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ORIGINAL ARTICLE

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Inhibition of frog skeletal muscle sodium channels by newly synthesized chiral derivatives of mexiletine and tocainide

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Abstract To search for potent use-dependent blockers of skeletal muscle sodium channels as potential antimyotonic agents, the actions of newly synthesized chiral analogs of mexiletine and tocainide were tested in vitro on sodium currents of single fibers of frog semitendinosus muscle by vaseline-gap voltage clamp method. The effect of each drug on the maximal peak Na^+ transient ($I_{Na\ max}$) was evaluated as both tonic and use-dependent block by using infrequent depolarizing stimulation and trains of pulses at 2–10 Hz frequency, respectively. The mexiletine analog 3-(2,6-dimethylphenoxy)-2-methylpropanamine (Me2), having an increased distance between the phenyl and the amino groups, was less potent than mexiletine in producing a tonic block but produced a remarkable use-dependent block. In fact, the half-maximal concentration (IC_{50}) for tonic block of S(–)-Me2 was 108 μM vs. 54.5 μM of R(–)-mexiletine, but the IC_{50} was 6.2 times lowered by the 10 Hz stimulation with respect to the 2.4fold decrease observed with mexiletine. The R(–)-mexiletine and the S(–)-Me2 were about twofold more potent than the corresponding enantiomers in producing a tonic block, but the stereoselectivity attenuated during use-dependent blockade. The more lipophilic 2-(4-chloro-2-methylphenoxy)-1-phenylethylamine (Me1), presently available as raceme, produced a potent and irreversible tonic block of the sodium currents with an IC_{50} of 29 μM, but had a less pronounced use-dependent inhibition, with a 1.9fold decrease of the IC_{50} at 10 Hz. The R(–) isomer of 2′,6′-valinoxylidide (To1), a tocainide derivative with an increased hindrance on the chiral carbon atom, was twofold (IC_{50} = 209 μM) and tenfold (IC_{50} = 27.4 μM) more potent than R(–)-tocainide in tonic and use-dependent block, respectively. Tocainide was almost devoid of stereoselectivity, whereas the eudismic ratio of To1 [(IC_{50} S(+)-To1/IC_{50} R(–)-To1] was 1.7. As for mexiletine and Me2, the stereoselectivity of To1 was the weaker the higher the frequency of stimulation. The cyclic pyrrolo-imidazolonic tocainide analog To2 produced a small tonic block at 500 μM, and 1 min stimulation at 10 Hz was needed to show up a 50% block of $I_{Na\ max}$. All the compounds produced a left-shift of the steady-state inactivation curve correlated positively with the extent of use-dependent inhibition, with the exception of the cyclic To2 that acted as an open-channel blocker. The highly use-dependent blockers Me2 and To1 might be promising drugs to solve high frequency discharges of action potentials typical of myotonic muscles. Concomitantly the high potency of Me1 and the open-channel block exerted by To2 can represent important features to get selective blockers for skeletal muscle sodium channels.

Key words Na^+ channels · Skeletal muscle · Mexiletine and tocainide analogs · Enantiomers · Use-dependent block · Inactivated- and open-channel block · Myotonia

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Introduction

Skeletal muscle can be affected by dominant and recessive forms of genetic channelopathies, such as the myotonic syndromes, which are responsible for abnormal membrane hyperexcitability. Combined studies of molecular biology and electrophysiology allowed to correlate the various clinical phenotypes of the disease with the specific genetic alteration of either sodium or chloride channels, as well as to get insight in the channel malfunction consequent to the gene mutation (Lehmann-Horn and Rüdel 1996; Cannon 1996; Fahlke et al. 1995). As far as voltage-gated sodium channels are concerned, the various mutations impair to a different extent the channel inactivation, leading to persistent inward sodium currents responsible for membrane depolarization (Lehmann-Horn and Rüdel 1996; Cannon 1996).

Drug therapy of myotonic syndromes

If available, drugs able to directly ameliorate the channel function altered by the genetic disease would be of first choice, but the studies about the drug sensitivity of the mutated channels have just began (Fan et al. 1996). The current therapy for all forms of myotonia is symptomatic and consists in the block of sodium channels for solving the membrane hyperexcitability (Rüdel et al. 1980; Jackson et al. 1994; Lehmann-Horn and Rüdel 1996). In particular, the orally effective lidocaine derivatives Mexiletine and Tocainide, have been found to be beneficial in myotonic patients (Rüdel et al. 1980; Streib 1986; Jackson et al. 1994). Most of their relevant clinical usefulness is due to the ability to block voltage-gated sodium channels in a use-dependent manner that results in a stronger potency of the drug in situations of excessive firing of action potentials and/or prolonged depolarization, as occurring in the diseased tissues, rather than on physiological excitability. The use-dependent block is due to a preferential drug binding to the receptor on the α subunit of the sodium channel when this latter is open and/or inactivated and to an accumulation of such a block in consequence of a slow recovery from inactivation of the drug-bound channels during membrane repolarization (Catterall 1987; De Luca et al. 1991; Sunami et al. 1993; Ragsdale et al. 1994). Nevertheless Mexiletine and Tocainide have relevant side effects, especially at the hematopoietic and central nervous system level (Roden and Woosley 1986). Also, the doses effective for antimyotonic activity are in the same range, if not higher, of those used to get antiarrhythmic action, with the possibility of adverse effects on cardiac function as well (Rüdel et al. 1980; Streib 1986).

Rationale to get more selective antimyotonic agents

In searching for more selective antimyotonic agents, one should take into account that sodium channels of the various tissues are genetically distinct and show different kinetic and pharmacological properties. Cardiac and skeletal muscle types of sodium channels, apart from the different sensitivity towards tetrodotoxin (Catterall 1987), are also differently affected by lidocaine, the cardiac type being intrinsically more sensitive than skeletal muscle one, implying possible structural differences in channel sites important for drug binding (Wang et al. 1996). The pharmacological profile is further exacerbated by the long lasting inactivated states of the cardiac channels that favour inactivated-state block by the drug, whereas skeletal muscle sodium channels are also sensitive to open channel block, in view of the faster recovery from inactivation and the brief action potential duration (Wang et al. 1996). All these observations support the possibility to design use-dependent blockers of sodium channels more selective on skeletal muscle than on heart.

