

Resistance to D-Tubocurarine of the Rat Diaphragm as Compared to a Limb Muscle

Influence of Quantal Transmitter Release and Nicotinic Acetylcholine Receptors

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Background: **The diaphragm is resistant to competitive neuromuscular blocking agents, as compared to peripheral muscles. The basis of this difference may be a higher concentration of acetylcholine released or higher number of postsynaptic nicotinic acetylcholine receptors in diaphragmatic neuromuscular junctions.**

Methods: **Nerve-evoked twitch-tension was measured in rat hemidiapragm as was *Extensor digitorum longus* (EDL) nerve-muscle preparation to determine the effective D-tubocurarine concentration that decreased twitch responses by 50%. The mean quantal content of endplate potentials was determined in single junctions in a low-Ca^{2+}, high-Mg^{2+} Krebs-Ringer medium. Strips of hemidiaphragm and EDL muscle, containing the endplate regions, were used to determine the number of nAChR nicotinic acetylcholine receptor binding sites with the aid of radiolabeled [^{125}I]α-bungarotoxin.**

Results: **The effective D-tubocurarine concentration that decreased twitch responses by 50% (median [interquartile range]) was seven-fold higher in the hemidiaphragm than in the EDL (1.82 μM [1.43–2.20] *vs.* 0.26 μM [0.23–0.29], $P < 0.01$). The median of the mean quantal content was higher in the hemidiaphragm than in the EDL (0.57 [0.44–0.84] *vs.* (0.14 [0.11–0.19], $P < 0.01$). The number of specific [^{125}I]α-bungarotoxin binding sites to junctional nicotinic acetylcholine receptors was higher in the diaphragm than in the EDL (1.15 fmol/mg [0.48–1.70] *vs.* 0.55 fmol/mg [0.23–0.70] , $P < 0.05$).**

Conclusion: **The current study indicates that the resistance of the diaphragm to neuromuscular blocking agents can be explained by both a higher mean quantal content of endplate potentials and a higher number of nicotinic acetylcholine receptor binding sites than in the peripheral EDL muscle.**

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THE diaphragm is resistant to the blocking effect of competitive neuromuscular blocking agents (NMBA), as compared to peripheral muscles. Dose-response curves demonstrated a shift to the right of the diaphragmatic response compared to the *Adductor pollicis* muscle in humans.[1–3] After an intubating dose of a competitive NMBA in anesthetized patients, the recovery of the diaphragm-evoked response occurs earlier than at the *Adductor pollicis* muscle.[4–7] The resistance of the diaphragm to NMBA is still poorly understood. Muscle type composition, which differs between the diaphragm and peripheral muscles, does not explain the difference in muscle relaxant effect.[8–9] In the cat, Waud and Waud demonstrated that the safety margin of neuromuscular transmission in the diaphragm was greater than in the *Tibialis anterior* muscle, but they could not provide any explanation for this difference.[10]

The mechanism of resistance may be either presynaptic or postsynaptic. Presynaptic factors include the modulation of acetylcholine release from motor nerve terminals. Postsynaptic factors include the density of nicotinic acetylcholine receptors (nAChR) and the rate of acetylcholine hydrolysis by acetylcholinesterase. We recently measured acetylcholinesterase activity of the different heterooligomers of the neuromuscular junction in the diaphragm and in a peripheral mouse limb muscle.[11] Although acetylcholinesterase activity was lower in the diaphragm than in the *Extensor digitorum longus* (EDL), this difference could not explain the diaphragmatic resistance to tubocurarine because specific inhibition of acetylcholinesterase did not change the four-fold effective D-tubocurarine dose-ratio between the diaphragm and the EDL observed in the mouse.[11]

In the current study, we investigated whether the diaphragmatic resistance to D-tubocurarine depends on the quantal content of endplate potentials and/or on the number of nAChR binding sites in the neuromuscular junctions of the diaphragm and the EDL.

Materials and Methods

Animals

The study was approved by the Animal Ethics Committee of the Centre National de la Recherche Scientifique. All experiments were performed in accordance with European Community guidelines for animal laboratory handling. This study, including care of animals, was conducted according to the official edict presented by

the French Ministry of Agriculture (Paris, France) and the recommendations of the Helsinski Declaration. Thus, these experiments were conducted in an authorized laboratory and under the supervision of an authorized researcher (Dr. Molgó). Sprague-Dawley female adult rats weighing 180–200 g were purchased from Iffa Credo (Saint Germain sur l'Arbresle, France). Rats were housed in groups of three, and food and water were provided *ad libitum*.

