Cholinesterase Activity of Motor End Plate in Human Skeletal Muscle

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ABSTRACT The activity and properties of cholinesterase of the motor end plate in human intercostal muscle were studied in the isolated muscle membrane. This preparation was used because cholinesterase activity of the membrane preparation was localized in the motor end plate without contamination of cholinesterase of other muscle components. Under the experimental conditions, cholinesterase in a human end plate hydrolyzed 1.21×10^8 molecules of acetylcholine per msec, which is smaller than hydrolysis of 2.69×10^8 by a motor end plate of rat intercostal muscle. Studies with cholinesterase inhibitors and specific substrates indicated that about 90% of cholinesterase of human motor endplates is acetylcholinesterase, and about 10% is pseudocholinesterase. The end plate cholinesterase had an optimal pH of 7.8 and a Michaelis-Menten constant of 4.15 mmoles/liter, and was stable at 4°C for at least 4 wk. Motor end plates were estimated to contain only about 2% of the total cholinesterase activity of human intercostal muscle, compared with about 20% in rat tibialis anterior muscle. The difference is due to the lower cholinesterase activity of the motor end plate and higher cholinesterase activity of non-end plate components in human muscle than in rat muscle. The isolated muscle membrane provides a useful preparation for the study of the properties of motor end plate in human skeletal muscle.

INTRODUCTION

Cholinesterase plays a cardinal role, together with acetylcholine and acetylcholine receptor, in cholinergic transmission including the neuromuscular transmission. Cholinesterase of the neuromuscular junction may play a role in the pathophysiology of diseases of neuromuscular transmission, and in the action of drugs which affect neuromuscular transmission. However, there has been no quantitative study of cholinesterase activity of the motor end plate of human skeletal muscle. In a previous communication, the authors reported cholinesterase activity of the motor end plate of rat skeletal muscle, by use of an isolated muscle membrane preparation (1). This preparation was used because cholinesterase activity of the muscle membrane is localized in motor end plates.

In the present study, the activity and properties of cholinesterase of the motor end plate of normal human intercostal muscle were estimated in the isolated muscle membrane. The intercostal muscle was chosen since this muscle is mainly used in microelectrophysiological studies of human motor end plates.

METHODS

Human intercostal muscle was obtained during postmortem examination performed within 5 hr of death from the fifth to eighth intercostal spaces of patients with no history of neuromuscular disease.

The muscle homogenate was prepared by homogenizing the muscle in a Waring Blendor with 50 mM $CaCl_2$ and then filtering through 18 mesh plastic net. In order to prepare the muscle membrane fraction, the sarcoplasmic components of the homogenate were dissolved and removed by repeated washing. Details of these methods have been described in a previous communication (1).

Cholinesterase activity was measured by the hydroxamic acid method (2). Incubation was performed at 37°C for 30 min in a medium which contained 4 mM acetylcholine bromide, 100 mM NaCl, 10 mM $MgCl_2$, and 30 mM sodium phosphate buffer, pH 7.5, unless otherwise indicated. Nitrogen was measured by the micro-Kjeldahl method.

Staining for cholinesterase activity in muscle sections or in the membrane fraction was performed using acetylthiocholine as the substrate (3). To visualize motor end plates of the muscle membrane, an aliquot of membrane suspension was spread on a Millipore filter, type SM (pore size, 5.0 ± 1.2 μ). The number of motor end plates was counted under a microscope, and the diameter of motor end plates was measured by an ocular micrometer with graduations of 1.25 μ. Statistical analyses were performed as described by Goldstein (4).

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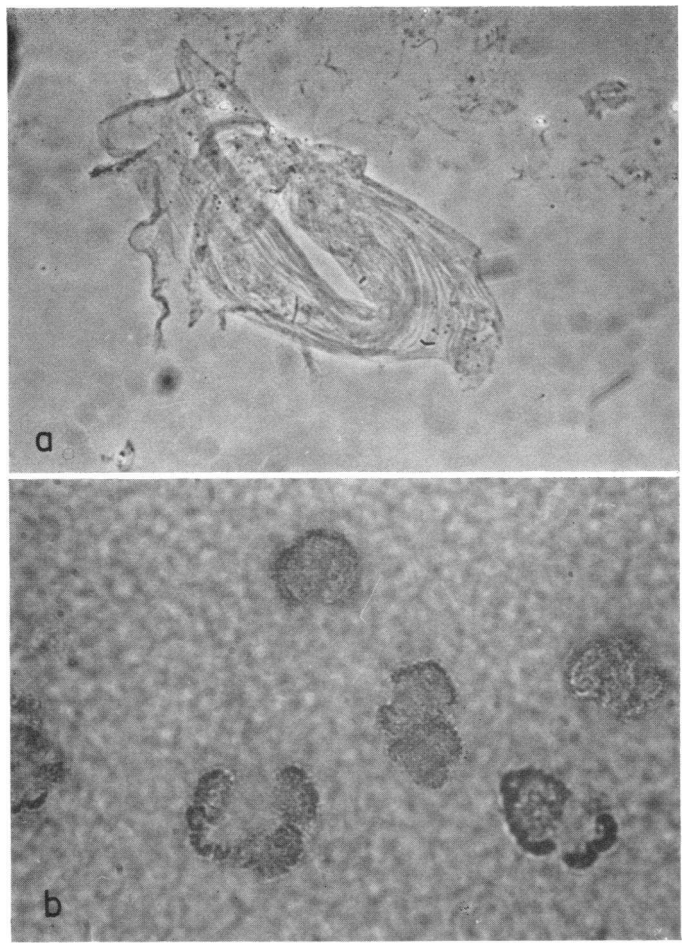