New chiral analogs of mexiletine and tocainide

The aim of the present study was to synthesize new chiral analogs of mexiletine and tocainide and to screen their effects on the sodium currents of frog muscle fibers recorded by means of the vaseline gap voltage clamp method. The choice of testing compounds in the enantiomeric pure form derives from the previous findings that the R enantiomers of this class of compounds are more potent than the S ones on sodium channels of various tissues (Yeh 1980; Sheldon et al. 1987; Hill et al. 1988; Tricarico et al. 1991). The presence of a stereospecific site on sodium channels of adult skeletal muscle fibers has been confirmed by us by using mexiletine enantiomers (De Luca et al. 1995). The possible use of compounds in enantiomeric pure form may be clinically useful in consideration of their enantioselective metabolism (Block et al. 1988; Knoche et al. 1996). Thus, the new analogs differed from the parent compounds for structural modifications at the level of the chiral carbon atom that is responsible for the spatial disposition of the molecule at the receptor. The newly synthesized compounds shown in Fig. 1 were chosen to evaluate the effects of i) the increased steric hindrance on the chiral center of both α-amino anilide (To1) and aryloxy-alkylamino series (Me1); ii) the increased distance between the chiral carbon atom and the amino terminal group (Me2) and iii) the introduction of the chiral atom in a rigid cyclic structure (To2). Other than modify the stereoselective behaviour, these changes can affect the physicochemical properties of the drug, such as lipophilicity and pKa (Byrnes et al. 1979). This latter parameter can affect the ratio between charged and uncharged form at physiological pH, the charged form being the one contributing to potency and use-dependent behaviour (Wang et al. 1993; 1994). Lipophilicity can be further increased by the presence of chlorine substituent on the aromatic ring and can also affect drug potency (Quan et al. 1996).

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 (21 ± 2° C). 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 70–100 μm and 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). 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 upon increasing the amplifier gain. Then the solution in the pool A was replaced with the external solution and after about 10 min of equilibration the recordings were performed at 10° C. The holding potential (h.p.) used was –100 mV. Sodium currents were recorded

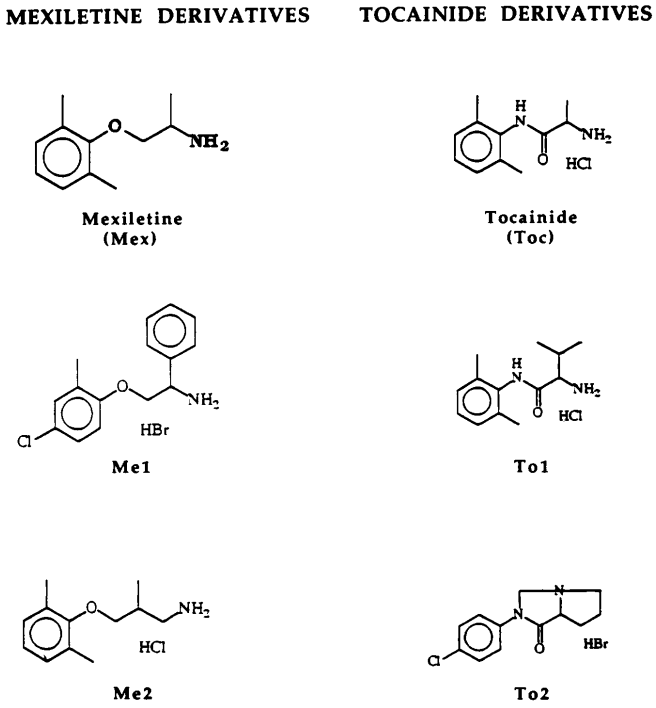

Fig. 1 Chemical structure of the chiral analogs of mexiletine and tocainide

using an amplifier connected via a A/D and D/A Digidata 1200 Interface (Axon) 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 (pClamp6 software package; Axon Instruments, Foster City, Calif., USA). The currents flowing in response to depolarizing command voltages were low pass filtered at 10 kHz (Frequency Devices, USA), visualized on an oscilloscope and sampled at 20 kHz. When necessary, leak and capacities currents were subtracted by P/4 method. Briefly the P/4 protocol applies 4 scaled subpulses each of 1/4th of the amplitude of the main stimulus (test pulse, see below) prior the application of this latter. The scaled subpulses produce only passive membrane responses that are then summed and subtracted from the response obtained with the test pulse, thus allowing only the recording of the active membrane response due to ionic currents (Bezanilla and Armstrong 1977). The acquired traces were analyzed later using Clampfit program (pClamp6 software package; Axon Instruments).

Drugs and solutions. The following solutions (mM) were used: Normal physiological solution: NaCl 115, $CaCl_2$ 1.8, Na_2HPO_4 2.15, NaH_2PO_4 0.85; External solution: NaCl 77, Choline-Cl 38, $CaCl_2$ 1.8, Na_2HPO_4 2.15, NaH_2PO_4 0.85; Internal solution: CsF 105, MOPS 5, $MgSO_4$ 2, EGTA 5, Na_2ATP 0.55; (pH was adjusted at 7.2 with a standard NaOH concentrated solution).

All the compounds tested (Fig. 1) were prepared in our laboratories in single enantiomeric forms as hydrochloride or hydrobromide salts according to procedures described in details elsewhere (Franchini et al. 1993, 1994). All compounds were fully characterized by routine spectroscopic analyses; analytical results for C, H and N, were within ± 0.4% of the theoretical values. It has to be noted that the absolute configuration of (–)- and (+)-Me2 enantiomers is S and R, respectively. In spite of this S(–)-Me2 has a conformation of the groups linked to the chiral center similar to that of R(–)-Mex (Fig. 2), the same being also true for R(+)-Me2 and S(+)-Mex. The enantiomers were stable under the condition used in the present study. Stock solutions of each compound were prepared by dissolving the compounds in external solution, whereas stock solutions in DMSO (100µl/mg) were used for Me1. DMSO, at the highest concentration used for dilution (0.2%), was without effect on the parameters recorded. All chemicals used were of analytical grade and obtained from Sigma Chemical Company (St. Louis, Mo., USA).

Pulse protocols and statistical analysis. The curves describing the voltage dependence of sodium current (I/V curve) were constructed with a cycle of 10 ms test pulses from the h.p. to increasing potentials (from –60 to +60 mV) and used to evaluate the tonic block exerted by each compound. Thus, the intervals between each test pulse were long enough (~ 3 s) to allow complete recovery of sodium channel from inactivation. The use-dependent block exerted by drugs was evaluated by using trains of 10 ms test pulses from the h.p. to –20 mV at 2 and 10 Hz frequency for 30 s–1 min. The 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 "activable" 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 18–20 times with the prepulse potential value increased each time by 5 mV steps. The evaluation of recovery from inactivation in the absence and presence of drugs was evaluated by applying 30 s trains of pulses (from h.p. to –20 mV) at decreasing frequencies from 10 Hz to 0.1 Hz. For each train the current peaks recorded at the steady-state was considered to be due to the fraction of channels that had recovered from inactivation during the intervals between the pulses (De Luca et al. 1991). The area under the inward sodium current transients, used to evaluate the open-channel block mechanism, has been obtained by routine data analysis performed by the Clampfit program.

