Evaluation of the Antimyotonic Activity of Mexiletine and Some New Analogs on Sodium Currents of Single Muscle Fibers and on the Abnormal Excitability of the Myotonic ADR Mouse

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ABSTRACT

To search for use-dependent sodium channel blockers to selectively solve muscle hyperexcitability in hereditary myotonia, mexiletine (MEX; compound I) and its newly synthesized analogs, 2-(4-chloro-2-methylphenoxy)-benzenethanamine (compound II) and (–)-S-3-(2,6-dimethylphenoxy)-2-methylpropahamine (compound III), were tested on intercostal muscle fibers from the myotonic ADR mouse through use of the standard current-clamp microelectrode technique. In parallel, the effects of these compounds on the sodium channels were measured on frog muscle fibers under voltage-clamp conditions. The tonic and use-dependent blocks of peak sodium currents (INamax) produced by each compound were evaluated by using a single depolarizing pulse and a pulse train at 10 Hz frequency, respectively. At 10 and 50 μM, MEX decreased the occurrence of spontaneous excitability in myotonic muscle fibers; 100 μM was required to decrease the amplitude of the action potential and the stimulus-induced firing of the membrane as well as to increase the threshold current to elicit a single action potential. MEX produced a tonic block of INamax with an half-maximal concentration (IC50) of 83 μM, but the IC50 value for use-dependent block was 3-fold lower. Compound III, which differs from MEX in that it has a longer alkyl chain, similarly blocked first the spontaneous and then the stimulus-evoked excitability of myotonic muscle fibers but at 2-fold lower concentrations than MEX. Compound III was less potent than MEX in producing a tonic block of INamax (IC50 = 108 μM) but was a strong use-dependent blocker with an IC50 close to 15 μM. The more lipophilic compound II irreversibly blocked both spontaneous and stimulus-evoked membrane excitability at concentrations as low as 10 μM and shortened the latency of the action potential in a concentration-dependent fashion. Compound II produced a potent tonic block of INamax (IC50 = 30 μM), and its potency increased 2-fold during high-frequency stimulation. Both of the new analogs (compound II in particular), but not MEX, were less effective on the excitability parameters of striated fibers of healthy vs. ADR mice, a characteristic that increases their interest as potential antimyotonic agents.

The clinical term of myotonia is used to identify a series of dominant and recessive forms of genetic diseases of skeletal muscle characterized by abnormal membrane excitability and delayed muscle relaxation after voluntary contraction. Sodium channel myotonias, paramyotonia congenita and hypokalemic periodic paralysis are due to mutations of the gene coding for the skeletal muscle type of voltage-gated sodium channels (SCN4). The mutated channels show different degrees of impairment of inactivation, which in turn causes membrane depolarization (Cannon, 1996; Lehmann-Horn and Rüdel, 1996). Mutations producing mild depolarization are responsible for the clinical phenotype of hyperexcitability, whereas those exerting sustained depolarizations lead to paralytic attacks (Cannon, 1996). In the dominant myotonia congenita and recessive generalized myotonia, the characteristic hyperexcitability and SpDs of action potentials are related to an abnormally low resting GCl (Adrian and Bryant, 1974; Bryant and Morales-Aguilera, 1971). The protein ClC-1 has been

ABBREVIATIONS: MEX, mexiletine; GCl, resting chloride conductance; ADR, arrested development of righting response phenotype; INa, sodium current; AP, action potential amplitude; Lat, latency of the action potential; Th, threshold potential; SpD, spontaneous discharge; ADR, afterdischarge; INa, sodium current; IC50, half-maximal blocking concentration; INamax, maximal peak sodium current; pKa, negative logarithm of acid dissociation constant; KATP, channels, ATP-dependent potassium channels.
claimed to be the putative channel responsible for the macroscopic \( G_{Cl} \); this hypothesis was supported by the finding of several mutations of the CIC-1 genes in patients and myotonic animals (Gronemeier et al., 1994; Lehmann-Horn and Rüdel, 1996; Steinmeyer et al., 1991). The attempts to restore the macroscopic \( G_{Cl} \) of genetically myotonic goats with various pharmaceuticals known to modulate this parameter in normal subjects have been unsuccessful (Bryant and Conte Camerino, 1991).

