Cholinesterases and the Resistance of the Mouse Diaphragm to the Effect of Tubocurarine

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Background: The diaphragm is resistant to competitive neuromuscular blocking agents. Because of the competitive mechanism of action of tubocurarine, the rate of hydrolysis of acetylcholine at the neuromuscular junction may modulate its neuromuscular blocking effect. The authors compared the neuromuscular blocking effect of isolated diaphragm and extensor digitorum longus (EDL) muscles and quantified the acetylcholinesterase activity in hetero-oligomers.

Methods: Adult Swiss-Webster and collagen Q-deficient (ColQ−/−) mice were used. The blocking effect of tubocurarine on nerve-evoked muscle twitches was determined in isolated diaphragm and EDL muscles, after inhibition of acetylcholinesterase by fasciculin-1, by butyrylcholinesterase by tetrasisopropylpyrophosphoramide, or both acetylcholinesterase and butyrylcholinesterase by neostigmine, and in acetylcholinesterase-deficient ColQ−/− mice. The different acetylcholinesterase oligomers extracted from diaphragm and EDL muscles were quantified in sucrose gradient.

Results: The EC50 for tubocurarine to decrease the nerve-evoked twitch response was four times higher in the diaphragm than in the EDL. The activity of the different acetylcholinesterase oligomers was lower in the diaphragm compared with the EDL. Inhibition of acetylcholinesterase by antagonists resulted in an increased dose of tubocurarine but an unchanged resistance ratio between the diaphragm and the EDL. A similar diaphragmatic resistance was found in ColQ−/− mice.

Conclusion: The current study indicates that, despite differences in acetylcholinesterase activity between the diaphragm and EDL, the diaphragmatic resistance to tubocurarine cannot be explained by the different rate of acetylcholine hydrolysis in the synaptic cleft.

IT has long been recognized that the diaphragm is more resistant to competitive neuromuscular blocking agents than peripheral muscles. The mechanism of the so-called respiratory-sparing effect of muscle relaxants remains unclear.1–5 The mechanism of resistance of respiratory muscles to competitive neuromuscular blocking agents may be either presynaptic or postsynaptic. Presynaptic factors include modulation of acetylcholine release from motor nerve terminals, and postsynaptic factors include the density of nicotinic acetylcholine receptors at the endplate and the rate of hydrolysis of acetylcholine by cholinesterases at the neuromuscular junction. This latter mechanism is considered in the current study.

In mammals, two cholinesterases, acetylcholinesterase and butyrylcholinesterase, may hydrolyze acetylcholine. They are organized in complexes that depend on the association with noncatalytic proteins, the collagen tail (ColQ),8 and a hydrophobic tail PRImA,9 as shown in figure 1. The rest of the cholinesterase activity is referred as due to soluble globular forms (monomer, dimer, and tetramer). At the neuromuscular junction of skeletal muscle, acetylcholinesterase is highly concentrated by ColQ. In the absence of ColQ due to point mutations in humans10 or obtained by homologous recombination in mice,11 acetylcholinesterase is absent.

The aim of the study was to test in vitro whether the resistance of the diaphragm to tubocurarine-induced neuromuscular blockade depends on the rate of hydrolysis of acetylcholine by acetylcholinesterase. For this purpose, first we compared the effects of tubocurarine on isolated diaphragm and extensor digitorum longus (EDL) muscles. Second, we quantified the acetylcholinesterase complexes containing ColQ or PRImA and soluble globular forms in diaphragm and EDL. Third, we determined whether acetylcholinesterase inhibition by fasciculin-1, butyrylcholinesterase inhibition by tetrasisopropylpyrophosphoramide (iso-OMPA), or complete inhibition of both acetylcholinesterase and butyrylcholinesterase by neostigmine12 alters differently the sensitivity of the diaphragm and EDL muscles to tubocurarine. Fourth, we compared the effect of tubocurarine in the diaphragm and EDL muscles during the chronic absence of acetylcholinesterase in collagen Q-deficient mutant mice,11 before and after acetylcholinesterase or butyrylcholinesterase inhibition.