The data were expressed as mean ± standard error of the mean (SEM). The estimates of SEM of normalized Na^+ currents (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 $I_{Na\ max}$ (IC_{50}) were determined by using a non-linear least squares fit of the concentration-response curves to the following logistic equation:

$$\text{Effect} = -100/\{1 + (K/[\text{drug}])^n\}$$

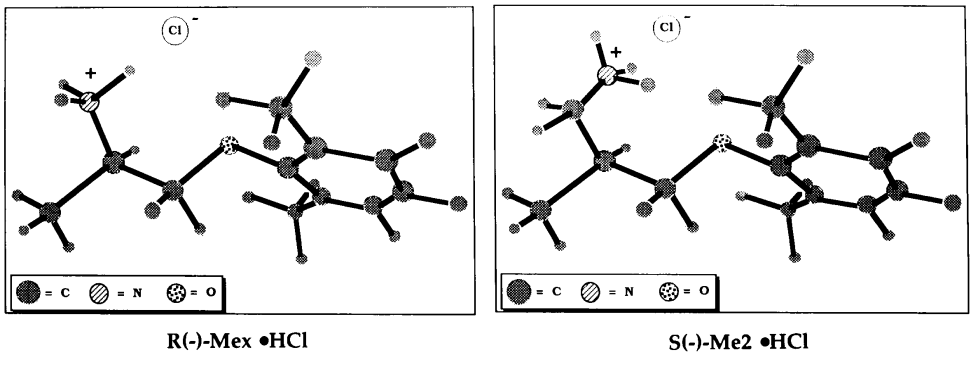

Fig. 2 Spatial conformation of R(–)-Mex and S(–)-Me2 derived from X-ray crystallographic analysis

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 h_∞ curves have been fitted with a single Boltzmann distribution and the potential at which 50% of the sodium channels were inactivated ($Vh_{1/2}$) was calculated at the inflection point of the curves (De Luca et al. 1991). Single or double exponential decay equations have been used to fit experimental points and calculate the time constants for either the development of use-dependent block or the recovery from inactivation of the drug-blocked channel.

Statistical significance of differences between mean values has been estimated by unpaired Student's *t*-test.

Results

Tonic and use-dependent block of Na^+ channels by chiral derivatives of mexiletine and tocainide

The tonic block exerted by each test compound was evaluated during the construction of the current-voltage (I/V) relationship and calculated as percent reduction of the inward sodium transient at the voltage of its maximum ($I_{Na\ max}$) (about –30 mV). The interval between each successive depolarizing step was kept long enough (~ 3 s) so to allow sodium channels to recover from previous block and to evaluate drug interaction with the channels at the holding potential, i.e. in the resting state. Afterwards the use-dependent behaviour of each compound was evaluated with trains of test pulses from the h.p. 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 sodium current over the tonic block was observed, that progressively cumulated until a new equilibrium was reached (Fig. 3). The value of the current at this 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).

Mexiletine derivatives

The elongation of the alkyl chain of Mex by the insertion of a methylene moiety, as in Me2, led to a decreased potency in producing a tonic block of the $I_{Na\ max}$ with respect to Mex, as shown in Fig. 3, in which the effects of 50 μM of R(–)-Mex and S(–)-Me2, the enantiomers with the same conformation (Fig. 2), are compared. On the contrary the high frequency protocol at 2 and 10 Hz showed a remarkable use-dependent behaviour of Me2. In fact at 10 Hz the final block produced by 50 μM S(–)-Me2 was comparable to that produced by the same concentration of R(–)-Mex (Fig. 3). Consequently, the concentration-response curve for tonic block of S(–)-Me2 was shifted to the right with respect to that of R(–)-Mex, but the curves of the two compounds became almost overlapping when the block was calculated at the end of the 10 Hz stimulation (Fig. 4A). The calculated value of IC_{50} of S(–)-Me2 was twofold higher than that of R(–)-Mex for tonic block

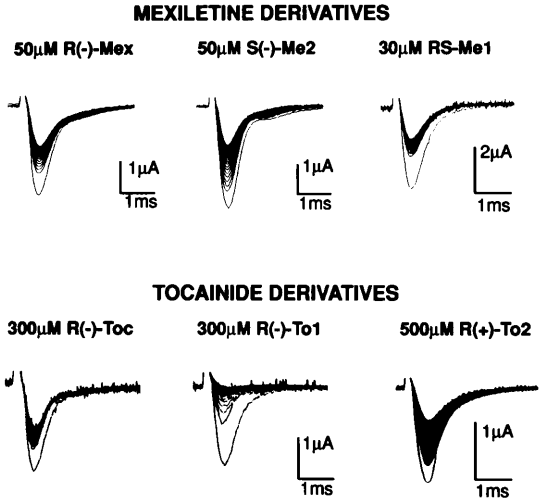

Fig. 3 Na^+ current transients recorded by the three-vaseline gap voltage clamp method from single fibers of frog semitendinosus muscle in the absence and in the presence of the test compounds. *In the upper panel* are shown the effects of 50 μM S(–)-Me2 and 30 μM RS-Me1 in comparison with the effects produced by 50 μM R(–)-mexiletine (Mex). *In the lower panel* are shown the effects of 300 μM R(–)-To1 and 500 μM R(+)-To2 in comparison with the effects produced by 300 μM R(–)-Tocainide (Toc). The control traces recorded in the absence of drug are clearly evident being the greatest ones and have been recorded with a 10 ms test pulse from the holding potential of –100 mV to –20 mV. A reduced Na^+ transient elicited by the same test pulse in the presence of the drug was due to the tonic block exerted by the compound. A repetitive stimulation at the 10 Hz frequency produced a cumulative reduction of the Na^+ currents over the tonic block, until a new equilibrium was reached, due to the use-dependent blockage of the channels

but decreased remarkably in a frequency-dependent manner (Table 1). At 10 Hz, the IC_{50} of S(–)-Me2 was 6.2 times lower with respect to that of tonic block vs. the 2.4fold lowering of IC_{50} observed for R(–)-Mex. Both Mex and Me2 produced a stereoselective block of the sodium currents, with the R(–)-Mex and the S(–)-Me2 more potent than the corresponding enantiomers (Table 1); however when increasing the stimulation frequency the stereoselectivity of the two compounds attenuated. In fact the eudismic ratios [IC_{50} Distomer/IC_{50} Eutomer] of Mex decreased from 2.1 found for tonic block to 1.8 and 1.2 at 2 Hz and 10 Hz, respectively, whereas those for Me2 decreased from 1.7 to about 1 at both 2 Hz and 10 Hz (Table 1). In the attempt to gain insight in the attenuation of stereoselectivity during use-dependent blockade the time course of development of block at 10 Hz frequency has been evaluated for both the enantiomers of Me2. As shown in Table 2, the attainment of the equilibrium for both the enantiomers of Me2 occurred with a double exponential kinetic. The process was the faster the higher the concentration of the drug, i.e. in the presence of 100 μM of S-(–)-Me2 the cumulative block occurred mostly (90%) with a fast kinetic and a reduction of both τ_{fast} and τ_{slow} was observed. Interestingly, in the presence of 100 μM of R(+)-Me2 the equilibrium was attained with a kinetic overlapping that observed with 50 μM of S(–)-Me2,