This result is in line with the recent findings that in the goat phenotype, the genetic mutation seriously impairs the function of the chloride channel in its physiological range (Beck et al., 1996). In fact, severe malfunctions up to a total loss of function of muscular chloride channel gene product (CIC-1) can result from different mutations (Fahlke et al., 1995; Gronemeier et al., 1994). Thus, the therapy for all the myotonic syndromes, whether related to sodium or chloride channels, is mainly symptomatic and addressed at relief of membrane hyperexcitability. Actually, the antimyotonic drugs that are used clinically are orally effective lidocaine derivatives such as MEX and tocainide (Jackson et al., 1994; Lehmann-Horn and Rüdel, 1996; Rüdel et al., 1980), which are able to block the generation and propagation of the action potential in skeletal muscle by blocking the voltage-gated sodium channels. Nevertheless, their use is restricted by their possible side effects, especially those produced at the hematopoietic and central nervous system levels (Rodén and Woosley, 1986). In addition, effective antimyotonic doses are as large as those used to obtain antiarrhythmic action, with a possible effect on cardiac function as well.

In the attempt to search for selective antimyotonic agents, two characteristics should be taken into account. First, the drugs should block the \( I_{Na} \) in a use-dependent manner. The use-dependent blockers stabilize the bound channels in the inactivated state from which the recovery is slowed down (Catterall, 1987; De Luca et al., 1991; Grant and Wendt, 1992). This mechanism ensures a stronger potency of the compound on tissues with excessive firing of action potentials and/or permanent depolarization, such as the muscles affected by sodium or chloride channel phenotypes of myotonia, than on tissues with a physiological excitability. Second, sodium channels of the various tissues are genetically distinct and show different kinetic and pharmacological properties. It was recently reported that in addition to having different sensitivities toward tetrodotoxin, cardiac and skeletal muscle types of sodium channels are affected differently by local anesthetic-like drugs; the pharmacological profile is further exacerbated by the different inactivation properties of the two channel types (Wang et al., 1996). These observations support the possibility of designing use-dependent blockers of \( I_{Na} \) to be more selective on skeletal muscle than on the heart and to be able to relieve sarcolemmal hyperexcitability in myotonic subjects with fewer side effects.

The aim of the present study was to screen the effects of newly synthetized analogs of MEX as potential antimyotonic agents by evaluating in vitro their ability to suppress the pathological hyperexcitability of skeletal muscle isolated from myotonic ADR mouse, a phenotype with a severe recessive form of low \( G_{Cl} \) myotonia (Gronemeier et al., 1994; Mehrke et al., 1988; Steinmeyer et al., 1991). The newly synthetized compounds were used to investigate the influence of some structural properties of the molecule on anti-myotonic activity and, in particular, (1) the role of the distance between the aromatic and amino-terminal groups (compound III, fig. 1) and (2) the increase in lipophilicity produced by the insertion of a chlorine atom in the para position of the aromatic ring and of a phenyl group on the carbon atom linked to the amino group (compound II, fig. 1). These two compounds were chosen on the basis of the effects they produced on skeletal muscle \( I_{Na} \) during preliminary voltage-clamp experiments and further verified in the present study. In fact, compound III showed noticeable use-dependent behavior, whereas compound II was a very potent \( I_{Na} \) blocker, two characteristics that may be of importance in obtaining selective antimyotonic agents.

**Methods**

Current-clamp recordings of macroscopic membrane excitability of ADR myotonic and healthy mice. Myotonic ADR (genotype \( adr/adr \)) mice and wild-type healthy littermates (genotype \( +/+ \) or \( adr/+ \)) that were 3 months old were used for all the experiments. Myotonia in ADR mice is a recessive disease due to the insertion of a transposon into the CIC-1 gene (Gronemeier et al., 1994; Steinmeyer et al., 1991). The diseased homozygous animals develop a severe and clearly distinguishable myotonic state, whereas the genotypic \( adr/+ \) animals do not show recognizable signs of myotonia and do not have an alteration of the macroscopic \( G_{Cl} \) with

![Fig. 1. Chemical structure of MEX and its newly synthetized analogs.](image)
respect to wild-type (+/+ ) despite the defect in one allele (Gronemeyer et al., 1994; Mehrke et al., 1988).