Materials and Methods

Mice
All experiments were conducted in adult (2- to 6-month-old) Swiss-Webster mice purchased from IFFA...
The preparations were mounted in Rhodorsil® silicone by immediate exsanguination. Isolated nerve–muscle preparations were isolated from mice of the Centre National de la Recherche Scientifique. The study was approved by the Animal Ethics Committee of the French Ministry for Research and Industry relating the use and storage of transgenic animals. Rearing conditions were in keeping with the guidelines of the French Ministry for Research and Industry. The diet (Renutryl; Nestlé Clinical, Sèvres, France) was provided ad libitum and water were provided. In addition, a liquid diet (Renutryl; Nestlé Clinical, Sèvres, France) under standard conditions (constant room temperature of 24°C and a 12:12 daylight cycle). Food and water were provided ad libitum. In addition, a liquid diet (Renutryl; Nestlé Clinical, Sèvres, France) was provided ad libitum. In addition, a liquid diet (Renutryl; Nestlé Clinical, Sèvres, France) was provided. Rearing conditions were in keeping with the guidelines of the French Ministry for Research and Industry relating the use and storage of transgenic animals. The study was approved by the Animal Ethics Committee of the Centre National de la Recherche Scientifique.

**Fig. 1.** Schematic representation of cholinesterase hetero-oligomers. The two enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are symbolized as a bifunctional protein containing the catalytic domain and the WAT domain, and the two known anchors ColQ or PRiMA. In addition to monomers, dimers, and tetramers of each enzyme (G), the hetero-oligomers result from the association of a short N-terminal domain (proline rich attachment domain [PRAD]) in ColQ or PRiMA with the C-terminal extension domain (tryptophan [W]) amphiphilic tetramerization (WAT) of AChE and BChE. ColQ targets the tetramers to the basal lamina and PRiMA to the plasma membrane.

CREDO (Saint Germain sur l’Arbresle, France) and ColQ-deficient mutant mice (ColQ<sup>−/−</sup>). The transgenic mice were housed in the transgenic facility unit (Centre National de la Recherche Scientifique, GIF sur Yvette, France) under standard conditions (constant room temperature of 24°C and a 12:12 daylight cycle). Food and water were provided ad libitum. In addition, a liquid diet (Renutryl; Nestlé Clinical, Sèvres, France) was provided. Rearing conditions were in keeping with the guidelines of the French Ministry for Research and Industry relating the use and storage of transgenic animals. The study was approved by the Animal Ethics Committee of the Centre National de la Recherche Scientifique.

**Recordings on Isolated Neuromuscular Preparations**

Left phrenic nerve–hemidiaphragm muscle and EDL nerve–muscle preparations were isolated from mice killed by dislocation of the cervical vertebrae followed by immediate exsanguination. Isolated nerve–muscle preparations were mounted in Rhodorsil<sup>®</sup> silicone (Rhône-Poulenc, St. Fons, France)–lined polymethyl methacrylate baths (4 ml volume) containing standard Krebs-Ringer’s physiologic solution (789). Trout liver was used as a standard solution. The Krebs-Ringer’s physiologic solution used in the experiment contained standard chloride concentrations of 154 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.0 mM HEPES buffer (pH 7.4), 11 mM glucose. The solution was gassed with pure oxygen.

For twitch tension measurements, one of the tendons of the hemidiaphragm or EDL muscle was tied with silk thread, via a suction microelectrode adapted to the diameter of the motor nerve. Current pulses of 0.15 ms duration and supramaximal intensity were supplied by an S-44 stimulator (Grass Instruments, Astro-Med Inc., West Warwick, RI), and the other tendon was pinned to the Rhodorsil<sup>®</sup>-lined chamber. Twitches were evoked by stimulating the motor nerve via a suction microelectrode. The stimuli were adjusted to obtain maximal contractile responses and was monitored during the duration of the experiment. The muscle was adjusted to obtain maximal contractile responses and was monitored during the duration of the experiment. The muscle was adjusted to obtain maximal contractile responses. The muscle was adjusted to obtain maximal contractile responses.