Neuromuscular Blocking Effect of D-Tubocurarine

Isolated rat EDL and left hemidiaphragm nerve-muscle preparations were mounted in silicone-lined organ baths superfused with an oxygenated standard Krebs-Ringer solution containing: 154 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 11 mM D-glucose (pH 7.4). Twitch tension measurements were performed to evaluate the effect of D-tubocurarine on both the EDL and the hemidiaphragm, using techniques previously described.[11] After preparations were equilibrated for 30 min with oxygenated physiologic solution, D-tubocurarine concentration-response curves were performed.

Measurement of the Mean Quantal Content of Endplate Potentials

In isolated nerve-muscle preparations, membrane potential and synaptic potentials were recorded at 22°C with intracellular microelectrodes filled with 3 M KCl (8–12 MΩ resistance) by using conventional techniques and an Axoclamp-2A system (Axon Instruments, Union City, CA). The motor nerve of isolated neuromuscular preparations was stimulated with a suction microelectrode adapted to the diameter of the nerve, with 0.1-ms pulses at 0.2 Hz and supramaximal voltage (typically 3–8 V) supplied by an S-44 stimulator (Grass Instruments, West Warwick, RI) The signals were acquired and digitized by a 12-bit A/D converter (Digidata 1200B, Axon Instruments). Computer analysis was performed using a suite of purposed-designed electrophysiological analysis programs.[12] The mean quantal content (m_o) of endplate potentials was determined in a low-Ca^{2+} (0.4 mM), high-Mg^{2+} (7.5 mM) Krebs-Ringer solution. The so called "method of failures"[13] was used in which $m_o = \ln (NT/N_o)$, where NT represents the total number of stimuli delivered to the motor nerve and N_o represents the number of failures of release (*i.e.*, number of stimuli not followed by an endplate potential). To calculate m_o in individual junctions, 100–150 consecutive stimuli were delivered to the motor nerve.

Nicotinic Acetylcholine Receptor Evaluation

Excised rat hemidiaphragm and EDL muscles were pinned in the silicone-lined organ bath with the aid of mini pins. A careful dissection was performed under the microscope to isolate small muscle strips containing the endplate regions. Also, regions containing no endplates were excised to validate the specific α-bungarotoxin binding. This dissection was performed in an oxygenated Ca^{2+}-free Krebs-Ringer solution in which (Ca^{2+} was replaced by Mg^{2+}) to prevent proteolysis. These strips were immediately frozen until use for determining nAChR ligand binding sites.

For determining the number of nAChRs binding sites present on diaphragm or EDL, the specific binding of radiolabeled [^{125}I]α-bungarotoxin was measured in a blind manner. A total of 10 or 20 mg of the junctional region of rat diaphragm and EDL muscles were prepared in 1 ml of Krebs-Ringer solution containing 0.5% bovine serum albumin. Total binding was obtained after 3 h of incubation with 2.5 nM [^{125}I]α-bungarotoxin (250 Ci/mmol; Amersham Biosciences, Orsay, France) under agitation. The nonspecific binding was determined after 1 h of preincubation with a large excess of nicotine (1 mM) or α-bungarotoxin (5 μM) before the addition of [^{125}I]α-bungarotoxin. The tissue samples were washed first for 3 h in cold buffer and then overnight. The total and nonspecific radioactivity fixed on the muscle preparations were counted on a gamma counter, and then the specific binding in fmol/mg of muscle was calculated. Experiments were made in triplicate.

Data Analysis and Statistics

The results are presented as the median and 25–75% interquartile range (IQR) or the mean ± SEM when appropriate. The number of separate experiments in different muscle is indicated. Sigmoidal nonlinear regression curve fitting for D-tubocurarine concentration-response data were performed to calculate the effective concentration that reduces 50% twitch tension. Comparison of data between the diaphragm and the EDL was performed using the Wilcoxon test. Differences between the diaphragm and the EDL were considered statistically significant at $P < 0.05$.

Results

Neuromuscular Blocking Effect of D-Tubocurarine

A concentation-response study was conducted in each preparation investigated to compare the activity of D-tubocurarine in the rat hemidiaphragm and the EDL muscle. As shown in figure 1, higher concentrations of D-tubocurarine were needed to block nerve-evoked muscle twitches in the diaphragm than in the EDL muscles. The median effective concentration of D-tubocurarine that reduces 50% twitch tension was of 1.82 μM (IQR, 1.43–2.20 μM) for the diaphragm and 0.26 μM (IQR, 0.23–0.29 μM) for the EDL ($P < 0.01$).

