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 tubocurarine on 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, butyrylcholinesterase by tetraisopropylpyrophosphoramide, or both acetylcholinesterase and butyrylcholinesterase by neostigmine, and in acetylcholinesterase-deficient ColQ−/− muscles. 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 as 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−/− muscles.

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.1–7 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 of 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 tetraisopropylpyrophosphoramide (iso-OMP), 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.
Two enzymes

| Acetylcholinesterase (AChE) | Butyrylcholinesterase (BChE) |

Two structural proteins

| CoQ | PRiMA |

Several oligomers

| Soluble AChE (G) | Soluble BChE (G) |

**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 CoQ 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 CoQ or PRiMA with the C-terminal extension domain (tryptophan (W) amphiphilic tetramerization [WAT]) of AChE and BChE. CoQ 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 Innovation 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® silicone (Rhône-Poulenc, St. Fons, France)–lined polymethyl methacrylate baths (4 ml volume) containing standard Krebs-Ringer’s physiologic solution of the following composition: 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 an adjustable stainless steel hook, to an FT03 isometric transducer (Grass Instruments, Astro-Med Inc., West Warwick, RI), and the other tendon was pinned to the Rhodorsil®-lined chamber. Twitches were evoked by stimulating the motor nerve 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.) at frequencies of 0.1 Hz. For each preparation investigated, the resting tension was adjusted to obtain maximal contractile responses and was monitored during the duration of the experiment. Tension signals from the isometric transducer were amplified, collected, and digitized with the aid of a computer equipped with a DT2821 analog-to-digital interface board (Data Translation, Marlboro, MA). Computerized data acquisition and analysis were performed with a program kindly provided by John Dempster, Ph.D. (Senior Research Officer, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, Scotland).

All experiments were performed at 22°C. After preparations were equilibrated for 30 min with oxygenated physiologic solution, tubocurarine concentration–response curves were performed in single nerve–muscle preparations from normal and ColQ<sup>−/−</sup> muscles. In some experiments, preparations were treated with either fasciculin-1 (specific inhibitor of acetylcholinesterase), or neostigmine (inhibitor of both acetylcholinesterase and butyrylcholinesterase). For inhibition of butyrylcholinesterase, preparations were first pretreated with iso-OPMA for 30 min followed by washout of the drug from the medium.

**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-X-100 inhibits mouse butyrylcholinesterase, we used 1% Brij-97 because it does not inhibit butyrylcholinesterase, and we found Brij-97 to be more efficient than Tween 20 to extract membrane anchored acetylcholinesterase. We eliminated bacitracin (often used in gradients) because we have observed a degradation of CoQ 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,
Fullerton, CA). Each gradient was collected in 48 fractions and was assayed for cholinesterase activities. 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-OMPA 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 Svedberg 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 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 tetramer9; 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-OMPA, 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 reproducibly 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>Diaphragm EC50 of Tubocurarine, μM</th>
<th>EDL EC50 of Tubocurarine, μM</th>
<th>Diaphragm/EDL EC50 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.42 ± 0.11*</td>
<td>0.36 ± 0.06</td>
<td>4.1 ± 0.8</td>
</tr>
<tr>
<td>Fasciculin-1</td>
<td>4.73 ± 0.35†</td>
<td>1.05 ± 0.10†</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Iso-OMPA</td>
<td>1.40 ± 0.10*</td>
<td>0.35 ± 0.50</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>3.87 ± 0.89†</td>
<td>0.86 ± 0.13†</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>ColQ−/−</td>
<td>1.89 ± 0.33†</td>
<td>0.41 ± 0.06</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>ColQ−/− + fasciculin-1</td>
<td>1.78 ± 0.29</td>
<td>0.43 ± 0.12</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>ColQ−/− + iso-OMPA</td>
<td>1.53 ± 0.37*</td>
<td>0.38 ± 0.10</td>
<td>4.1 ± 0.3</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.

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...
Table 2. Quantification of Acetylcholinesterase Activity Extracted from Diaphragm and EDL Muscles in Control and ColQ^-/- Mutant Mice

<table>
<thead>
<tr>
<th></th>
<th>Acetylcholinesterase Activity, (\text{nmol ACh} \cdot \text{min}^{-1} \cdot \text{mg}^{-1})</th>
<th>% of Each Oligomer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diaphragm</td>
<td>EDL</td>
</tr>
<tr>
<td>(G^*) Control</td>
<td>0.76 ± 0.20</td>
<td>2.11 ± 0.64*</td>
</tr>
<tr>
<td>ColQ^-/-</td>
<td>1.05 ± 0.75</td>
<td>1.50 ± 0.94†</td>
</tr>
<tr>
<td>(P) Control</td>
<td>0.34 ± 0.07</td>
<td>0.83 ± 0.13*</td>
</tr>
<tr>
<td>ColQ^-/-</td>
<td>0.22 ± 0.14</td>
<td>0.42 ± 0.09†</td>
</tr>
<tr>
<td>(Q) Control</td>
<td>2.05 ± 0.43</td>
<td>2.74 ± 0.63*</td>
</tr>
<tr>
<td>ColQ^-/-</td>
<td>—</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^-/-).