**Tissue Extraction of Cholinesterases**

Muscles were prepared in the conditions required for electrophysiologic experiments. The muscles were frozen and ground in liquid nitrogen. The powder was homogenized in a glass–glass handheld homogenizer in five volumes of buffer containing 25 mM Tris-HCl, pH 7.4, 10 mM EDTA, 800 mM NaCl, 1% Brij-97 (Sigma-Aldrich Chimie, St. Quentin-Fallavier, France) 2 mM benzamidine, 20 μg/ml pepstatin, 40 μg/ml leupeptin. Sedimentation analyses of acetylcholinesterase forms were performed in 5–20% (wt/vol) sucrose gradients containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 800 mM NaCl, and 1% Brij-97. Because it was previously noted that Triton-X100 inhibits mouse butyrylcholinesterase, we used 1% Brij-97 because it does not inhibit butyrylcholinesterase, and we found that the enzyme is more efficient than Tween 20 to extract membrane anchored acetylcholinesterase. We eliminated bacitracin (often used in gradients) because we have observed a degradation of ColQ complexes in its presence during centrifugation. The gradients were centrifuged at 38,000 rpm at 7°C for 17 h 30 min, using a SW41 rotor (Beckman Instruments,
Acetylcholinesterase and butyrylcholinesterase activities were assayed using 0.7 mM acetylthiocholine, and 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of 50 μM iso-OMP A or 10 μM 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one (BW284c51), respectively. Optical density was measured at 414 nm several times. A part was assayed for internal sedimentation markers (alkaline phosphatase [6.1 S] and β-galactosidase [16 S] from Escherichia coli). Their activity profiles were used to establish a linear relation between the fraction numbers and Sedvberg units. Each gradient normalized to the activity and milligrams of protein (estimated by BCA protein assay; Pierce Biotechnology Inc., Rockford, IL) was fitted by seven gaussian peaks using Rockford, IL) was fitted by seven gaussian peaks using PeakFit (Systat Software, Inc., Richmond, CA) corresponding to six major classes of acetylcholinesterase oligomers, peak 16.5 S: 3 ColQ and 3 acetylcholinesterase tetramers (12 acetylcholinesterase); peak 12.5 S: 3 ColQ and 2 acetylcholinesterase tetramers (8 acetylcholinesterase); peak 10.5 S: soluble acetylcholinesterase tetramer; peak 8.5 S: PRiMA and one acetylcholinesterase tetramer; peaks 3.8 S and 1.8 S: acetylcholinesterase dimer and/or monomer. The data are presented as ColQ:acetylcholinesterase complex, PRiMA:acetylcholinesterase complex, and soluble globular acetylcholinesterase.

Staining of Nicotinic Acetylcholine Receptors and Acetylcholinesterase

For staining nicotinic acetylcholine receptors and acetylcholinesterase in whole-mount preparations, the thin flat levator auris longus muscle and the hemidiaphragm were used. The muscles were fixed with 4% paraformaldehyde (30 min, room temperature) in phosphate-buffered saline (pH 7.4) and rinsed several times with the same buffer. To label nicotinic acetylcholine receptors, preparations were incubated for 45 min with fluorescein isothiocyanate–conjugated α-bungarotoxin (1 mg/ml; 1:500; Molecular Probes, Europe BV, Leiden, The Netherlands). To stain acetylcholinesterase, preparations were incubated for 1 h with tetramethylrhodamine isothiocyanate–conjugated fasciculin-1 (1:500 dilution) in phosphate-buffered saline. Fluorescent fasciculin-1 was prepared using the FluorReporter Protein Labeling Kit from Molecular Probes. Labeled preparations were mounted on glass slides with Vectashield antifading mounting medium (Vector Laboratories, Inc., Burlingame, CA).

