). To better evaluate the role of the substituents on the aromatic ring for the drug potency, we evaluated the effects of two analogs of the potent phenyl derivative Me4 in which one (Me7) or both (Me8) methyl groups were replaced by hydrogen. It was quite interesting to find that the effect of these substitutions on the phenyl analogs of Mex was not as dramatic as that observed with Mex. In fact both Me7 and Me8 were still very potent sodium channel blockers; in particular, Me7 showed IC_{50} values for both tonic and use-dependent block even lower with respect to that of Me4 (Table 1; Figs. 2B and 4). In agreement with such a strong potency, it was very difficult to calculate the IC_{50} values for Me7 and Me8 from the h.p. of −75 mV. For comparison we found that 1 μM *R*-(−)Me7 and 3 μM *R*-(−)Me8, able to produce a 26 ± 6% (n = 4) and a 15 ± 4% (n = 3) reduction of the I_{Na} from the h.p. of −140 mV, respectively, produced about a 70% decrease of the current at −75 mV. During the 10-Hz stimulation the potency of both compounds increased by 2-fold versus the tonic block, showing a less remarkable use-dependent behavior with respect to Me4. In fact, as far as the *R*-(−) enantiomers are concerned, the ratio between IC_{50} Me4/IC_{50} Me7 decreased from 5 of tonic block to 2.7 during use-dependent block, suggesting that the higher potency of Me7 versus Me4 slightly attenuated during the high frequency of stimulation. Nonetheless, at 10 Hz *R*-(−)Me7 was almost 30-times more potent than *R*-(−)Mex (Table 1). Me3, Me7, and Me8 were stereoselective, the *R* enantiomers being twice as potent as the *S* ones (Table 1). Interestingly, opposite to what was observed with Mex and Me5, the stereoselectivity of Me7 and Me8 was maintained during the use-dependent block (Table 1). This observation, along with the less remarkable use-dependent behavior described above, allows us to propose a fast kinetic of drug binding-unbinding that does not curtain the stereoselective interaction with the receptor. Similar to what observed with Me4, the effects of both Me7 and Me8 were slowly reversible on washout.

TABLE 1

Concentrations for half-maximal tonic and use-dependent block of sodium currents by chiral analogs of mexiletine

The columns from left to right are as follows: Drug used; Concentrations able to produce the half-maximal response (IC_{50}) in producing a tonic block (calculated during infrequent depolarizing stimulation from −100 to −20 mV) and a use-dependent block calculated by using trains of depolarizing pulses at the frequency of 2 and 10 Hz. The IC_{50} values have been obtained during nonlinear least squares fit of the concentration-response data to the logistic equation described in *Materials and Methods*.

Compound	Tonic Block IC_{50}	Use-Dependent Block	
		2 Hz IC_{50}	10 Hz IC_{50}
	μM	μM	μM
R(−)-Mex	74 ± 1.7	35 ± 5.4	31.0 ± 7.6
S(+)-Mex	127 ± 2.8	49 ± 4.5	32.5 ± 6.6
Substitutions on Asymmetric Carbon Atom			
R(−)-Me4	11.1 ± 0.3	7.6 ± 0.6	3.2 ± 0.3
S(+)-Me4	9.3 ± 0.8	6.7 ± 0.6	3.0 ± 0.4
R(−)-Me5	25.2 ± 0.6	10.0 ± 0.4	5.7 ± 0.5
S(+)-Me5	46.1 ± 1.1	15.2 ± 2.1	5.8 ± 0.6
R(−)-Me6	6.9 ± 0.5	6.5 ± 0.4	2.4 ± 0.1
Substitutions on Aromatic Ring			
R(−)-Me3	497 ± 25	344 ± 34	208 ± 16
S(+)-Me3	600 ± 61	386 ± 60	236 ± 37
R(−)-Me7	2.2 ± 0.4	2.08 ± 0.3	1.2 ± 0.1
S(+)-Me7	7.5 ± 0.4	5.7 ± 0.4	3.1 ± 0.2
R(−)-Me8	7.8 ± 1.4	6.9 ± 0.7	4.1 ± 0.5
S(+)-Me8	29.8 ± 0.6	27.9 ± 0.6	15.8 ± 0.4