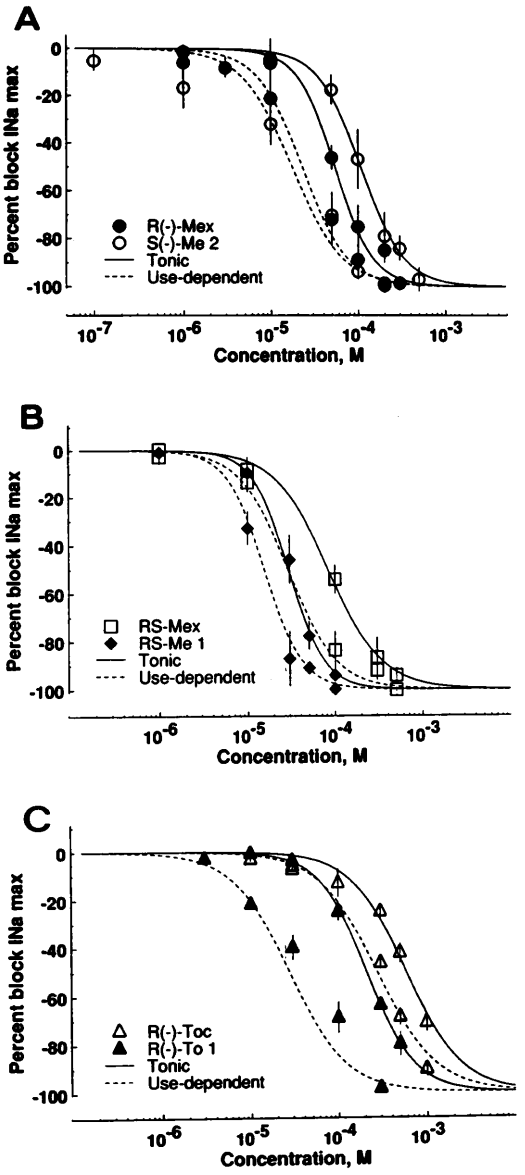

Fig. 4 Concentration-response curves for tonic (*continuous line*) and use-dependent block of the Na^+ currents (*dashed lines*) obtained with **A** R(–)-Mex and S(–)-Me2; **B** RS-Mex and RS-Me1 and **C** R(–)-Toc and R(–)-To1. Each value is the mean ± SEM from 3 to 7 fibers of the percent block of $I_{Na\ max}$ observed in the presence of each concentration of the drugs vs. $I_{Na\ max}$ in the absence of a drug in the same fiber. The amount of block has been evaluated in conditions of infrequent depolarizing pulsing (tonic block) or after 30 s of a repetitive stimulation at 10 Hz. The curves fitting the experimental points were obtained using the logistic equation described in the Methods. The mean values of $I_{Na\ max}$ in the absence of drugs were 9.0 ± 1.6 mA/cm^2 (n = 14), 10.1 ± 1.7 mA/cm^2 (n = 9) and 9.4 ± 0.5 mA/cm^2 (n = 13) for **A**, **B** and **C**, respectively

Table 1 Concentrations for half-maximal tonic and use-dependent block of sodium currents by chiral analogs of mexiletine and tocainide

Compound	Tonic block IC_{50} (μM)	Use-dependent block	
		2 Hz IC_{50} (μM)	10 Hz IC_{50} (μM)
R(–)-Mex	54.5 ± 12	24.6 ± 3.2	22.8 ± 3.6
S(+)-Mex	114 ± 21	43.5 ± 6.5	27.4 ± 5.7
S(–)-Me2	108 ± 13	35.0 ± 7.0	18.1 ± 3.9
R(+)-Me2	179 ± 22	40.5 ± 14	23.7 ± 13
RS-Mex	83.3 ± 14.1	37.4 ± 7.8	28.9 ± 4.9
RS-Me1	29.2 ± 3.2	24.6 ± 3.2	15.5 ± 1.8
R(–)-Toc	583 ± 11	380 ± 7.0	274 ± 5.0
S(+)-Toc	523 ± 8.0	302 ± 7.0	222 ± 5.0
R(–)-To1	209 ± 9.0	69.0 ± 8.2	27.4 ± 1.7
S(+)-To1	355 ± 27	85.5 ± 5.4	30.4 ± 4.5
R(+)-To2	> 1 mM		~ 500 μM
S(–)-To2	> 1 mM		> 500 μM

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) and a use-dependent block calculated by using trains of depolarizing pulses at the frequency of 2 Hz and 10 Hz. The IC_{50} values have been obtained during non-linear least squares fit of the concentration-response data to the logistic equation described in the Methods

corroborating that the accumulation of block follows the same stereoselective behaviour observed during tonic block. The attenuation of stereoselectivity could also be due to the time course of recovery from inactivation of the drug-blocked channels that can be influenced by the unblocking kinetic of the enantiomers. This has been confirmed in Fig. 5 that shows the recovery from inactivation, measured as residual current at the end of trains of pulses at decreasing frequency from 10 Hz to 0.1 Hz (De Luca et al. 1991), in the absence and presence of Me2 enantiomers (100 μM). The recovery occurred as a monoexponential process for both the enantiomers. The time course of the recovery was similar for the two enantiomers, the time constant being 1.8 and 1.5 s for the S(–) and the R(+)-Me2, respectively. Thus at the interpulse duration of the 10 Hz frequency the channel block predominates over recovery leading to the apparently similar potency of the opposite enantiomers during use-dependent blockade. Similar results have been obtained with the enantiomers of mexiletine and of the other analogs (data not shown). Furthermore, the R(+)-Me2 showed a peculiar behaviour in the low range of concentrations (0.1–10 μM) in that it produced a slight but detectable increase of the peak $I_{Na\ max}$ that reached a maximum of +7.7 ± 3.9% (n = 9) at 1 μM, an effect only occasionally observed with the S(+)-Mex (data not shown).