Neuromuscular preparations were observed with a laser scanning confocal multiphoton system (Leica TCS SP2; Leica Microsystems, Mannheim, Germany) mounted on an upright microscope controlled through the manufacturer-supplied software and workstation. Images were collected using an oil-immersion lens (×40, numerical aperture = 1.25). The 488 nm wavelength line of an Argon-ion laser and the 543 nm wavelength lines of an He-NE laser were used for fluorescein and tetramethylrhodamine excitation, respectively. Series of optical sections were collected using a standard scanning mode format of 1,024 × 1,024 pixels, and three-dimensional projections of the images were constructed and analyzed. The area of nicotinic acetylcholine receptor staining was measured using ImageJ, a public domain image analysis software package, using standard procedures of threshold-selection for identifying regions with higher fluorescence than the surrounding ones.

Drugs

Neostigmine methylsulphate was purchased from France Biochem (Meudon, France); (+)-tubocurarine hydrochloride, iso-OMP A, BW284c51, and DTNB were obtained from Sigma-Aldrich Chimie. Fasciculin-1 was purified and kindly provided by Evert Karlsson, Ph.D. (Professor, Biomedical Centre, Uppsala University, Uppsala, Sweden).

Data Analysis and Statistics

The results are presented as the mean ± SD. The number of separate experiments in different muscles is indicated. To evaluate the resistance to tubocurarine between the diaphragm and the EDL muscles, concentration–response curves were generated in individual muscles and expressed as percent reduction of the maximal twitch response. Each drug concentration was applied by perfusion and allowed to equilibrate for 25 min. Sigmoidal nonlinear regression curve fitting for concentration–response data and estimation of the effective concentration that reduces 50% twitch tension (EC50) was calculated using Origin 6 software (Microcal Software Inc., Northampton, MA). The diaphragm/EDL EC50 ratio was calculated from data obtained at individual muscles. Data were analyzed using the Student t test (two tailed), and differences between the control and experimental values were considered statistically significant at P < 0.05.

Results

Neuromuscular Blocking Effect of Tubocurarine in Isolated Hemidiaphragm and EDL Nerve–Muscle Preparations from Normal Mice

Concentration–response studies were conducted in each preparation to compare the activity of tubocurarine in the mouse hemidiaphragm and EDL muscles. As shown in figure 2, higher doses of tubocurarine were needed to block nerve evoked muscle twitches in the hemidiaphragm than in the EDL muscle. From dose–response curves, the effective concentration that inhibited 50% twitch height (EC50) was calculated and found to be 1.42 ± 0.11 μM for the hemidiaphragm and 0.36 ±
Biochemical Quantification of Cholinesterases in Hemidiaphragm and EDL Muscles

Cholinesterase oligomers in diaphragm and EDL muscles from normal, and ColQ−/− mutant mice were quantified by sucrose gradient. As shown in figure 3, six peaks corresponding to three classes of oligomers were reproducitively observed. The ColQ-containing oligomers (Q) correspond to the 16.5 and 12.5 S peaks in which a triple helix of ColQ is associated with three or two tetramers, respectively; these oligomers are absent in ColQ−/− mutant muscles (fig. 3). The PRIMA-containing oligomer (P) corresponds to the 8.5 S peak, in which an acetylcholinesterase tetramer is linked to a transmembrane protein. The soluble globular forms (G) correspond to the 3.8 and 1.8 S peaks (monomer and/or dimer) and to the 10.5 S (a soluble tetramer). Acetylcholinesterase activity (determined in nmol acetylthiocholine hydrolyzed per minute per mg protein) seems more important in the EDL than in the diaphragm, as shown in table 2. In our experimental conditions, butyrylcholinesterase could not be quantified.