2. Effect of Chiral Derivatives of Mex on Steady-State Inactivation

Several pieces of information confirmed that Mex is an inactivated- but not an open-channel blocker (Sunami et al., 1993; De Luca et al., 1997a; Desaphy et al., 1999). Inactivated channel blockers shift the steady-state inactivation curves toward more negative potentials in a manner dependent on the duration of the depolarizing prepulse, implying a voltage- and time-dependent reduction of the number of channels available for opening due to drug-favored inactivation (Tricarico et al., 1991; De Luca et al., 1995, 1997a). In frog striated fibers the steady-state inactivation is fully reached with prepulse duration of 50 ms; in fact, the $Vh_{1/2}$

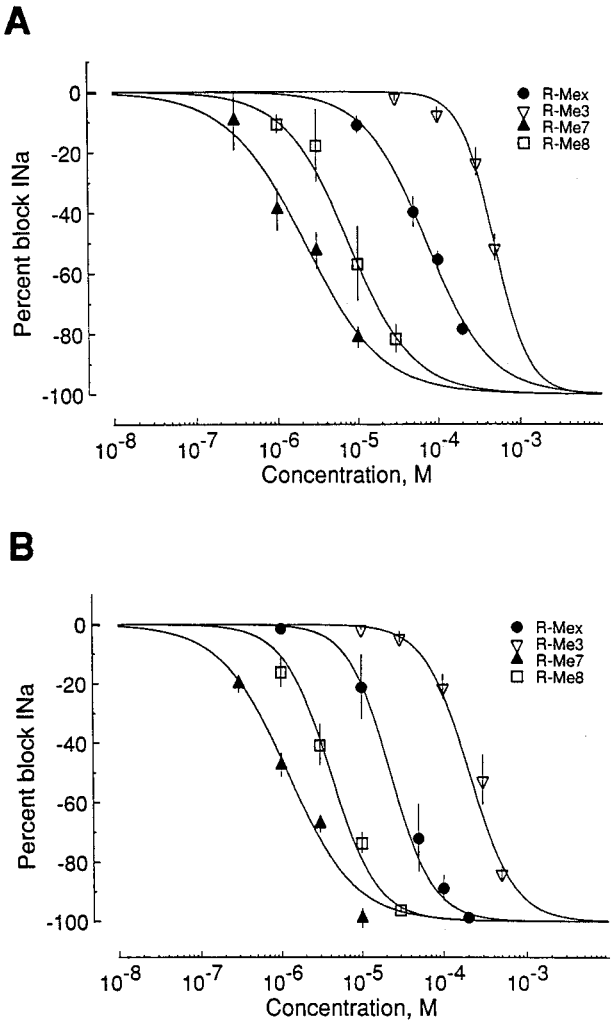

Fig. 4. Concentration response curves for Mex and Me4 derivatives obtained by replacing one (Me7) or both (Me3 and Me8) methyl groups from the aromatic ring. In A are shown the curves for the tonic block at the h.p. of –100 mV, whereas in B are shown the curves for the use-dependent block at 10 Hz. Each point is the mean ± S.E. from three to seven fibers. The curves have been obtained by fitting the experimental points to the logistic equation described in *Materials and Methods*.