The intercostal muscle was dissected with the animals under urethane anesthesia (1.2 g/kg i.p.) and immediately placed in a muscle bath at 30°C. Soon after the removal of the muscle, the still-anesthetized animals were killed with an overdose injection of urethane. The two-microelectrode technique was used for the electrophysiological recordings (Bryant and Conte Camerino, 1991; De Luca et al., 1992, 1995). A voltage-sensing electrode and a current-passing electrode were placed 50 μm apart in the central region of randomly selected superficial fibers. After recording of the resting membrane potential, the fibers were current-clamped at −80 mV for evaluation of the membrane excitability parameters. The excitability characteristics of the sampled fibers were determined by recording the intracellular membrane potential response to square-wave depolarizing constant current pulses of 100 msec at a frequency of 1 Hz. The current intensity was gradually increased until the depolarization was just sufficient to elicit a single action potential. The current intensity was then further increased in an attempt to generate two or more action potentials. If these could not be elicited by a current pulse that was ≥3-fold the pulse that elicited a single action potential, we considered this to be a failure to fire repetitively. In this way, it was possible to record and measure the parameters of minimum current intensity that would elicit a single action potential (Ith), the membrane potential at which a single action potential could be elicited (Th), the AP, the Lat (membrane potential at which a single action potential could be generated (N spikes) (De Luca et al., 1992, 1995).

Most of the myotonic fibers showed characteristic SpD of action potentials on the insertion of the voltage-sensing microelectrode and self-sustained ADs (i.e., discharges of action potential after the end of the application of the depolarizing stimulus) (Adrian and Bryant, 1974; Mehrke et al., 1988). The high electrical activity of myotonic muscle fibers was often accompanied by visible contraction (never observed in muscle fibers of healthy animals), which could cause either the dislodgement of the electrode from the fibers or a postcontraction depolarization affecting the quality of the current-clamp procedure. To avoid such mechanically induced artifacts, most of the myotonic preparations were pretreated in vitro with dantrolene sodium (2 mg/liter). As already described (Bryant and Conte Camerino, 1991; Morgan and Bryant, 1977), this compound avoided fiber contraction due to inhibition of calcium release from intracellular stores but did not affect either the occurrence of spontaneous electrical activity of sarclemma or the characteristics of the stimulus-induced action potential. Thus, in each preparation, the number of fibers showing either SpDs or ADs was calculated as a percentage of the total number of fibers sampled both in the absence and presence of the drugs to evaluate their ability to suppress these pathological manifestations in each individual muscle preparation.

**Recordings of I_{Na} using the three vaseline gap voltage-clamp technique.** Voltage-clamp recordings of I_{Na} were performed on 1–2-cm-long segment of single muscle fibers obtained by microsurgery from the ventral branch of semitendinosus muscle of rana esculenta. The cut end fiber was superfused with an internal solution consisting of 148 mM NaCl, 4.5 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgCl₂, 12 mM NaHCO₃, 0.44 mM NaH₂PO₄ and 5.55 mM glucose. The solutions were continuously gassed with 95% O₂/5% CO₂ (pH 7.2, 7.4). For I_{Na} recordings, the semitendinosus muscle fibers were perfused with an internal solution consisting of 77 mM NaCl, 38 mM coline-Cl, 1.8 mM CaCl₂, 2.15 mM Na₂HPO₄ and 0.85 mM NaH₂PO₄ and dialyzed with an internal solution consisting of 105 mM CsF, 5 mM 3-(N-morpholino)propanesulfonic acid, 2 mM MgSO₄, 5 mM ethylene glycol bis(α-aminoethyl ether)-N,N,N′,N″-tetraacetic acid and 0.55 mM Na₂ATP (pH 7.2 with NaOH concentrated solution). The compounds tested were synthesized in our laboratories. Briefly, MEX (compound I) was synthesized according to procedures previously detailed (Franchini et al., 1994); compound II [2-(4-chloro-2-methylphenylo)-benzenethanamine] was obtained through condensation of bromoketon with the opportune phenolic derivative and transformation of the product to oxime through reaction with hydroxyamine and successive reduction to amine II and compound III (–)-S-3-(2,6-dimethylphenox)-2-methylpropanamine was obtained in the optically active form through condensation of the chiral alcohol with the opportune phenolic derivative after protection of the nitrogen atom (Duranti et al., 1995) (fig. 1). All compounds were fully characterized by routine spectroscopic analyses; analytical results for C, H and N were within ±0.4% of the theoretical values. Stock solutions of MEX and compound III were prepared in physiological or external solutions for current-clamp and voltage-clamp experiments, respectively, whereas stock solutions in dimethylsulfoxide (100 μl/g) were used for compound II. All the stock solutions were prepared daily, and the final concentrations to be tested in vitro on isolated muscle were obtained by further dilution of the stock solution as needed. Dimethylsulfoxide at the highest concentration used (0.2%) was without effect on any of the parameters recorded. On both intercostal muscle and frog muscle fibers, no more than three concentrations of the same compound were tested,
and the preparations were exposed to each concentration for \( \approx 10 \) min before recording to allow the maximum effect of the drug to be reached.