Table 1. Comparison of the EC50 of Tubocurarine Blocking Nerve-evoked Muscle Twitch on Isolated Diaphragm and EDL Muscles from Normal and ColQ−/− Mutant Mice under Various Experimental Conditions

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>EC50 of Tubocurarine, µM</th>
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<tbody>
<tr>
<td></td>
<td>Diaphragm</td>
</tr>
<tr>
<td>Control</td>
<td>1.42 ± 0.11*</td>
</tr>
<tr>
<td>Fasciculin-1</td>
<td>4.73 ± 0.35†</td>
</tr>
<tr>
<td>Iso-OMPA</td>
<td>1.49 ± 0.10*</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>3.97 ± 0.89†</td>
</tr>
<tr>
<td>ColQ−/−</td>
<td>1.89 ± 0.33*</td>
</tr>
<tr>
<td>ColQ−/− ± fasciculin-1</td>
<td>1.78 ± 0.29</td>
</tr>
<tr>
<td>ColQ−/− ± iso-OMPA</td>
<td>1.53 ± 0.37*</td>
</tr>
</tbody>
</table>

Fasciculin-1 was used to selectively inhibit acetylcholinesterase, iso-OMPA was used to inhibit butyrylcholinesterase, and neostigmine was used to inhibit both enzymes. ColQ−/− mutant neuromuscular preparations were also used because they do not express acetylcholinesterase. Values are presented as mean ± SD (n = 6–8 for each condition).

* P < 0.01 vs. extensor digitorum longus (EDL). † P < 0.01 vs. control.

Fig. 2. Concentration–response curves for tubocurarine in isolated extensor digitorum longus (EDL circles) and hemidiaphragm (DIA; squares) muscles from wild-type (WT) mice, under control conditions (filled circles and squares) and after treatment with 10 µM neostigmine (open circles and squares). Points are mean values ± SDs obtained in 6–8 different muscles.

0.06 µM for the EDL (table 1). The comparison of the EC50 indicates that a 4.1 ± 0.78-fold higher concentration of tubocurarine is needed in the hemidiaphragm than in the EDL muscle to obtain a similar degree of neuromuscular blocking effect.

Neuromuscular Blocking Effect of Tubocurarine in Nerve-Muscle Preparation after Inhibition of Cholinesterases

To determine whether the sensitivity to tubocurarine of diaphragm and EDL muscles was affected differently by cholinesterase inhibitors, preparations were equilibrated with 350 nM fasciculin-1 or 10 µM neostigmine for 30 min to totally inhibit acetylcholinesterase, and both acetylcholinesterase and butyrylcholinesterase, respectively, before tubocurarine was added to the bathing
medium. As shown in figure 2, in the presence of 10 μM neostigmine, the concentration–response curves for tubocurarine were shifted to the right in both hemidiaphragm and EDL muscles when compared with preparations in which cholinesterases were active. Under this condition, the calculated EC50 for tubocurarine in the hemidiaphragm and EDL were significantly (P < 0.01) higher (table 1), but the diaphragm/EDL EC50 ratio was not significantly different with respect to the control. Similarly, with fasciculin-1 (fig. 4), which selectively inhibits only acetylcholinesterase, the concentration–response curve was also shifted to the right in both hemidiaphragm and EDL when compared to controls. Their EC50 was significantly higher than in controls (table 1), but the diaphragm/EDL EC50 ratio was not significantly modified. In contrast, when neuromuscular preparations were pretreated with 100 μM iso-OMPA to inhibit butyrylcholinesterase (open circles and squares) to inhibit acetylcholinesterase, Points are mean values ± SDs obtained in 6–8 different muscles.