values were −74.8 ± 1.07 and −76 ± 1.1 mV (n = 47) with prepulse durations of 50 and 1000 ms, respectively, and the difference was not statistically significant (P = .44). Nonetheless, as already anticipated, Mex produced a greater decrease of the steady-state channel availability on increasing the length of the inactivating prepulse to 1000 ms (De Luca et al., 1995, 1997a; see also Fig. 5). In Fig. 6, the effects of the R-(−) enantiomers of the new Mex analogs on the h_∞ curves constructed with the protocol of 1000 ms are shown; all of them produced a significant and concentration-dependent shift of the curves toward more negative potentials, with a potency related to their ability to block peak $I_{Na.}$ Thus R-(−)Me7 was the most potent in this respect, producing a clear shift at concentrations as low as 1 μM, whereas R-(−)Me3 was the least potent. By comparing in Fig. 5 the shifts of $Vh_{1/2}$ produced by the drugs at a concentration close to the calculated IC_{50}, it results that Mex and its analogs with substitutions on the chiral carbon atom (Me4, Me5, and Me6) produce a greater shift than Me3, Me7, and Me8, likely for the stronger use-dependent behavior of the former group of compounds. This consideration is rather qualitative, because it was not possible to use for all the compounds a constant ratio between the real concentration used experimentally and the calculated IC_{50} value (concentration/IC_{50}), thus leading to over- or underestimation of the drug potency in producing the h_∞ shift. Taking this consideration into account, the above analysis is validated quantitatively if one compares in Fig. 5 the $Vh_{1/2}$ shifts produced by compounds having the same ratio concentration/$IC_{50,}$ such as Me5 and Me8 (ratio = 1.2), Me6 and Me7 (ratio = 1.4), and Mex and Me3 (ratio = 0.6). Accordingly, all the compounds were more effective in producing the left shift at 1000 ms than at 50 ms of prepulse duration (Fig. 5) and the difference in the amount of shift produced with the two protocols seemed also to be related to the use-dependent behavior of each compound. All of these observations suggest that the new analogs maintain the inactivated-channel blocking activity of Mex and that this accounts for the use-dependent mechanism of block. A certain stereoselective behavior of the compounds was also observed on the left-shift of the h_∞ at 1000 ms. For instance, 3 μM R-(−) Me7 produced a 12.6 ± 2.8 mV (n = 5) shift that was significantly greater than the 5.8 ± 0.25 mV (n = 5) shift produced by the same concentration of S-(+) enantiomer (P < .05), 50 μM R-(−) Mex, and 30 μM Me5 were 1.2 and 1.5 times more potent than the S-(+) isomers at the same concentrations, respectively, whereas no stereoselectivity was found for Me4.

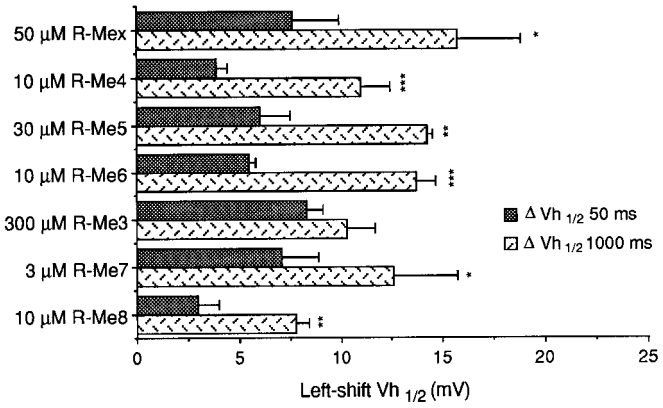

Fig. 5. Shift of the steady-state inactivation curves, calculated at the $Vh_{1/2}$ value, produced by the test compounds at concentrations close to the half-maximal effective ones in relation to the prepulse duration. For each drug the left shift produced has been calculated in any individual experiments versus the related $Vh_{1/2}$ control value in the absence of drug at either 50 or 1000 ms prepulse duration. The individual values have been averaged. Each group of bars shows the shift of the h_∞ curve produced by each compound in the two experimental conditions and each point is the mean ± S.E. of three to six individual determinations. Significant difference between left shift values at 50 and 1000 ms, $^*P$ < .05; $^{**}P$ < .01; $^{***}P$ < .005.