**Statistical analysis.** Data are expressed as mean \( \pm \) S.E.M. The statistical significances of the differences between groups of mean values were calculated by unpaired Student's *t* test. The molar concentrations of the drugs producing a 50% block of firing or of \( I_{\text{t, max}} \) (IC\(_{50}\)) were determined by using a nonlinear least-squares fit of the concentration-response curves to the following logistic equation: Effect = \(-100/1 + \{K/(\text{drug})\}^n\), where Effect is percent change of the parameter, \(-100\) is the maximal effect, \(K\) is the IC\(_{50}\) value of the tested drug, \(n\) is the logistic slope factor and [drug] is the molar concentration of the tested drug (De Luca et al., 1992, 1995). The estimates of S.E.M. and \(n\) for normalized percent values were obtained as described by Green and Margerison (1978).

**Results**

**Excitability characteristics of muscle fibers of myotonic ADR mice.** The excitability characteristics of intercostal muscle of myotonic ADR mice are detailed in table 1, and representative recordings are shown in figure 2. Spontaneous myotonic activity, such as high-frequency discharges of action potentials (>10 Hz) on insertion of the recording electrode, was detected in \( \sim 60\% \) to 80% of fibers in each ADR muscle preparation (table 1 and fig. 2). The myotonic muscle fibers needed very little depolarizing current to generate one action potential and, as expected on the basis of the low \( G_{\text{Cl}} \) myotonic phenotype, the action potential showed a significantly longer Lat than muscle fibers from healthy mice. However, no significant differences with respect to normal animals were observed in AP or values of Th. As is typical of myotonic state, the muscle fibers of ADR mice were able to generate a significantly higher number of action potentials than healthy ones when the depolarizing stimulus was just slightly increased over the \( I_{\text{th}} \) (table 1). In myotonic muscle fibers, the stimulus-evoked train of action potentials was followed by self-sustained ADs at the end of the depolarizing current pulse in \( \sim 75\% \) to 100% of the fibers sampled (table 1 and fig. 2).

**Effects of MEX and its analogs on excitability characteristics of muscle fibers of myotonic ADR mouse.** All of the tested compounds affected the excitability characteristics of myotonic muscle fibers, and we took care to evaluate the ability of each compound to relieve at low concentrations than healthy ones when the depolarizing stimulus was just slightly increased over the \( I_{\text{th}} \) (table 1). In myotonic muscle fibers, the stimulus-evoked firing and SpDs on insertion of the voltage-sensitive microelectrode.