Quantal Transmitter Release in Hemidiaphragm and EDL Junctions

To compare the mean number of quanta released by nerve stimulation in hemidiaphragm and EDL muscles,

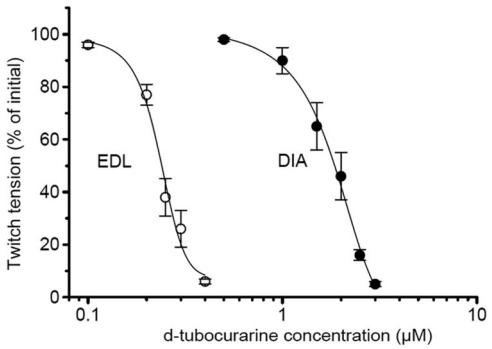

Fig. 1. Concentration-response curves for D-tubocurarine in isolated rat *Extensor digitorum longus* (EDL; *white circles*) and hemidiaphragm (DIA; *black circles*). Points are the mean values ± SEM obtained in paired muscles from seven rats.

the mean quantal content of endplate potentials was determined at individual junctions of the two muscles in a low-Ca^{2+}, high-Mg^{2+} medium. Figure 2 shows the results obtained in 30 hemidiaphragm and EDL junctions from eight rats. The median value of the mean quantal content of endplate potentials in the hemidiaphragm was 0.57 (IQR, 0.44–0.84), significantly higher ($P < 0.01$) than the 0.14 (IQR, 0.11–0.19) value obtained in the EDL.

Muscle Membrane Nicotinic Acetylcholine Receptors

No specific binding was found in excised muscle strips devoid of endplates. As shown in table 1, the median number of specific nAChR binding sites was significantly higher ($P < 0.05$) in the diaphragm (1.15 fmol/mg [IQR 0.48–1.70 fmol/mg]) than in EDL (0.55 fmol/mg [IQR, 0.23–0.70 fmol/mg]).

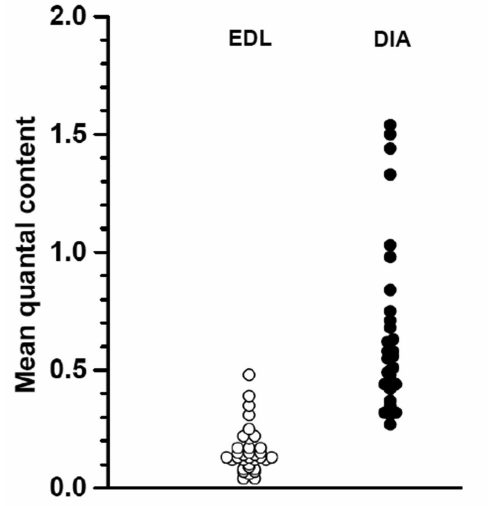

Fig. 2. Evaluation of the mean quantal content of endplate potentials in junctions from rat *Extensor digitorum longus* (EDL; *white circles*) and hemidiaphragm (DIA; *black circles*) bathed in a low-Ca^{2+} (0.4 mM), high-Mg^{2+} (7.5 mM) Krebs-Ringer solution. Thirty paired measures were performed in EDL and DIA muscles obtained from eight rats. Each point represents data obtained in a single junction. Note that the mean quantal content of endplate potentials is higher in the DIA than in the EDL muscles.

Table 1. Comparison of the Specific [^{125}I]α-Bungarotoxin Binding Sites (fmol/mg) on the Rat Diaphragm and EDL Muscles

	Diaphragm (n = 7)	EDL (n =7)
Specific binding sites	1.55* (0.48–1.70)	0.55 (0.23–0.70)

Data are presented as median (interquartile range). * $P < 0.05$ *vs. Extensor digitorum longus* (EDL).

Discussion

In the current study, we observed from the concentration–response curves that the effective concentration of D-tubocurarine that reduces 50% twitch tension in the isolated rat hemidiaphragm was approximately 7-fold higher than in the EDL. In addition, we have demonstrated that both the mean quantal content of endplate potentials, which provides an indication of evoked quantal transmitter release, and the nAChR-specific binding sites are in higher numbers in the diaphragm than in EDL muscles of the rat.

The different potency of D-tubocurarine presently observed *in vitro* in the rat between the diaphragm and the EDL corroborates our previous findings in the mice.[11] In comparison, a 2.5- to 3-fold potency ratio of steroidal NMBA was observed *in vivo* in the rat,[14] whereas a 7-fold D-tubocurarine effective concentration ratio was observed in the current study. This difference can probably be explained by differences between the *in vivo* and *in vitro* conditions.