Discussion

Newly synthesized derivatives of Mex were tested on sodium channels of native skeletal muscle fibers to clarify the structural requirements necessary to get potent and use-dependent channel blockers, potentially useful for a safer treatment of the hyperexcitability of the myotonic syndromes. We found that the groups on the asymmetric carbon atom are pivotal for the drug potency. The replacement of the methyl group of Mex with apolar ones, such as a phenyl, a benzyl, or an isopropyl moiety, led to a 3- to 10-fold increase in the potency, and we found a clear correlation between the calculated K_d for both voltage- and use-dependent block and the lipophilicity of the above substituents. Similar results have been observed previously with tocainide analogs (Tricarico et al., 1991; De Luca et al., 1997a), corroborating the idea of a common binding site and that the asymmetric center strongly influences the disposition of the molecule at the receptor. Our finding is in line with the first view of Wang et al. (1993), that the receptor for LA-like drugs has one or two large hydrophobic domains that can accommodate up to a 12-carbon atom chain or a phenyl group near the terminal amino group. These results also corroborate the presumed LAs binding site drawn with mutagenesis experiments (Ragsdale et al., 1994, 1996; Wright et al., 1998; Li et al., 1999) and in turn strengthen the idea that the molecular characterization of the receptor can lead to a rational design of more effective therapeutic agents. The presumed receptor for LA-like drugs is on the D4-S6 segment of the α-subunit, a region highly conserved between channel types and species, because change in amino acid hydrophobicity in this region greatly modifies the potency and the use-dependent block by LA-like drugs (Ragsdale et al., 1994, 1996; Marban et al., 1998; Wright et al., 1998; Li et al., 1999). Ragsdale et al. (1994) first proposed a key role of two aromatic amino acids lining the pore, the Phe 1764 and Tyr 1771, in type II brain channel, whose distance on the α helix matches that between the amino group and the aromatic ring of LAs. Corresponding residues in the skeletal muscle sodium channels (μl) (Phe 1579 and Tyr 1586) account for cocaine-blocking activity (Wright et al., 1998). The Phe residue, and especially its aromatic nature, seems to be pivotal for open and inactivated channel block by drugs (Ragsdale et al., 1996; Li et al., 1999). Accordingly, we found that Mex derivatives with an aromatic ring near the terminal amino group, as Me4 and Me6, are potent blockers, likely for a stronger intermolecular force versus the methyl group with the aromatic moiety of the amino acid (Phe), due to an aromatic-aromatic (π-π) interaction. The cation-π interaction, proposed to occur between the aromatic ring of the amino acid residue and the protonated amino group of the drug (Dougherty, 1996; Li et al., 1999), seems less likely to account for the increased potency found

in the present study. In fact, Mex is present almost completely in the charged form at physiological pH; a likely increase in the p*K*a resulting from substitutions will therefore effect slight modification in the proportion of charged molecule in the physiological environment used. Accordingly, the ability of Me5 to be more potent than Mex but less potent than Me4 and Me6 can be due to the intermediate physicochemical characteristic of the isopropyl group. It has to be underlined that stereoselectivity, due to the carbon atom on which the substitutions are made, is detectable with Mex and Me5, whereas Me4 is poorly stereoselective. The lack of stereoselectivity in this highly potent analog may be explained by assuming two different ways of binding, depending on the nature of the substituents on the stereogenic center. When the group linked to the asymmetric center is small or poorly hydrophobic, as the methyl one of Mex, the pivotal interaction is mediated by the aryloxy moiety that is thought to influence the disposition of the molecule at the receptor (Hill et al., 1988; Zamponi and French, 1994). The consequent alignment of the molecule will lead to the second hydrophobic interaction (CH_3) and then to the third interaction (NH_2), this latter important for stereoselectivity. However, the presence of an aromatic group on the asymmetric center will allow another tight π-π interaction; thus the two lipophilic parts are preferentially accommodated, achieving a strong complimentary two-point interaction that minimizes the stereospecific requirements of the receptor, especially if these are not very stringent (Fig. 7). This is indeed the case of sodium channels for which the difference in potency between opposite enantiomers amounts at 2- to 5-fold (Hill et al., 1988; Tricarico et al., 1991; De Luca et al., 1995, 1997a). Hence, the nature of the groups on the asymmetric center can modulate the effect of the structural changes on the distal aryloxy group and likely the interaction between this latter and the other aromatic amino acid in the binding site (Tyr). In fact the simple removal of the two *ortho*-methyl groups from the phenyl ring of Mex, as in Me3, strongly decreases the drug potency, likely for a weakening of the interaction with the receptor due either to a withdrawal of electronic cloud or to a greater number of conformations that the less constrained ring can assume. However, the same chemical modification on the phenyl derivative of Mex, as in Me7 and Me8, is not as dramatic as expected, being minimized by the critical strong interaction occurring at the former hydrophobic site. Actually, Me7 was the most potent among the new derivatives and also showed a maintained stereoselectivity during use-dependent blockade, the *R*-(−) enantiomer being in this condition up to 26-fold more potent than *R*-(−)Mex. Thus the concomitant presence of the phenyl group on the chiral center and of only one methyl on the aromatic ring may favor a steric conformation of the molecule that allows an optimal and fast interaction with the receptor. A likely hypothesis is that the absence of the methyl group may allow the drug molecules to be accommodated more fully in the