**TABLE 1**

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Preparations/fibers</th>
<th>AP</th>
<th>(I_{\text{th}})</th>
<th>Lat</th>
<th>Th</th>
<th>N spikes</th>
<th>Fibers with SpD</th>
<th>Fibers with AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>4/20</td>
<td>98\pm 2.3</td>
<td>102\pm 5.8</td>
<td>12.8 \pm 0.7</td>
<td>27.0 \pm 1.2</td>
<td>5.1 \pm 0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ADR</td>
<td>4/25</td>
<td>95.3 \pm 4.7</td>
<td>22.4 \pm 2.7 (^*)</td>
<td>92.3 \pm 2.4 (^*)</td>
<td>25.7 \pm 1.1</td>
<td>11.7 \pm 0.7 (^*)</td>
<td>74 \pm 2</td>
<td>87 \pm 7</td>
</tr>
</tbody>
</table>

\( I_{\text{th}}\), current intensity at the base to elicit one action potential. N spikes, maximum number of action potentials elicitable. The percent of fibers showing SpDs just on the insertion of the voltage-sensitive microelectrode was obtained by calculating in each preparation the fibers with SpDs divided by the total number of fibers sampled (from 20 to 30 fibers) and then averaging the individual values. The percent of fibers with ADs after the stimulus-induced firing was calculated in a similar manner. Values are mean \( \pm \) S.E.M. from \( n \) fibers.

\(^*\) Significantly different from the same parameter of control animals by Student’s *t* test (\( P < .001 \)).
TABLE 2
Effects of MEX and its analogs on excitability characteristics of myotonic and healthy mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>AP</th>
<th>Ith</th>
<th>Lat</th>
<th>Th</th>
<th>SpD</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>mV</td>
<td>nA</td>
<td>msec</td>
<td>mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADR mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEX</td>
<td>10</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>-35±8</td>
<td>N.C.</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>+9.0±7</td>
<td>-90±10</td>
<td>-68±9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-110±7</td>
<td>N.C.</td>
<td>N.C.+22.7±8</td>
<td>-100</td>
<td>-100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>-130±6</td>
<td>+100±37</td>
<td>-64.1±7</td>
<td>+33.9±9</td>
<td>-100</td>
<td>-100</td>
</tr>
<tr>
<td>Compound III</td>
<td>5</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>-7.0±6</td>
<td>-25±9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-9.1±11</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>-34±9</td>
<td>-80±6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-18.8±6</td>
<td>+37.8±21</td>
<td>N.C.</td>
<td>+13±4</td>
<td>-100</td>
<td>-100</td>
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<tr>
<td></td>
<td>100</td>
<td>-28.8±3</td>
<td>+235±80</td>
<td>N.C.</td>
<td>+47.2±7</td>
<td>-100</td>
<td>-100</td>
</tr>
<tr>
<td>Compound II</td>
<td>10</td>
<td>N.C.</td>
<td>+156±22</td>
<td>-28.6±17</td>
<td>+5.2±9</td>
<td>-25±6</td>
<td>-80±8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-12.6±8</td>
<td>+78±14</td>
<td>-63.5±8</td>
<td>+29.4±12</td>
<td>-100</td>
<td>-100</td>
</tr>
<tr>
<td>Healthy mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEX</td>
<td>300</td>
<td>-13.8±4</td>
<td>+53.8±9</td>
<td>N.C.</td>
<td>+30.0±11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound III</td>
<td>100</td>
<td>-13.6±4a</td>
<td>-17.0±13c</td>
<td>N.C.</td>
<td>+8.5±6c</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-11.4±3</td>
<td>-3.5±12c</td>
<td>N.C.</td>
<td>+15.3±6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a = Significant difference between the effects produced by the same drug at the same concentration in ADR and control mouse muscles by Student’s t test (*P < .01, *P < .005 and **P < .001).