To the best of our knowledge, no studies have been reported comparing quantal transmitter release between rat diaphragm and peripheral muscles. Differences in quantal transmitter release have been reported between nerve terminals innervating rat fast and slow muscles.[15] The increase in quantal release presently detected in the diaphragm with respect to the EDL may be due either to differences in the available number of nerve terminal release sites and/or nerve terminal extension[16] or to differences in calcium channels available for triggering evoked quantal acetylcholine release.[17] In the current study quantal transmitter release was measured in low-calcium, high-magnesium medium. By reducing the ratio of Ca^{2+} to Mg^{2+} concentrations in the bathing fluid, transmitter release is diminished to levels that are too low to generate an action potential,[13] allowing electrophysiological recordings. Although this method has some limitations, it is useful for comparing relative values of the quantal content of endplate potentials between muscles.[18]

The use of μ-conotoxin GIIIB, a specific skeletal muscle sodium channel blocker[19] that abolishes muscle contraction at low concentration, allows the measurement of evoked acetylcholine release in more physiologic conditions. However the concentration of μ-conotoxin GIIIB necessary to abolish muscle contraction in the

diaphragm[20] appears to be higher than in peripheral muscles.[15] Therefore the use of this latter technique requiring higher μ-conotoxin GIIIB concentration that may lead to partial block of nerve impulse requires further assessment to compare acetylcholine release at the neuromuscular junction of the diaphragm and the EDL muscles.

A 2-fold increase in the specific [^{125}I]α-bungarotoxin binding to nAChRs was observed in the diaphragm with respect to the EDL muscle, despite a large scattering among data from different animals. This difference could be attributed to natural variable density of nAChRs and/or to variation in the dissection/preparation of muscle trips. In contrast to the results of our study, Ibebunjo *et al.* observed that the nAChR density did not vary in the cat and in the goat between different muscles, including the diaphragm and limb muscles.[21–22] This difference with our present results may be explained by the different methodology used. Ibebunjo *et al.* measured the number of binding sites per endplate,[21–22] whereas it was quantified per mg of muscle in the current study. Another difference may be due to the different species studied, as Ibebunjo *et al.*, did not observe any significant difference in the potency of vecuronium between the diaphragm and peripheral muscles in the goat, as well as in the cat.[9,21]

The 3.5-fold higher quantal content of endplate potentials and the 2-fold higher number of nAChRs sites in the diaphragm may provide an explanation of the 7-fold difference in sensitivity to D-tubocurarine when compared to the EDL, although there is no clear linear relationship between these physiologic parameters and the effect of NMBA. Non-depolarizing NMBA act by occupying nAChR competitively with acetylcholine. A higher acetylcholine release at the neuromuscular junction of the diaphragm will diminish the neuromuscular blocking effect of nondepolarizing NMBA. The diaphragm resistance to NMBA may be also explained by its high nAChR density. The nAChR density is a major determinant of the muscle response to NMBA. Increase in receptor density will decrease the neuromuscular blocking effect of competitive agents.[23] A 3- to 5-fold increase in receptor density, mostly extrajunctional, was associated with resistance to NMBA after burn.[24] However, most studies about nAChR density and muscle relaxant effect focused on disease states,[24–26] and the relationship between receptor density and muscle relaxant response established during pathologic states does not necessarily apply to the comparison between normal muscle groups.

Among skeletal muscles, the diaphragm differs by its rate of activation. The ratio of active to inactive times (*i.e.*, "the daily duty cycle") is of 45%, whereas it varies between 2 and 14% in peripheral muscles.[27] The neuromuscular junction of the diaphragm has been extensively studied because of the easiness to set up the phrenic nerve–diaphragm preparation, but there is no study comparing the neuromuscular junction of the diaphragm to that of peripheral muscles to delineate their functional neurophysiological characteristics. It was stated that the resistance of the diaphragm to NMBA was due to the greater safety margin of its neuromuscular junction,[10] but this statement was not supported by an electrophysiological analysis. Our study provides an explanation to these phenomena and will require further investigations to complete the present findings.

In conclusion, by comparing the neuromuscular junction of the rat diaphragm and a limb muscle, we observed that the mean quantal content of endplate potentials and the number nAChR binding sites were greater in the diaphragm muscle. These findings may explain why this muscle is resistant to competitive neuromuscular blocking agents.

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ANESTHESIOLOGY REFLECTIONS

Connell Gas-Oxygen Apparatus, Brass Model War SP

A New York surgeon and manufacturer, Dr. Karl Connell (1878–1941) produced his War SP Model in a brass-and-copper-appointed version (the pretty "Officer's" one seen here) and in a duller nickel-plated finish (presumably the "Enlisted Man's" version). Used by Allied Forces in France and Belgium, and neutral services in the Netherlands during World War I, Connell's "Special" SP featured a round brass plaque listing instructions in both English and French. The War SP Model exemplified the "rotary vane" type of flowmeter which succeeded the Connell Anesthetometer's piston-type and which preceded the inclined double-ball-bearing flowmeters of later Connell cabinet models. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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