FIGURE 1 Isolated membrane fraction of human intercostal muscle. (*a*) unstained sample under phase contrast microscopy, × 125, and (*b*) motor end plates demonstrated by cholinesterase staining. × 500.

RESULTS

Properties of isolated human muscle membrane. The muscle membrane was recovered as a white, fluffy suspension, which under the phase-contrast microscope consisted of semitransparent or granular membranes of various shapes (Fig. 1 *a*). The appearance was not altered by storage in suspension at 4°C for 6 months. Numerous motor end plates were visualized in the membrane by staining for cholinesterase activity (Fig. 1 *b*). The fine structures of these end plates were similar to structures of end plates demonstrated in muscle sections, including the presence of isolated sole plates. This appearance is in contrast to the continuous network of the sole plates in rat motor end plates (1). The end plates appeared swollen in the membrane stored at 4°C for 3 months, and became diffuse cholinesterase-active spots in the membrane stored for 6 months, probably due either to inactivation or diffusion of the enzyme. Motor end plate was the only structure demonstrated by staining the membrane for cholinesterase.

Cholinesterase activity of human muscle membrane. The cholinesterase activity of human skeletal muscle membrane per unit nitrogen was 3.86 times greater ($P < 0.001$) than the cholinesterase activity of the total muscle homogenate (Table I). This yield was disappointingly low, compared with the 27.1 times greater concentration of activity achieved by isolation of rat muscle membrane. The low yield was due mainly to the high cholinesterase activity of non–end plate components and partly to low cholinesterase activity of motor end plates. Whereas the cholinesterase activity per nitrogen was 4.67 times greater in human muscle homogenate compared with rat muscle homogenate, the cholinesterase activity of human muscle membrane was only 64.5% of the activity of rat muscle membrane.

TABLE I

Cholinesterase Activity of Intercostal Muscle

	Membrane* (No.)	End plate‡ (No.)	Homogenate* (No.)	Membrane/ homogenate
Human	8.61 ±0.442 (125)	0.362 ±0.130 (92)	2.24 ±0.072 (53)	3.86 ±0.253
Rat§	13.00 ±0.764 (53)	0.805 ±0.047 (53)	0.48 ±0.002 (55)	21.1 ±1.68
Human/rat	0.645 ±0.049	0.455 ±0.027	4.67 ±0.195	

Mean ±SE.

* μmoles acetylcholine hydrolyzed/mg nitrogen per 30 min.

‡ Nanomoles acetylcholine hydrolyzed/end plate per 30 min.

§ From Namba and Grob (1).

These results were substantiated by histochemical studies. Slight overstaining for cholinesterase activity of human skeletal muscle resulted in precipitation of reaction products in the entire muscle (Figs. 2 *a* and *b*), whereas simultaneous staining of rat skeletal muscle produced reaction products only in the end plate area (Figs. 2 *c* and *d*).

From the number of motor end plates in human skeletal muscle membrane, a single motor end plate was calculated to have hydrolyzed 0.362 nmoles of acetylcholine