the Lat to 33.5 ± 7.1 msec (n = 9) (P < .001) and a 40-nA increase in Ith (table 2). Compound III was able to decrease dose-dependently both SpDs and ADs in the range of 5 to 10 µM, being more potent than MEX on the ADs. At the above concentrations, the firing capability of the membrane was also slightly reduced (fig. 3). Compound III at 50 µM completely suppressed spontaneous myotonic manifestations and significantly reduced the maximum number of spikes that the membrane could generate from 13 ± 0.8 (n = 6) to 8.3 ± 0.8 (n = 9) (P < .005). In fact, compound III inhibited the firing capability of the membrane more effectively than MEX, with an IC50 value of 66 vs. 214 µM (fig. 3). Compound III was also more potent than MEX in reducing the AP and in increasing both Ith and Th (fig. 2 and table 2). The effects of this compound increased at 100 µM, and at 180 µM we observed a complete block of the excitability in that none of the sampled fibers were able to generate one action potential (data not shown). Compound II was very potent in depressing myotonic hyperexcitability but showed a different pattern of effects compared with MEX and compound III. In fact, at concentrations as low as 10 µM, this compound not only depressed the occurrence of SpD and ADs but also produced a reduction of the firing capability comparable to that observed with 100 µM MEX, with the half-maximal concentration on this parameter being 24 µM (fig. 3 and table 2). Furthermore, at 10 µM it decreased the latency of the action potential to 66 ± 16 msec (n = 6), increased significantly Ith from 19 ± 1.5 (n = 7) to 48.7 ± 4.5 (n = 6) nA (P < .001) and slightly shifted Th toward more positive potentials (fig. 2). All these effects were concentration dependent, being more pronounced at 50 µM. At this concentration, compound II significantly shortened the Lat to 33.7 ± 7.4 msec (n = 7) (P < .001) and almost completely blocked the ability of the fiber to generate more than two action potentials. A decrease in the amplitude of the action potential was also observed (table 2). At 100 µM, compound II completely blocked the ability of the fibers to generate even one action potential. In contrast to MEX and compound III, the effects of compound II were not reversible on washout. None of the compounds tested significantly modified the resting membrane potential.

Effects of MEX and its analogs on excitability parameters of muscle fibers of healthy mice. The effects of the test compound on the excitability of intercostal muscles of healthy mice were studied to better evaluate the specificity of the antymyotonic activity. As shown in table 2, MEX modified the depolarization-induced excitability parameters of healthy muscle fibers at concentrations similar to those effective on the same parameters in myotonic ones. Different effects were observed with the two analogs. Compound III at 100 µM was much less effective on the excitability parameters of healthy muscle fibers than on those of ADR muscle (table 2), with the exception of the membrane firing capability, which was similarly reduced in both cases (by 70 ± 3% in

![Fig. 3. Concentration-response curves showing the percent inhibition of the maximum number of spikes elicitable (firing) produced by MEX, compound III and compound II on the intercostal muscle of myotonic ADR mice. Each value is the mean ± S.E.M. from 8 to 12 normalized fibers. The curves fitting the experimental points were obtained using the logistic function detailed in the text. The IC50 value for each compound calculated from the fit was 214 ± 26, 66 ± 6 and 24 ± 4 µM for MEX, compound III and compound II, respectively.](image-url)
healthy and by 68.5 ± 5% in ADR muscle fibers). Compound II at 50 μM reduced the amplitude of the action potential of healthy muscle fibers in a manner similar to the effect produced in myotonic fibers. However, the decrease in membrane firing capability was much less evident on normal muscle fibers. At 50 and 100 μM, compound II reduced the firing capability by 51 ± 6% and 68.3 ± 6%, respectively, effects that were much less pronounced than those observed with 50 μM in myotonic fibers. At 50 and 100 μM, a concentration-dependent decrease in I_{Na} and a small increase in T_{Na} were observed. Under this experimental condition, the Lat was little modified by compound III; this parameter was only slightly reduced at 100 μM (table 2). In addition, on healthy muscle fibers, the test compounds did not produce any remarkable effect on resting membrane potential.