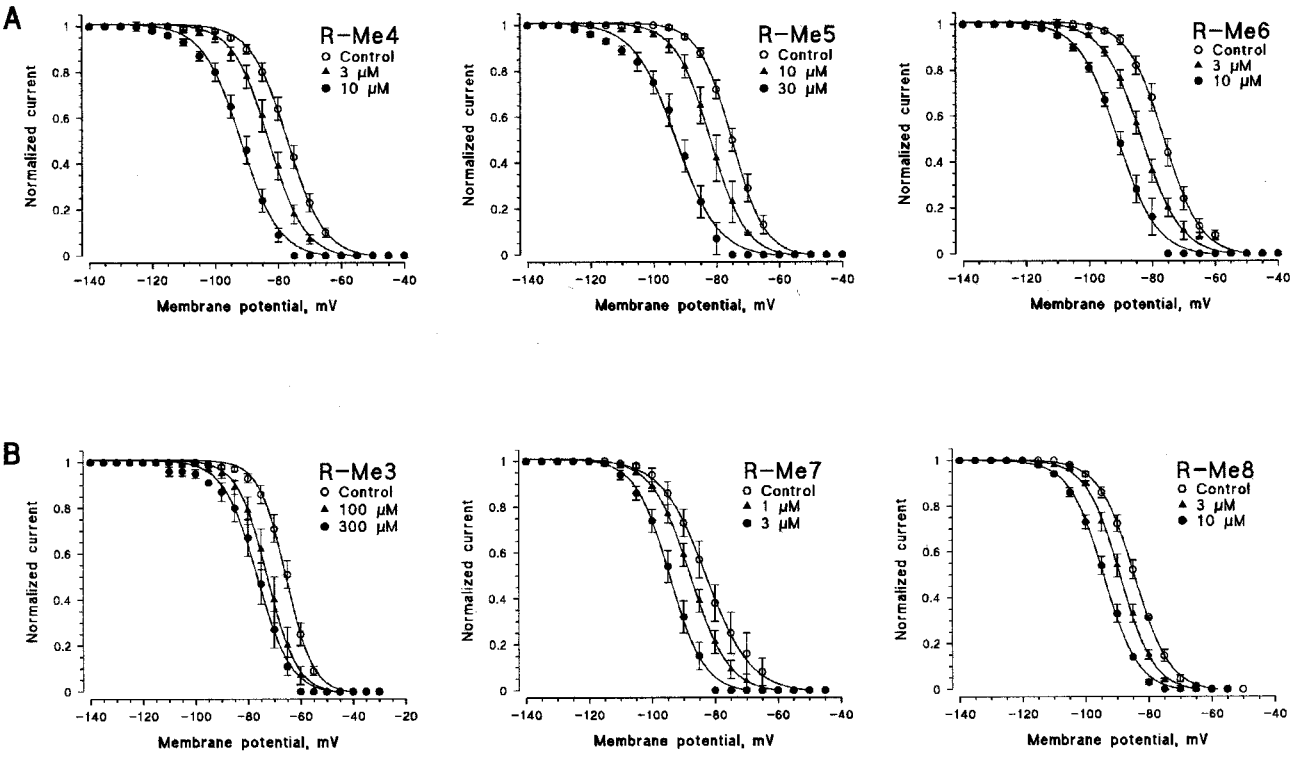

Fig. 6. Steady-state inactivation curves constructed with prepulse duration of 1000 ms, and effect of increasing concentrations of the *R*-(−) enantiomers of Me4, Me5, and Me6 (A) and Me3, Me7, and Me8 (B). At each membrane potential is shown the current amplitude normalized to the maximal I_{Na} value obtained at −140 mV. Because a certain variability exists between controls, the effect of each drug has been compared with the control h_∞ curve recorded in the same set of experiments. Each point is the mean ± S.E. of three to seven experiments. The curves have been fitted by a single Boltzmann distribution as described in *Materials and Methods* and allowed the calculation of the membrane $Vh_{1/2}$. The calculated $Vh_{1/2}$ for the curves shown in the figure are the following (in mV): Me4: Control = −76.9 ± 0.19, 3 μM = −82.9 ± 0.2, 10 μM = −91.8 ± 0.3; Me5: Control = −75 ± 0.16, 10 μM = −81.8 ± 0.17; 30 μM = −92.8 ± 0.4; Me6: Control = −76.2 ± 0.16, 3 μM = −83.3 ± 0.13, 10 μM = −90.9 ± 0.3; Me3: Control = −65.5 ± 0.2, 100 μM = −72.6 ± 0.2, 300 μM = −76.4 ± 0.4; Me7: Control = −82.8 ± 0.2, 1 μM = −87.8 ± 0.1, 3 μM = −94.4 ± 0.23; Me8: Control = −84.5 ± 0.1, 3 μM = −89.0 ± 0.14, 10 μM = −94.2 ± 0.19.

channel receptor site and thereby further enhance the interaction of the newly added aromatic ring with its binding site, because only small changes in the distance of π-π interactions would have a significant effect on affinity.