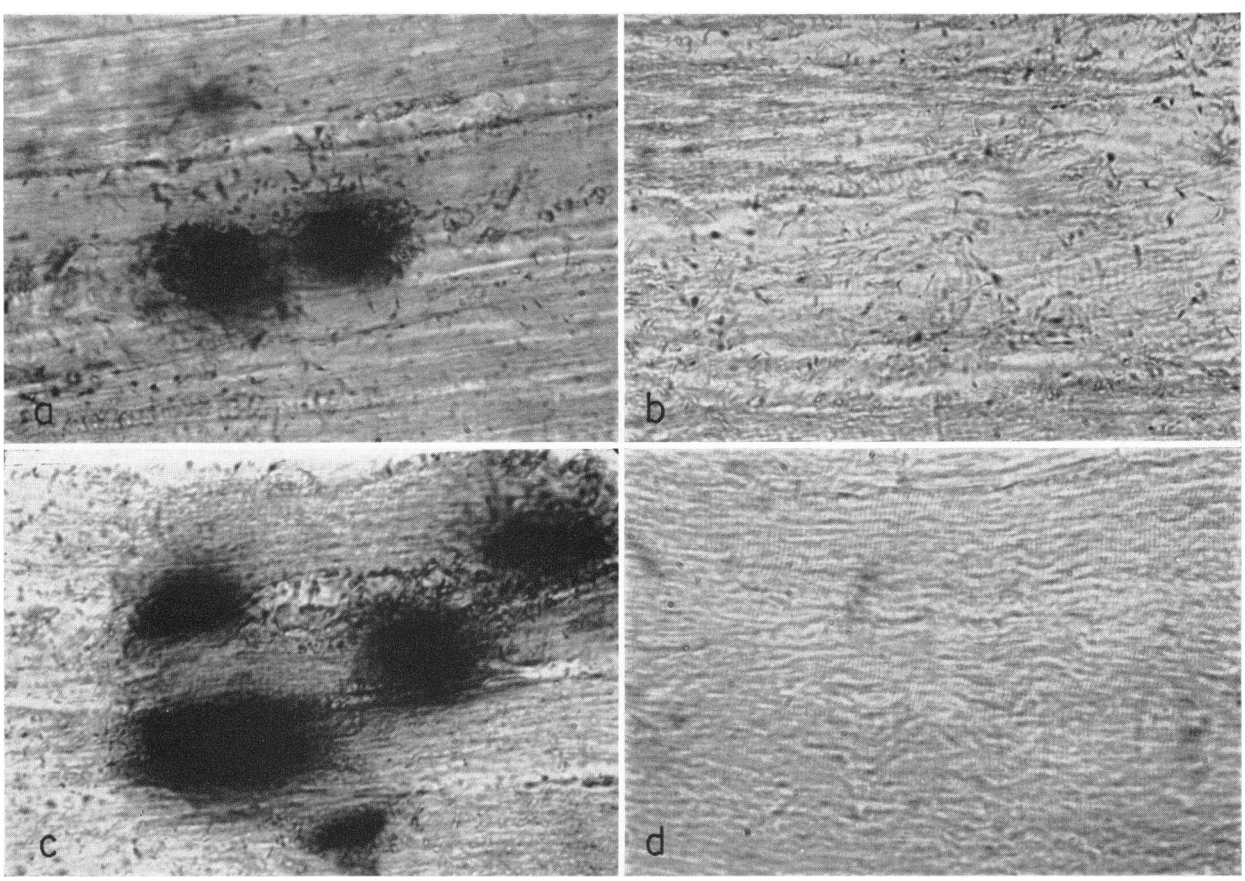

FIGURE 2 Sections of intercostal muscle overstained for cholinesterase by incubation at 37°C for 20 min. In human muscle, overstaining of motor end plates (*a*) is accompanied by precipitation of the reaction products in the non-end plate area (*b*). In rat muscle, motor end plates were greatly overstained (*c*) but there was no reaction product in the non-end plate area (*d*). Frozen sections, cholinesterase staining. × 465.

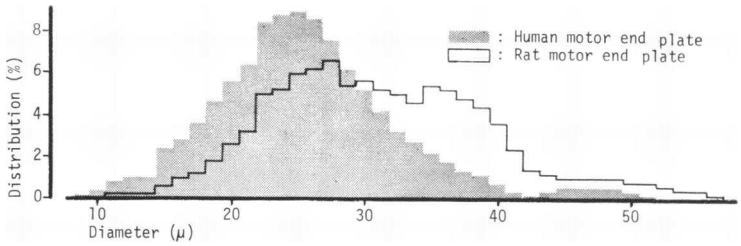

FIGURE 3 Diameter of motor end plates in the isolated membrane of human and rat intercostal muscles.

in 30 min, or 1.21×10^8 molecules of acetylcholine per msec.

The mean cholinesterase activity of the human motor end plate was 45.5% of the mean activity of the rat motor end plate ($P < 0.001$) (Table I). However, individual estimations indicated considerable overlap in activities since the 95% tolerance limits of acetylcholine hydrolyzed in 30 min were 0.362 ±0.252 nmoles in the human motor end plate and 0.805 ±0.689 nmoles in the rat motor end plate. The diameter in the largest dimension was 25.94 ±0.317 μ in the human motor end plate compared with 31.25 ±0.363 μ in the rat motor end plate (mean ±SE in 500 motor end plates, respectively, Fig. 3). Assuming that the shape of motor end plates is similar, the area of human motor end plate was 68.3 ±2.86% (mean ±SE in 500 end plates) of the area of rat motor end plate. Actually the synaptic area of human motor end plate consists of isolated sole plates whereas rat motor end plate is made up of a continuous sole plate. Therefore, cholinesterase activity per unit synaptic area appears to be in the same range in both human and rat end plates.