† Not significantly different (\(P > 0.05\)).

EDL = extensor digitorum longus.

### Discussion

medium. As shown in figure 2, in the presence of 10 \(\mu M\) 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 \(EC_{50}\) for tubocurarine in the hemidiaphragm and EDL were significantly \((P < 0.01)\) higher (table 1), but the diaphragm/EDL \(EC_{50}\) 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 \(EC_{50}\) was significantly higher than in controls (table 1), but the diaphragm/EDL \(EC_{50}\) ratio was not significantly modified. In contrast, when neuromuscular preparations were pretreated with 100 \(\mu M\) iso-OMPA (followed by wash-up of this agent), which allowed complete and irreversible inhibition of butyrylcholinesterase activity, the calculated \(EC_{50}\)s 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 \(EC_{50}\) ratio was not significantly different from the control. Furthermore, the \(EC_{50}\) for tubocurarine in ColQ^-/- muscles was significantly modified neither by treatment with 350 \(\mu M\) fasciculin-1 (fig. 5) nor by pretreatment with 100 \(\mu M\) 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 \(\alpha\)-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 \(\alpha\)-bungarotoxin staining in randomly selected endplates revealed a mean surface area of 412.1 ± 188.8 \(\mu m^2\) (\(n = 42\) junctions examined) for wild-type endplates and 212.0 ± 96 \(\mu m^2\) (\(n = 46\) 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.

![Fig. 4. Concentration–response curves for tubocurarine in isolated extensor digitorum longus (EDL; circles) and hemidiaphragm (DIA; squares) muscles from wild-type (WT) mice after pretreatment with 100 \(\mu M\) iso-OMPA to inhibit butyrylcholinesterase. Points are mean values ± SDs obtained in 6–8 different muscles.](https://example.com/fig4)

![Fig. 5. Concentration–response curves for tubocurarine in isolated extensor digitorum longus (EDL; circles) and hemidiaphragm (DIA; squares) muscles from ColQ^-/- mice (open circles and squares) and after treatment with 350 \(\mu M\) fasciculin-1 (filled circles and squares). Points are mean values ± SDs obtained in 5–8 different muscles.](https://example.com/fig5)
Fig. 6. Confocal micrographs of nicotinic acetylcholine receptor staining and acetylcholinesterase staining, obtained in whole-mounted wild-type (WT) and ColQ⁺/⁻ muscles. (A) Representative staining of endplate nicotinic acetylcholine receptors with fluorescein-conjugated α-bungarotoxin, in WT and ColQ⁺/⁻ hemidiaphragm muscles. Note the smaller area distribution of the staining in the ColQ⁺/⁻ endplate. (B) Staining of nicotinic acetylcholine receptors, from WT (a) and from ColQ⁺/⁻ (c) levator auris longus muscles, and acetylcholinesterase staining with tetramethylrhodamine-conjugated fasciculin-1 (b and d) from the same muscles. Note the absence of fasciculin-1 labeling in the ColQ⁺/⁻ junction (d), in contrast to the WT junction (b). Images represent projections of series of 15 optical sections spaced 0.1 μm apart from each muscle. (C) Relation between the nicotinic acetylcholine receptor staining area and the muscle fiber diameter for WT (empty circles) and ColQ⁺/⁻ junctions (filled circles). The linear relations for WT and ColQ⁺/⁻ data were estimated by linear regression.

Discussion

In the current study, we observed that the activity of the different acetylcholinesterase oligomers was lower in the diaphragm as compared with the EDL (table 2). Furthermore, concentration–response studies in control preparations revealed that the concentration of tubocurarine necessary to decrease by 50% nerve-evoked muscle twitch of the isolated mouse diaphragm was approximately four times higher than that required in the isolated EDL muscle (table 1). Moreover, inhibition of acetylcholinesterase by antagonists resulted in an increased concentration of tubocurarine and a displacement of the concentration–response curves to the right but an unchanged resistance ratio between the diaphragm and the EDL. A similar unchanged resistance ratio was observed in ColQ⁻/⁻ muscles.