Other parts of the channel protein might equally contribute to the drug binding site. A point-mutation study by Nau et al. (1999) proposes that residue N434 of the D1-S6 segment of μ1 sodium channel, known to account for batrachotoxin binding, plays a role in the potency of bupivacaine enantiomers, supporting our view of a third point of interaction for chiral compounds. Also, other amino acids in D4-S6 not facing the pore, as well as charged residues in the selectivity filter able to modulate the movement of the charged drug to and from the receptor in the hydrophilic pathway of the open channel, can influence drug binding (Sunami et al., 1997; Wright et al., 1998). However, alteration of channel gating and kinetic often results from mutagenesis; because the drug effect is strongly channel-state dependent, the hypothesis drawn from mutagenesis experiments have to be verified with structure-activity relationship studies of drugs on native channel (Kuo, 1998).

All of the compounds tested showed a use-dependent behavior that was comparable (Me3, Me7, and Me8) or higher (Me4, Me5, and Me6) than that of Mex. Apart from Me3, all of the other compounds have higher molecular weight, lipophilicity, and higher affinity toward the receptor with respect to Mex. Lipophilicity seems unlikely to account for the high use-dependent behavior, because lipophilic drugs should rapidly equilibrate with the membrane phase and give a rapid recovery from block (Liu et al., 1994). The maintained or increased use-dependent behavior can be due to a concomitant increase of molecular weight and to the high p*K*a values that favor the presence of the drug in the charged form (Yeh and TenEick, 1987; Ehring et al., 1988; Wang et al., 1993; Liu et al., 1994). The high affinity toward the receptor of our inactivated channel blockers can also contribute to use-dependent behavior by slowing the unbinding kinetic (Courtney, 1990; De Luca et al., 1997a; Quan et al., 1996).