### Table 2: Quantification of Acetylcholinesterase Activity Extracted from Diaphragm and EDL Muscles in Control and ColQ⁻/⁻ Mutant Mice

<table>
<thead>
<tr>
<th>Acetylcholinesterase Activity, nmol ACh·min⁻¹·mg⁻¹</th>
<th>% of Each Oligomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm</td>
<td>EDL</td>
</tr>
<tr>
<td>G*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.76 ± 0.20</td>
</tr>
<tr>
<td>ColQ⁻/⁻</td>
<td>1.05 ± 0.75</td>
</tr>
<tr>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.34 ± 0.07</td>
</tr>
<tr>
<td>ColQ⁻/⁻</td>
<td>0.22 ± 0.14</td>
</tr>
<tr>
<td>Q</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.05 ± 0.43</td>
</tr>
<tr>
<td>ColQ⁻/⁻</td>
<td>—</td>
</tr>
</tbody>
</table>

* Each of the oligomers were separated in sucrose gradients and quantified, as described in Tissue Extraction of Cholinesterases (Materials and Methods section). Values are presented as mean ± SD. Activity units are in nmol acetylthiocholine (ACh) hydrolyzed per minute and per milligram protein; the proportion of oligomers represents the percentage of each population (n = 4 or 5 for controls; n = 3 for ColQ⁻/⁻).

The calculated EC50 from concentration–response curves for tubocurarine (fig. 4) were not significantly different from controls in both hemidiaphragm and EDL muscles (table 1).

**Blockade Induced by Tubocurarine on Isolated Neuromuscular Preparations from ColQ⁻/⁻ Mutant Mice**

Concentration–response curves for tubocurarine in ColQ⁻/⁻ muscles revealed that the curves were shifted to the right in the hemidiaphragm when compared with the EDL, and the diaphragm/EDL EC50 ratio was not significantly different from the control. Furthermore, the EC50 for tubocurarine in ColQ⁻/⁻ muscles was significantly modified neither by treatment with 350 nm fasciculin-1 (fig. 5) nor by pretreatment with 100 μM iso-OMPA (table 1).

**Distribution of Nicotinic Acetylcholine Receptors in ColQ⁻/⁻ Endplates**

The distribution of nicotinic acetylcholine receptors was investigated in muscles removed from age-matched wild-type and ColQ⁻/⁻ mice. As shown in figure 6, staining with fluorescein-conjugated α-bungarotoxin revealed that nicotinic acetylcholine receptors distributed in a smaller and fragmented endplate area in ColQ⁻/⁻ muscles, when compared with wild-type endplates. This effect was not only observed on the diaphragm (fig. 6A), but also on the thin and flat levator auris longus muscle (fig. 6B). Quantitative image analysis of α-bungarotoxin staining in randomly selected endplates revealed a mean surface area of 412.1 ± 188.8 μm² (n = 42 junctions examined) for wild-type endplates and 212.0 ± 96 μm² (n = 46 junctions) for ColQ⁻/⁻ endplates, indicating in the latter a significant (P < 0.05) decrease in the mean surface area occupied by nicotinic acetylcholine receptors. As shown in the scatter diagram of figure 6C, the surface area of nicotinic acetylcholine receptors staining was well correlated to the diameter of the muscle fibers both in wild-type and in ColQ⁻/⁻ endplates, but the surface area was smaller for most ColQ endplates.
tubocurarine (4.73 \text{ M}) in the diaphragm as compared with the EDL cannot be extrapolated to in vivo studies, it is worth noting that the EC_{50} of nondepolarizing muscle relaxants in humans are also 1.5–2 times higher for the diaphragm than for the adductor pollicis muscle.2,3,5

To determine whether differences in the cholinesterase activity exist between the diaphragm and EDL muscles, we have analyzed the three main acetylcholinesterase oligomers, including the globular oligomers, the ColQ-acetylcholinesterase, and the PRIIMA-acetylcholinesterase hetero-oligomers (fig. 3 and table 2), and found that the activity of these oligomers was lower in the diaphragm as compared with the EDL. In ColQ^{-/-} mutants, acetylcholinesterase is not accumulated at the neuromuscular junction, suggesting that ColQ-acetylcholinesterase is the main oligomer in the synaptic cleft.11