The insertion of a phenyl group on the chiral carbon atom and of a chlorine atom in para position of the aromatic ring, along with the removal from this latter of one of the two ortho-methyl groups, led to the bulky and lipophilic Me1. This compound, available and tested as raceme, produced a remarkable tonic block of the $I_{Na\ max}$, as at 30 μM it was more effective than 50 μM of R(–)-Mex (Fig. 3). However, the use-dependent block produced by Me1 was much less pronounced with respect to both Mex and Me2. At the frequency of 10 Hz the further

Table 2 Time course parameters for the development of use-dependent block at 10 Hz frequency by enantiomers of the mexiletine analog Me2

Drug		*n*	A_{fast}	τ_{fast} (s)	A_{slow}	τ_{slow} (s)
S(–)-Me2	50 μM	4	0.65 ± 0.09	0.510 ± 0.1	0.35 ± 0.09	83 ± 31
	100 μM	6	0.91 ± 0.07	0.289 ± 0.07	0.09 ± 0.07	34 ± 16
R(+)-Me2	100 μM	4	0.61 ± 0.1	0.432 ± 0.04	0.39 ± 0.1	86 ± 35

The time course of block development by drug during the 10 Hz stimulation frequency has been evaluated by measuring the progressive decrease of the current amplitude at each successive pulse with respect to the current amplitude at the first pulse of the stimulation train. The full attainment of equilibrium during use-dependent blockade mostly occurred with a double exponential kinetic. The fast component (A_{fast}) predominates and occurred with time scale of hundreds of milliseconds (τ_{fast}). The residual slow component (A_{slow}) occurred with time course of seconds (τ_{slow}). S(–)-Me2, almost twice more potent than R(+)-Me2 on tonic block, reached the equilibrium the faster the higher the concentration used, so that at 100 μM the block occurred for the most with the fast process, both time constants being markedly decreased. On the contrary the kinetic found for 100 μM of R(+)-Me2 overlapped that of 50 μM S(–)-Me2. Each point is the mean ± SEM from n number of fibers

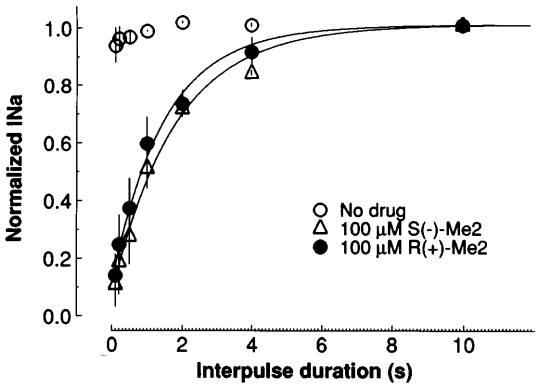

Fig. 5 Recovery from inactivation in the absence and presence of 100 μM of either S(–) or R(+)-Me2. *Each point* has been obtained by measuring the residual current at the end of 30 s trains pulses of decreasing frequencies from 10 Hz to 0.1 Hz. At each frequency the residual current at the steady state is a function of the number of channels that recovered from inactivation during the interval between pulses. The longer the interval between pulses the greater the number of channels that recovers, until an equilibrium is reached according to the time course of the process. Thus each point has been obtained by normalizing the residual current at the end of each train to the $I_{Na\ max}$ obtained at the longest interpulse used (0.1 Hz). The values expressed as mean ± SEM from 2–3 fibers have been plotted against the duration of the interval between pulses and fitted to a single exponential function

reduction of the $I_{Na\ max}$ over tonic block was modest and rapidly reached a new equilibrium. As shown in Fig. 4B, the concentration-response curve for tonic block of Me1 was clearly shifted to the left with respect to that of RS(±)-Mex, so that the calculated IC_{50} of Me 1 was almost three times lower than that of the parent compound (Table 1). However, in agreement with the less pronounced use-dependent behaviour of this analog with respect to Mex, the curve obtained at 10 Hz was also shifted to the left but to a less amount, the calculated IC_{50} value being 1.9 times lower than that of RS(±)-Mex. In contrast with Mex and Me2, the effects of Me1 were slightly reversible upon washout.

Tocainide derivatives

The tocainide derivative To1, with an increased steric hindrance on the chiral carbon atom due to the introduction of an isopropyl group in place of the methyl group, was more potent than the parent compound in producing both a tonic and a use-dependent block, as can be seen in Fig. 3 in which the effects of 300 μM of the R(–)-isomers of Toc and To1 are compared. Consequently, the concentration-response curves of To1 for both tonic and use-dependent block were shifted to the left with respect to those of Toc (Fig. 4C). The higher potency of To1 vs. Toc was particularly evident during the high frequency of stimulation. In fact the IC_{50} of R(–)-To1 was 2.7 times lower than that of R(–)-Toc for tonic block, but up to 10 times lower during the 10 Hz frequency of stimulation (Table 1). Under our experimental conditions, Toc showed almost no stereoselectivity for both tonic and use-dependent block. To1 was more stereoselective than Toc in producing the tonic block, the R-(–) enantiomer being the eutomer (e.r. = 1.7), although, as above described for Mex and Me2, the eudismic ratio decreased to 1.2 and 1.1 for use-dependent block at 2 Hz and at 10 Hz, respectively (Table 1).

The introduction of the chiral carbon atom in a rigid pyrrolo-imidazolonic cycle led to the derivative To2. This compound that differs from tocainide also for the presence of a chlorine atom in para position and no ortho-methyl groups on the aromatic ring to allow a certain degree of flexibility to the cyclic molecule, exhibited very low potency. In fact the R(+)-To2 produced at 500 μM only a 13 ± 0.7% (n = 2) tonic block of $I_{Na\ max}$ (Figs. 3 and 6). To2 produced a use-dependent block of $I_{Na\ max}$ related to the frequency of stimulation, but to show up this mechanism it was necessary to prolong the stimulation to 1 min (Fig. 3). The slow use-dependent behaviour was able to significantly increase the drug potency in concentration-dependent manner, so that 500 μM produced a nearly 50% block of $I_{Na\ max}$ (Figs. 3 and 6). Such a slow cumulative block was even slower to remove, the recovery being still uncomplete after either 10–15 min of rest at the h.p. or after a stimulation protocol with 500 ms hyperpolarizing pulses from the h.p. to –140 mV before eliciting the transients with the –20 mV test pulse.

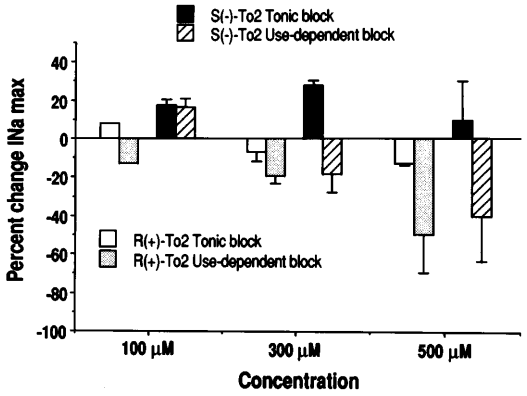

Fig. 6 Percent change of $I_{Na\ max}$ produced by R(+)-To2 and S(–)-To2 in the concentration range of 100–500 μM. Tonic block refers to the changes of $I_{Na\ max}$ observed during infrequent depolarizing stimulation and use-dependent block to the changes of $I_{Na\ max}$ observed after 1 min stimulation at 10 Hz. It has to be noted that, especially with S(–)-To2 and mostly during infrequent depolarizing stimulation, an increase rather than a block of $I_{Na\ max}$ has been observed. *Each bar* is the mean ± SEM from 2–3 fibers. $I_{Na\ max}$ in the absence of drug was 12 ± 1.2 mA/cm^2 (n = 5)

Although difficult to appreciate from a quantitative point of view due to the low potency, a certain stereoselectivity was also present in the cyclic tocainide derivative. Thus S(–)-To2 was almost devoid of tonic block up to 500 μM, but it produced an increase of the $I_{Na\ max}$, with a maximum increase of +27.5 ± 2.6% at 300 μM (Fig. 6). However, it similarly showed a slow use-dependent block that reached a 40% block at 500 μM after 1 min of stimulation at 10 Hz (Fig. 6).