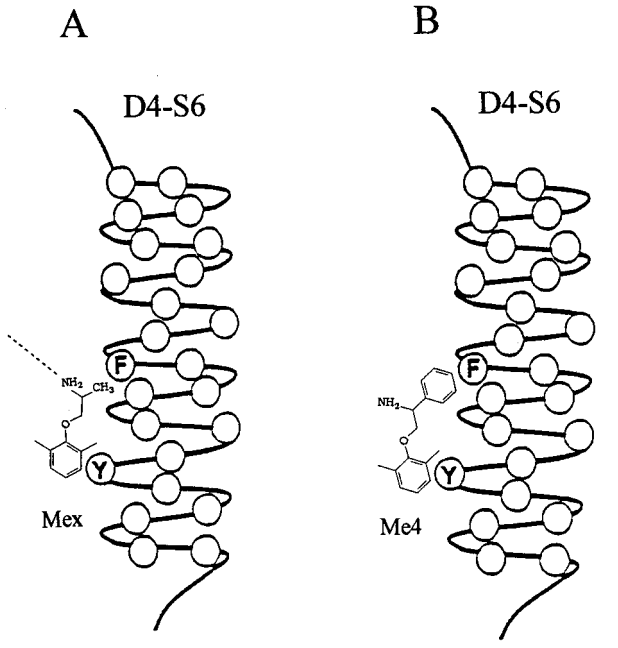

Fig. 7. A model for binding of Mex (A) and its phenyl derivative (Me4) (B) on transmembrane segment D4-S6 of sodium channel, according to the previously known location of the LA receptor (Ragsdale et al., 1994, 1996). For Mex, a strong hydrophobic interaction occurs between the aryloxy moiety of the drug and the aromatic Tyr amino acid that influences the position of the molecule, driving the following hydrophobic interaction of the methyl group with the Phe residue and a third interaction of the amino group with a point that is not yet precise (dotted line; direction is arbitrary). Such a three-point interaction will lead to stereoselective behavior. For Me4, the presence of the phenyl group favors another tight π-π interaction, the two hydrophobic parts of the drug being preferentially accommodated in a two-point binding. The consequent change in drug conformation can weaken the third interaction, minimizing the stereospecific requirement of the receptor. Although speculative, the model is based on the results obtained with the whole series of Mex derivatives tested here.

We have previously demonstrated a correlation between the potency of Mex and some related compounds in blocking voltage-gated sodium channels and their ability to solve in vitro the abnormal hyperexcitability of intercostal muscle fibers of myotonic adr/adr mouse (De Luca et al., 1997b). The antimyotonic activity was positively correlated with both use-dependent behavior and tonic block, the former accounting for a selective action, i.e., a higher potency on myotonic than on healthy mice muscle (De Luca et al., 1997b). The compounds described in this study may therefore represent beneficial antimyotonic agents because the increase in tonic block is accompanied by an increased or maintained use-dependent behavior. However, additional experiments are required to establish the possible tissue selectivity of the new Mex derivatives, because another crucial point for a safer treatment of muscle disorders is the use of drugs with fewer side effects on cardiac and neuronal excitability (Wang et al., 1996; Wright et al., 1997).

Recent evidence has shown that Mex or related compounds are effective in reducing sodium currents flowing through sodium channels in which inactivation is impaired from genetic mutations leading to either myotonia in skeletal muscle or inherited arrhythmia in heart (Dumaine and Kirsch, 1998; Sah et al., 1998). We also found that Me5 is more potent than Mex in reducing the persistent sodium current flowing through ATX-II-modified noninactivating channels, a pharmacological model of sodium channel myotonia (Desaphy et al., 1999a). Therefore, the new potent inactivated-channel blockers can have interesting therapeutic potential both for treating symptoms (hyperexcitability) and for directly counteracting the channel malfunction in the above pathologies.

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