**Effects of MEX and its analogs on I_{Na}s of single muscle fibers.** The effects of in vitro application of MEX and its analogs on I_{Na}s of frog semitendinosus muscle fibers are illustrated in figure 4. As detailed in the text, I_{Na_max} were elicited with 10-msec test pulses from the holding potential of −100 to −20 mV. Such a test pulse was applied as a single stimulus in both the absence and presence of the test drugs to evaluate the amount of tonic block (i.e., block exerted by the drug during the resting state of the channel and the membrane). After evaluation of the tonic block, the test pulse was repetitively applied at the frequency of 10 Hz for 30 sec to evaluate the ability of the test compounds to produce a use-dependent block (i.e., a block of the I_{Na} in a situation of high-frequency stimulation such as that occurring in the pathological myotonic state). MEX produced a tonic block of the I_{Na} in a concentration-dependent fashion with a calculated IC_{50} value of 83 μM. As expected, in the presence of MEX, the repetitive stimulation at 10-Hz frequency produced a further cumulative reduction of the I_{Na} due to use-dependent block. After 30 sec of such a stimulation, the use-dependent block by MEX fully attained the equilibrium, and the residual current normalized with respect to that in the absence of drug allowed the calculation of the amount of use-dependent blockade. The IC_{50} value for use-dependent block was three times lower than that for tonic block. Compound III was less potent in producing a tonic block of the currents with respect to MEX; in figure 4A, the effects of 50 μM MEX and compound III on inward sodium transients are compared. The calculated IC_{50} value of compound III for tonic block was 108 μM. However, the 10-Hz stimulation showed a remarkable use-dependent blockade by this compound so that 50 μM compound III or MEX produced a comparable block of I_{Na}. Thus, the IC_{50} value of compound III, calculated with the high-frequency stimulation protocol, was almost six times lower than that for obtaining the tonic block. Different behavior was observed with compound II; this compound produced a remarkable and irreversible tonic block of the I_{Na}s, with IC_{50} values as low as 30 μM. Nevertheless, little cumulative block was observed during the 10-Hz stimulation.

![Fig. 4. A, I_{Na} transients recorded by the three vaseline gap voltage-clamp method from single muscle fibers of frog semitendinosus muscle and from left to right) effects of MEX (50 μM), compound III (50 μM) and compound II (10 μM), respectively. After recording the control trace elicited by a single test pulse from −100 to −20 mV (a), the test compounds were applied. After 10 min of incubation, a single test pulse was again applied. The reduction in the current under this condition was the result of the tonic block exerted by the drug (b), and its amount was quantified by percent normalization with respect to the current in the absence of drug. A train of pulses at a frequency of 10 Hz was then applied for 30 sec, and a further cumulative block of the I_{Na}s over the tonic block was due to the use-dependent behavior of the compound (c). The amount of use-dependent block was calculated from percent normalization of the residual current at the end of the 30-sec stimulation with respect to the relative current in the absence of drug. As it can be seen, 50 μM MEX produced a greater tonic block of the current with respect to the same concentration of compound III; however, this latter was more use dependent, so at the end of the stimulation protocol, the percent reduction by compound III was comparable to that produced by MEX. In contrast, compound II was much more potent than both MEX and compound III in blocking I_{Na}, although this compound showed less use-dependent behavior. This protocol was repeated with various concentrations of the test compounds to construct concentration-response relationships (B). Each point is the mean ± S.E.M. of percent block of I_{Na_max} from three to five fibers. The curves fitting the experimental points led to the calculated values of IC_{50} for both tonic and use-dependent block that were 83 ± 14 and 30 ± 5 μM for MEX; 108 ± 13 and 17 ± 4 μM for compound III and 30 ± 3 and 15 ± 2 μM for compound II, respectively.

B
protocol, with the calculated IC$_{50}$ being twice as low as that for tonic block (fig. 4).

**Discussion**

The present study was aimed at evaluating the ability of MEX and its newly synthesized analogs to abolish the abnormal membrane excitability of myotonic muscle fibers of ADR mouse. In this phenotype, the pathological hyperexcitability is due to an abnormally low macroscopic $G_{Cl}$, the parameter that ensures the electrical stability of the membrane under resting conditions and maintenance of this latter after the voluntary excitation-contraction cycle. As a consequence of their low resting $G_{Cl}$, myotonic muscles spontaneously generate trains of action potentials, which trigger involuntary spasms; the self-sustained ADs of action potentials occurring in myotonic fibers are responsible for the delayed relaxation after voluntary movements and are caused by the low $G_{Cl}$-induced potassium accumulation in the t-tubules (Adrian and Bryant, 1974; Bryant and Morales-Aguilera, 1971; Lehmann-Horn and Rüdel, 1996).