Our study provided evidence that ColQ-acetylcholinesterase is significantly different in the EDL when compared with the diaphragm. In addition, the globular oligomers and PRIIMA-acetylcholinesterase hetero-oligomers were more abundant in the EDL than in the diaphragm. This higher activity would be expected to shift the concentration–response curve for tubocurarine to the right in the EDL when compared with the diaphragm. However, the opposite was observed. The concentration of globular oligomers in rat muscles has been reported to be higher in the fast EDL than in the slow soleus muscle.16 Also, PRIIMA-acetylcholinesterase is mainly produced by fast muscles (rat EDL) and is regulated by training.17 Despite the fact that the mouse diaphragm and EDL muscles are mainly composed of fast-contracting-type fibers, we observed differences in the activity of globular oligomers and PRIIMA-acetylcholinesterase hetero-oligomers.

A lower acetylcholinesterase activity would tend to increase acetylcholine concentrations in the synaptic cleft of the neuromuscular junction and may therefore increase the quantity of acetylcholine competing with tubocurarine molecules. In accord with this hypothesis, when acetylcholinesterase was inhibited either by fasciculin-1 (350 nM), which only inhibits acetylcholinesterase, or by neostigmine (10 \text{ nM}), which inhibits both acetylcholinesterase and butyrylcholinesterase,12 the EC_{50} of tubocurarine increased significantly in diaphragm and EDL muscles, compared with untreated muscles. In this context, the increase in the EC_{50} of tubocurarine (4.73 \pm 0.35) in the diaphragm after fasciculin-1, when compared with neostigmine (3.97 \pm 0.89) treatment (table 1), can be explained by the fact that fasciculin-1 not only inhibits acetylcholinesterase but also has been reported to enhance evoked quantal trans-
mutter.\textsuperscript{12} In contrast, neostigmine, at the concentration used in the current study (10 μM), has been shown to reduce evoked quantal release.\textsuperscript{12}

However, after complete acetylcholinesterase inhibition, the diaphragm/EDL \( EC_{50} \) ratio for tubocurarine remained unchanged and was not significantly different from controls (table 1).

The current experimental conditions did not allow us to quantify butyrylcholinesterase despite that the enzyme is clearly accumulated at the neuromuscular junction,\textsuperscript{12} possibly because of the threefold to fourfold lower catalytic turnover of butyrylcholinesterase \textit{versus} acetylcholinesterase.\textsuperscript{18} At the neuromuscular junction of skeletal muscle, the physiologic role of butyrylcholinesterase in acetylcholine hydrolysis is not well established.\textsuperscript{19} The observation that humans who lack butyrylcholinesterase activity are healthy supports the belief that butyrylcholinesterase is unessential.\textsuperscript{20,21} Consistent with this view, in the current study, the selective and irreversible inhibition of butyrylcholinesterase activity and quantal transmitter release at normal and acetylcholinesterase \textit{versus} butyrylcholinesterase in the diaphragm/EDL ratio for tubocurarine. The activity of acetylcholinesterase forms was lower in the diaphragm than in the EDL muscle, implying that there are fewer acetylcholine molecules hydrolyzed in the diaphragm than in the EDL during the same period of time. However, the diaphragmatic resistance to tubocurarine cannot be explained by the different rate of acetylcholine hydrolysis in the synaptic cleft.

In conclusion, we have investigated whether acetylcholinesterase of the neuromuscular junction may contribute to the resistance of the diaphragm to tubocurarine. The activity of acetylcholinesterase forms was lower in the diaphragm than in the EDL muscle, implying that there are fewer acetylcholine molecules hydrolyzed in the diaphragm than in the EDL during the same period of time. However, the diaphragmatic resistance to tubocurarine cannot be explained by the different rate of acetylcholine hydrolysis in the synaptic cleft.

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