Evaluation of the inactivated-channel block and of the open-channel block by chiral derivatives of tocainide and mexiletine

Mexiletine and tocainide are considered inactivated-channel blockers. On macroscopic currents this mechanism is observable as a shift of the steady state inactivation curves (h_∞) towards more negative potentials (Tricarico et al. 1991; De Luca et al. 1995), which implies a voltage-dependent reduction of the number of channels available for opening due to drug-favoured inactivation. Use-dependent block can be related to a preferential binding of both drugs when the channel enters states of inactivation occurring with time scales of hundreds of ms or seconds and thus slower than the fast inactivation (normally complete within 30–50 ms) responsible for the termination of the sodium transients (Collins et al. 1982). This mechanism can be evidenced as a greater drug-induced decrease of the steady state channel availability upon increasing the length of the inactivating prepulse beyond 50 ms (De Luca et al. 1991, 1995). Thus we tested the effects of the analogs on the steady-state inactivation curves constructed with prepulse duration of both 50 ms and of 1000 ms. Furthermore, it has been recently described that on skeletal muscle type of sodium channel, but not on the cardiac type, lidocaine exerts an additional

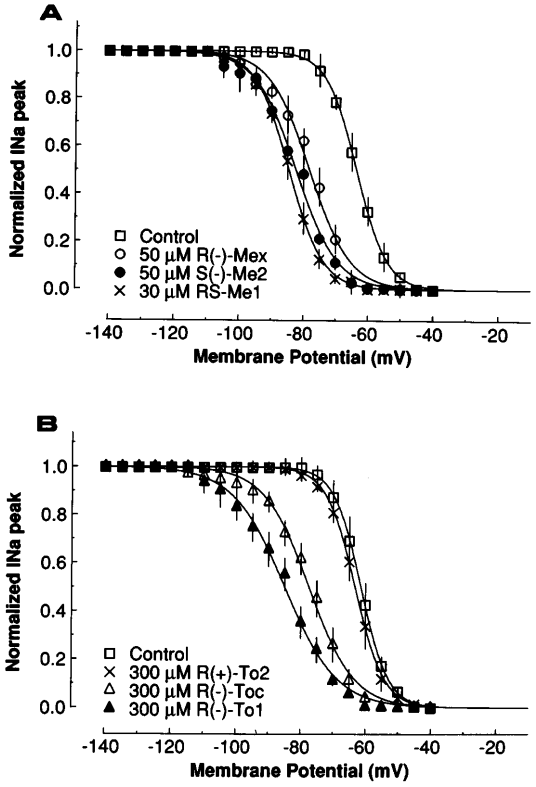

Fig. 7 Effect of **A** 50 μM R(–)-Mex and S(–)-Me2 and 30 μM RS-Me1; and **B** 300 μM of R(–)-Toc, R(–)-To1, and R(+)-To2 on steady-state inactivation curves constructed with a 1000 ms prepulse of variable value. The curves have been fitted by a Boltzmann distribution. The inflection point of the curves allowed to calculate the potential at which half of the channels are inactivated ($Vh_{1/2}$) both in the absence and in the presence of the drug. *Each point* is the mean ± SEM from 2 to 14 experiments

block during channel opening, a characteristic that may be of advantage to get drugs more selective on skeletal muscle (Wang et al. 1996). To assess if the new compounds could exert an open channel block a further analysis was performed on the sodium transients elicited with the protocol used for evaluating the tonic and the use-dependent block (single or trains of pulses from h.p. to –20 mV). In particular we evaluated the possible differences in the amount of block of sodium currents calculated on the area under the transients (which is a measure of open-channel probability) with respect to that calculated on the peak of $I_{Na\ max}$ as suggested by Wang et al. (1996). A greater block on the area vs. peak would be predictive of an additional block by drug occurring during channel opening (i.e. during the test pulse).

Mexiletine derivatives

Figure 7A shows the h_∞ curves obtained with 1000 ms prepulse in the presence of either 50 μM R(–)-Mex, 50 μM S(–)-Me2 or 30 μM of RS(±)-Me1. The three compounds produced a comparable left-shift of the h_∞ curve, as expected by taking into account the potency for use-de-

Table 3 Effects of chiral analogs of mexiletine and tocainide on the steady-state inactivation curves (h_∞) of sodium channels

Drug	Concentration (μM)	*n*	$\Delta Vh_{1/2}$ 50 ms (mV)	$\Delta Vh_{1/2}$ 1000 ms (mV)
R(–)-Mex	50	5	7.6 ± 2.3	17.1 ± 13.6
S(+)-Mex	50	7	7.4 ± 2.3	13.7 ± 2.7
S(–)-Me2	50	5	8.6 ± 2.5	16.3 ± 3.9
R(+)-Me2	50	3	6.4 ± 2.0	11.5 ± 3.5
RS-Mex	30	4	3.4 ± 1.6	6.2 ± 1.1
RS-Me1	30	3	11.3 ± 3.8	18.3 ± 2.8*
R(–)-Toc	300	7	6.1 ± 2.1	9.6 ± 1.1
S(+)-Toc	300	7	5.6 ± 1.2	9.9 ± 1.7
R(–)-To1	300	3	10.3 ± 2.6	25 ± 3.8*, **
S(+)-To1	300	3	7.3 ± 2.3	21.2 ± 2.2*, **
R(+)-To2	300	2	1 ± 1	2.8 ± 1.8
S(–)-To2	300	3	0.5 ± 5.4	0.2 ± 4.9