In previous studies, higher doses of tubocurarine have been shown to be needed in nerve–muscle preparations of young mice, in which the EC₅₀ of tubocurarine was reported to be approximately 2.4 times higher in the diaphragm than in the EDL muscle. Although current findings of the high resistance of the diaphragm to tubocurarine compared with the EDL cannot be extrapolated to in vivo studies, it is worth noting that the ED₅₀ of nondepolarizing muscle relaxants in humans are also 1.5–2 times higher for the diaphragm than for the adductor pollicis muscle.

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 PRiMA-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. Our study provided evidence that ColQ-acetylcholinesterase is significantly different in the EDL when compared with the diaphragm. In addition, the globular oligomers and PRiMA-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. Also, PRiMA-acetylcholinesterase is mainly produced by fast muscles (rat EDL) and is regulated by training. 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 PRiMA-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 μM), which inhibits both acetylcholinesterase and butyrylcholinesterase, the EC₅₀ of tubocurarine increased significantly in diaphragm and EDL muscles, compared with untreated muscles. In this context, the increase in the EC₅₀ of tubocurarine (4.73 ± 0.35) in the diaphragm after fasciculin-1, when compared with neostigmine (3.97 ± 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 release. In contrast, neostigmine, at the concentration used in the current study (10 μM), has been shown to reduce evoked quantal release.

However, after complete acetylcholinesterase inhibition, the diaphragm/EDL EC50 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, possibly because of the threefold to fourfold lower catalytic turnover of butyrylcholinesterase versus acetylcholinesterase. At the neuromuscular junction of skeletal muscle, the physiologic role of butyrylcholinesterase in acetylcholine hydrolysis is not well established. The observation that humans who lack butyrylcholinesterase activity are healthy supports the belief that butyrylcholinesterase is unessential. Consistent with this view, in the current study, the selective and irreversible inhibition of butyrylcholinesterase activity of normal diaphragm and EDL muscles by iso-OMPA did not significantly modify the EC50 of tubocurarine when compared with untreated muscles. These results further suggest that butyrylcholinesterase does not contribute to acetylcholine hydrolysis in the synaptic cleft. Because one would otherwise expect after inhibition of butyrylcholinesterase a change in the EC50 of tubocurarine. It is worth noting that in contrast to ColQ-acetylcholinesterase, which is primary present in the basal lamina of the neuromuscular junction, butyrylcholinesterase is localized in perisynaptic Schwann cells.

Interestingly, in ColQ−/− mutant muscles in which acetylcholinesterase is not accumulated at the neuromuscular junction (table 2), neither the diaphragm/EDL-EC50 ratio nor the EC50 for tubocurarine was significantly different from those obtained in normal muscle. In addition, there were no longer significant differences in globular oligomers and PRiMA-acetylcholinesterase hetero-oligomer between ColQ−/− diaphragm and EDL muscles. The equipotent effect of tubocurarine in ColQ−/− when compared with control muscles was striking and was further investigated. For this, we performed experiments on nerve-evoked muscle contraction to determine whether fasciculin-1 could shift the concentration-response of tubocurarine. However, the EC50 were not significantly different in the presence or absence of fasciculin-1. These results may indicate that molecular forms other than ColQ do not contribute to acetylcholine hydrolysis to any significant extent in ColQ−/− muscles. In addition, we stained postsynaptic nicotinic acetylcholine receptors with fluorescent-labeled α-bungarotoxin and quantified the surface area of the endplates. The results clearly showed that the α-bungarotoxin stained surface area of ColQ−/− endplates was reduced by 56.3% with respect to wild-type endplates. These results could explain that despite the lack of acetylcholinesterase activity in ColQ−/− endplates, EC50 of tubocurarine was not significantly different from that of controls.

The current study focused on the possibility that cholinesterase activity would differ between the diaphragm and EDL, thereby influencing the competitive neuromuscular blockade of tubocurarine. However, despite differences in acetylcholinesterase activity between the diaphragm and EDL, there are other mechanisms that have not been explored currently and could account for the diaphragmatic resistance. Other possible presynaptic or postsynaptic mechanisms may involve differences in quantal acetylcholine release from motor nerve terminals or differences in acetylcholine-receptor density in the muscle membrane. This is currently being explored in our laboratory.

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.

The authors thank Patricia Villeneuve and Valérie Lavallée (Technicians, Transgenic facility, Gif-sur-Yvette Campus, France) for taking care of the collagen Q−/− deficient mutant mice used in the current study.

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