Particular attention has therefore been devoted to evaluating the effects of MEX and its derivatives on these peculiar myotonic signs of the ADR phenotype and to correlate them with the ability to block the $I_{Na}$,s in a use-dependent manner. All the three compounds tested were effective, although appreciable differences were observed between them. As far as MEX is concerned, we observed that it started to reduce the abnormal SpDs of myotonic fibers at concentrations as low as 10 $\mu$M, a concentration close to that described to be clinically effective for this compound as an antiarrhythmic (Sato et al., 1992) and in good agreement with those able to produce a use-dependent block of the $I_{Na,s}$ (Sunami et al., 1993). The increased distance between the aromatic ring and the amino-terminal group by the insertion of a methylene moiety in the alkyl chain, in compound III, doubled the potency in reducing the firing capability of myotonic muscle fibers and slightly decreased the ability to modify the excitability characteristics of healthy muscle fibers. The stronger potency of compound III than MEX as an antmyotonic drug could be explained taking into account the results on the blocking mechanism of this compound on the $I_{Na,s}$. Compound III was less potent than MEX in producing a tonic block of the $I_{Na,s}$ (i.e., a block of the sodium channels during the resting state), but it caused a stronger use-dependent accumulation of $I_{Na}$ block during high-frequency stimulation.

The weak use-dependent behavior, irreversible action and lipophilic properties made compound III appear to be of little interest from a therapeutical point of view. One would predict a toxicological potential due to poor discrimination between pathological and physiological excitability patterns and long-lasting accumulation in various tissues. Nevertheless, compound II shows interesting features; in particular, (1) it was effective at low concentrations, (2) in contrast to MEX, it was more effective on myotonic fibers than on healthy ones and (3) it was able to shorten the Lat in a concentration-dependent manner in the myotonic but not the healthy fibers. This latter effect, which was also observed with MEX at doses as high as 300 $\mu$M, may be beneficial for therapeutic interventions on the low-$G_{Cl}$ myotonic state, such as the ADR mouse. In fact the long latency, and the consequent prolongation of action potential duration, is a characteristic feature of the decrease in $G_{Cl}$ (Adrian and Bryant, 1974). The Lat reflects the electrotonic response of the fibers before reaching the threshold, and it is mainly due to the resting ionic conductances to chloride and potassium ions. In the absence of $G_{Cl}$, as in ADR fibers, a shortening of the latency can be mediated by increasing potassium conductance through a modulation of potassium channels active at resting potential. It has been recently shown that MEX, at high concentrations, can open $K_{ATP}$ channels in the heart and thus shorten cardiac action potential duration (Sato et al., 1995). It is tempting to speculate that a similar mechanism can account for the decrease in latency observed with MEX and compound II in ADR.
fibers. K\textsubscript{ATP} channels are abundantly present on sarcolemma of striated fibers and are predisposed to opening at resting potential when the level of intracellular ATP falls or the cytoplasmic pH decreases (Longman and Hamilton, 1992; Tricarico and Conte Camerino, 1994), metabolic situations likely to occur in the myotonic muscle fibers because of the intensive muscle work.

The hypothesis that compound II can act on K\textsubscript{ATP} channels at concentrations close to those effective on sodium channels has yet to be validated through more direct experimental evidence. However, it is a particularly attractive hypothesis because the classic openers of K\textsubscript{ATP} channels have been proposed as alternative antimiotoytic agents (Longman and Hamilton, 1992). Furthermore, the above hypothesized mechanism would be specifically active in myotonic fibers, since the healthy fibers have a large G\textsubscript{Cl} and lack the metabolic alterations that facilitate opening of the K\textsubscript{ATP} channels. Other different mechanisms can also account for the effectiveness of compound II on the myotonic fibers at lower concentrations than on healthy ones. It is possible that in the healthy fiber, the compound acts with additive mechanisms able to counteract the block of I\textsubscript{Na,L} or, instead, that compound II has a very high affinity for inactivated sodium channels, which would predominate in the high discharging low-G\textsubscript{Cl} fibers. For instance, riluzole, a novel psychotropic agent with anticonvulsant properties, has been found to have a high affinity for inactivated sodium channels despite its weak use dependency (Hebert et al., 1994). Further investigation into the mechanisms underlying the effects of compound II, such as verification of its ability to open K\textsubscript{ATP} channels, and on its structure-activity relationship is of interest to improve the therapeutic potential of this mexiletine derivative. Finally, our results show that the newly synthesized analogs of MEX have different features from the parent compound and are of interest in the development of more potent blockers of the skeletal muscle sodium channels and potential antimiotoytic agents.

References