The columns from left to right are as follows: Drug used; Concentration of the drug in μM; *n*, number of fibers sampled; $\Delta Vh_{1/2}$, shift of the steady state inactivation curve produced by the drug. This has been calculated in each fiber as the differences at the inflection point ($Vh_{1/2}$ control – $Vh_{1/2}$ drug) of the h_∞ curves obtained with 50 ms and with 1000 ms prepulse, respectively. Each value is the mean ± SEM of the individual shifts from *n* fibers. The mean values of $Vh_{1/2}$ of h_∞ in the absence of drugs were –61.6 ± 0.79 mV and –64 ± 0.83 mV ($n = 84$) at 50 ms and 1000 ms, respectively
* Significantly different with respect to the related shift at 50 ms ($P < 0.05$ for R(–)-To1 and $P < 0.02$ for S(+)-To1)
** Significantly different with respect to the optically related parent compound ($P < 0.01$ for RS-Me1 vs. RS-Mex and for S(+)-To1 vs. S(+)-Toc; $P < 0.005$ for R(–)-To1 vs. R(–)-Toc)

Table 4 Evaluation of the contribution of an open-channel block mechanism on the tonic and use-dependent block of sodium currents by chiral analogs of mexiletine and tocainide

Drug	Concentration (μM)	Tonic block Peak/Area	Use-dependent block Peak/Area
R(–)-Mex	50	0.87	1
S(+)-Mex	50	1.1	1
S(–)-Me2	100	0.75	0.98
R(+)-Me2	100	0.82	0.94
RS-Mex	100	1	0.92
RS-Me1	30	0.96	0.96
R(–)-Toc	300	0.87	0.83
S(+)-Toc	300	0.68	0.86
R(–)-To1	100	1	0.91
S(+)-To1	100	0.67	0.94
R(+)-To2	300	0.21	0.6
S(–)-To2	300	< 0.1	0.53

The column from left to right are as follows: Drug used; Concentration of the drug used, in μM; Ratios between the mean value of the block of the sodium currents (elicited with 10 ms test pulses from h.p. to –20 mV) calculated at the transients peak and that calculated on the area under the transients (each mean value from 2–7 fibers) are shown for tonic and use-dependent block, respectively. For the use-dependent block the values have been calculated on the transients at the end of the 10 Hz stimulation frequency (i.e. when the steady-state is reached). A block calculated on the area higher than that at the peak is indicative of a certain amount of open channel block. Thus the lower the ratio the higher is the amount of open-channel block produced

pendent block. As shown in Table 3 the shift was greater with prepulse of 1000 ms than with that of 50 ms. This phenomenon was more pronounced with the eutomers than with the distomers of Mex and Me2, although for both compounds it did not reach statistical significance, in agreement with the small eudismic ratios found for both compounds during use-dependent block. On the contrary 30 μM of Me1 produced a significantly greater shift to the left than the same concentration of RS(±)-Mex with both 50 ms and 1000 ms prepulses. The shift was concentration dependent for all the compounds (data not shown). Mex and its derivatives were prevalently inactivated channel blockers. In fact the ratios between the percent block of $I_{Na\ max}$ calculated at the peak and that calculated on the area (Peak/Area) were always close to unity, indicating that very little open channel block occurs for these compounds during both tonic and use-dependent block (Table 4). Values slightly lower than unity were only observed for R(–)-Mex and for both enantiomers of Me2 on tonic block.

Tocainide derivatives

In Fig. 7B it is shown that 300 μM R(–)-To1 produced a greater shift of h_∞ than 300 μM R(–)-Toc, the shift produced by 300 μM R(+)-To2 being negligible. The quantitative evaluation of the effects produced by the various compounds is shown in Table 3. The difference in the h_∞ shift between 1000 ms and 50 ms prepulse was significantly different for both the enantiomers of To1, but it was slightly greater with the S(+) enantiomer. These data can account for the decreased eudismic ratio found with To1 during use-dependent block. In fact the R(–)-To1 produced a greater but not significant shift than the S(+)-one, whereas Toc showed again little stereoselectivity. The shift of h_∞ produced by R(–)- and S(+)-To1 vs. the same concentration of the related enantiomers of Toc was highly significant, in line with the higher potency and use-dependence of this derivative. The shift produced by 300 μM R(+)-To2 was small, in agreement with the little effect produced at this concentration, however it was greater than that observed with 300 μM S(–)-To2 (Table 3). The shift by R(+)-To2 was concentration dependent being larger at 500 μM ($\Delta V_{1/2} = 10 \pm 2$ with 1000 ms prepulse). For both enantiomers of To2 the difference in h_∞ shift between 50 ms and 1000 ms was not appreciable, suggesting that the slow development of use-dependent block was not due to the block of the channels during long-lasting inactivated states. The possibility that To2 may rather act as open-channel blocker is supported by the finding that the ratios peak/area calculated for 300 μM of R(+)- and S(–)- To2 were much smaller than unity (Table 4). The small ratios were found for both tonic and use-dependent block, the phenomenon being more evident on the former. The open-channel block mechanism can ac-

count for the apparent low potency of both enantiomers of To2 in producing a tonic block and for the need of a prolonged repetitive stimulation to show up use-dependent block. This analysis revealed that a certain amount of open-channel block did occur also for Toc and To1 especially during tonic block (Table 4). As can be seen in Table 4 the ratio between peak and area was always smaller for the S isomers vs. the R ones, suggesting that the two enantiomers can have different affinity for the resting and for the open channel state, a phenomenon that can play a role in the modest stereoselectivity of these compounds.

Discussion

The present study was aimed at evaluating the effects of chiral derivatives of mexiletine and tocainide on sodium channels of skeletal muscle fibers in order to clarify the structural requirements that are necessary to get a molecule useful for a safer treatment of the hyperexcitability of the myotonic syndromes. In agreement with previous findings (De Luca et al. 1995), we confirmed that in skeletal muscle mexiletine is a more potent sodium channel blocker than tocainide, this order of potency being maintained also with the related analogs tested in the present study. However the chemical modifications strongly modified the ability of the compounds to produce a tonic and/or a use-dependent block of the channels, and likely the mechanism by which these effects are brought about.

Potency in producing tonic and use-dependent block

As far as mexiletine is concerned, the introduction of a methylene group in the alkyl chain as in Me2 reduced by about twofold the ability of the compound to produce a tonic block of the channels. These data are in general agreement with the finding of Sheldon et al. (1991) showing that an optimal binding with the receptor is obtained with lidocaine derivatives with a two carbon alkyl chain, the affinity being reduced with longer chain links. Nevertheless, the derivative Me2 showed a stronger use-dependent behaviour so that it was equieffective, if not even more potent, than mexiletine at high frequency of stimulation. The elongation of the alkyl chain in Me2 led to two main physico-chemical changes: an increase of molecular weight and a possible increase of pKa, due to a smaller electron withdrawing effect on the amine group by the xyliloxy substituents (Byrnes et al. 1979). Both changes can play a role in the high use-dependent behaviour. In fact Yeh and TenEick (1987), by using a series of disopyramide derivatives, showed that the use-dependent behaviour is directly correlated to the molecular weight. Also, a higher pKa value accounts for a greater amount of charged molecule at physiological pH. It has been long claimed that the charged fraction of a local anesthetic-like compound contributes to its use-dependent behaviour because it can gain access to the receptor in the sodium channel by permeating the hydrophilic pathway of the open channel from the cytoplasm (Schwarz et al. 1977). In this way the charged moiety would be driven toward and away from the binding site not only in relation to the channel state, but also to the voltage gradient across the membrane (Wang et al. 1993, 1994). In fact the presumed receptor for the local anesthetic-like compounds is on the S6 segment of the IV domain, nearby the inactivation gate of the III-IV cytoplasmic linker, located halfway the electric field and modification of the amino acid hydrophobicity in this region greatly modified the use-dependent block by lidocaine derivatives (Ragsdale et al. 1994). The use-dependent behaviour is also due to the time constant governing the dissociation rate of the drug from the receptor, a yet undefined process that, according to the modulated receptor hypothesis should mainly take place during membrane repolarization for the transition of the channel from the high affinity inactivated-state to the low-affinity resting state (Catterall 1987). According to this view, the cumulative use-dependent block develops until the rate at which the drug binds is equal to the rate for drug dissociation. The slower the dissociation the later the equilibrium is reached, giving rise to a remarkable use-dependent behaviour. Although not accurately measured in our experiments, it is important to underline that both an increase of molecular weight and of pKa (Ehring et al. 1988), as well as a particularly high affinity to the receptor (Quan et al. 1996), can slow the rate of drug dissociation from the sodium channels. Similar considerations can be drawn for the high use-dependence of To1, the tocainide derivative in which the methyl group on the chiral carbon atom is replaced with an isopropyl group. Also, To1 produced a stronger tonic block with respect to tocainide probably due to a better interaction of the isopropyl group at the level of one of the two large hydrophobic domains on the receptor proposed by Wang et al. (1993), that can accommodate up to 12 carbon atom chains or a phenyl group. This can also account for the strong tonic block produced by the mexiletine derivative Me1 having a phenyl group on the chiral carbon atom and a chlorine atom on the other aromatic ring, that can further increase the hydrophobicity. Interestingly, the same structural changes of Me1 made on the tocainide molecule led to similar large increase in the potency for blocking the sodium channels of human myoballs (Tricarico et al. 1991). However, Me1 showed a less pronounced use-dependent block probably due to the ability of this highly lipophilic compound to rapidly equilibrate with the receptor through the hydrophobic phase of the membrane in spite of channel state that is the speed-limiting step for highly use-dependent blockers.

It has been extensively demonstrated that several compounds can interfere with sodium channels (Catterall 1987; Taylor and Meldrum 1995), corroborating the hypothesis that a flexibility of both receptor and drug can allow the accommodation of very different structures. The derivative To2 differed from the other derivatives for a more planar conformation due to the introduction of the chiral carbon atom and the amide function in a rigid

pyrrolo-imidazolonic cycle. This compound was characterized by a marked decrease in the potency for producing a tonic block and by a slowly developing use-dependent block mostly due to an open-channel block mechanism. Since the chemical changes introduced in this compound did not produce alteration of pKa and of lipophilicity remarkably different with respect to the other derivatives, it is likely that the open-channel block mechanism is related to the smaller number of conformations it can generate due to the rigidity and to the consequently more stringent conformational requirements at the receptor site.

Stereoselectivity

Stereoselectivity correlated quite well with drug potency, so that again mexiletine and Me2 were more stereoselective than tocainide and To1. This was true also within the two groups, in fact mexiletine, that on tonic block was more potent than its analog Me2, was also more stereoselective with an eudismic ratio of 2 vs. 1.6, whereas tocainide, less potent than To1 was also less stereoselective. The different stereoselectivity can be due to different drug-receptor interaction, in relation to the drug structure, or rather to other mechanisms. For instance we observed that in the amount of tonic block mediated by an open-channel block mechanism R(–)-Mex and S(–)-Me2 were, as expected, the eutomers, but the opposite was observed with Toc and To1, as the open channel block was mostly observed with the distomers. Interestingly we found that for all the compounds the stereoselectivity attenuated in a frequency dependent manner, the higher the stimulation frequency the smaller the stereoselectivity. Apart from To2, the use-dependent behaviour of all the compounds is mainly due to a greater interaction with the inactivated channels, since no open channel block occurred in this situation. All these results suggest that the receptor on the sodium channel has different stereospecific requirements in the various channel states; i.e. more stringent during the resting state but attenuated when the channel is inactivated. However, this explanation is not in line with the theory that a high affinity receptor-state (as the inactivated one) should also have more stringent stereospecific requirements. In fact other compounds such as RAC 109 showed little stereoselectivity during tonic block, but reached eudismic ratios of about 9 during high frequency of stimulation (Yeh 1980). Based on our preliminary analysis a current explanation for the dissipation of stereoselectivity is a slow recovery of the drug-bound channels from inactivation during the interval between the pulses. In fact as shown for Me2 enantiomers, the time course of use-dependent blockade follows a stereoselective behaviour, whereas the recovery from inactivation in the presence of either enantiomers occurs with time constants longer than the interpulse time interval used at 10 Hz. We propose that an accumulation of drug-bound channels due to a slow recovery can influence the IC_{50} and the apparent affinity constant, no matter if the drug binds in a stereoselective manner. From a therapeutic point of view, the dissipation of stereoselectivity observed with our experimental protocols has to be taken with caution, as other factors may rather enhance stereoselectivity on overall muscle excitability of myotonic muscle. In fact in a preliminary study we found that R(–) isomer of tocainide was fivefold more potent than S(+) one in solving in vitro the hyperexcitability of intercostal muscle of congenitally myotonic goats (Conte Camerino and Bryant 1994). The different disposition of the enantiomers in vivo has also to be taken into account (Block et al. 1988).

Conclusions

From this preliminary screening it is possible to conclude that the highly use-dependent Me2 and To1 may have improved antimyotonic activity with respect to the parent compounds. Both Me2 and To1, showing a reduction of the IC_{50} from the tonic to the use-dependent block ranging from 6 to 10 times, could be effective on a diseased tissue discharging at high frequency or depolarized at doses absolutely ineffective on quiescent tissues. In fact in a preliminary study on genetically myotonic ADR mouse we found that Me2 was twice as potent as mexiletine in reducing in vitro the abnormal sarcolemma hyperexcitability, but little effective on the normal excitability of healthy muscle (De Luca et al., 1997). At the same time also the other synthesized compounds may have interesting properties as antimyotonic agents. In fact the analog Me1 may represent a compound with a very high affinity for sodium channel, whereas the cyclic derivative of Toc in consequence of the pronounced open channel block, can fulfill the requirement for discriminating the action on skeletal over cardiac muscle (Wang et al. 1996) and represent a lead molecule for getting selective blockers for skeletal muscle sodium channels.

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