Properties of cholinesterase activity of human skeletal muscle membrane. There was no change in cholinesterase activity during storage of the suspension of skeletal muscle membrane at 4°C for 4 wk. The activity in 24 samples was 6.41 ±0.734 initially and 6.65 ±0.847 at 4 wk (mean ± SE, μmoles acetylcholine hydrolyzed in 30 min per mg of nitrogen).

Diisopropyl fluorophosphate (DFP) inhibited cholinesterase activity of the muscle membrane only slightly, while 1,5-bis-(4-trimethylammoniumphenyl)-*n*-pentane-3-one (62C47) and 1,5-*d*-(4-alyl-dimethylammoniumphenyl)-pentane-3-one (284C51) greatly inhibited the enzyme activity (Table II). DFP is an inhibitor of pseudocholinesterase, inhibiting less than 5% of acetylcholinesterase at 10^{-7} mole/liter, and 100% of pseudocholinesterase at 10^{-8} mole/liter or higher concentrations (5); 62C47, an acetylcholinesterase inhibitor, inhibits 50% of acetylcholinesterase at $10^{-7.2}$ mole/liter and 50% of pseudocholinesterase at $10^{-2.45}$ mole/liter (6); and 284C51, also an inhibitor of acetylcholinesterase, inhibits more than 95% of acetylcholinesterase and less than 5% of pseudocholinesterase at concentrations between 10^{-6} and 10^{-5} mole/liter (7). The muscle membrane hydrolyzed only 0.42 ±0.053 μmoles (mean ±SE in 59 measurements) of butyrylcholine per mg of nitrogen in 30 min. Butyrylcholine is a substrate for pseudocholinesterase. Cholinesterase activity of human skeletal muscle membrane therefore consists of more than 90% acetylcholinesterase and less than 10% pseudocholinesterase.

The amount of acetylcholine hydrolyzed by skeletal muscle membrane increased with increasing concentration of acetylcholine (substrate) between 0.5 mmole/liter and 8.0 mmoles/liter (Fig. 4). When the results were plotted by the method of Woolf (8), a Michaelis-Menten constant of 4.15 mmoles/liter and a maximum velocity of 15.8 μmoles of acetylcholine hydrolyzed per mg of nitrogen in 30 min were obtained. Using the number of motor end plates counted in individual membrane preparations used in the study in Fig. 4, we calculated the maximum velocity of cholinesterase per motor end plate to be 0.754 nmoles acetylcholine hydrolyzed in 30 min, or 2.52×10^8 molecules of acetylcholine hydrolyzed in 1 msec.

The optimal pH of the incubation medium for cholinesterase activity of human skeletal muscle membrane was 7.8 (Fig. 5).

TABLE II

Effect of Inhibitors on Cholinesterase Activity of Human Intercostal Muscle Membrane

Inhibitor		Cholinesterase activity*	Per cent inhibition
None		13.71 ±1.924	0
DFP	10^{-7} M	13.12 ±1.826	4.3
	10^{-6} M	11.00 ±1.098	19.7
62C47	10^{-6} M	2.80 ±0.337	79.5
	10^{-5} M	1.14 ±0.329	91.6
284C51	10^{-6} M	1.45 ±0.357	89.4
	10^{-5} M	1.34 ±0.836	90.1

* Mean ±SE in 10 samples. μmoles of acetylcholine hydrolyzed/mg nitrogen per 30 min.

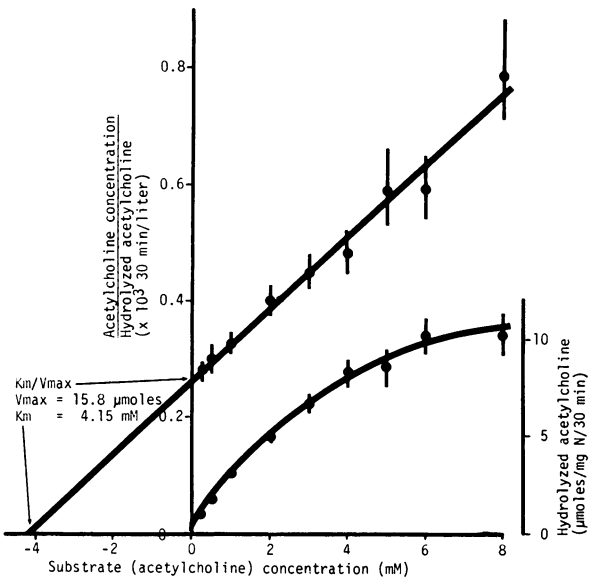

FIGURE 4 Effect of substrate concentration on cholinesterase activity of the isolated human skeletal muscle membrane. The circles indicate the mean, and the vertical lines the standard error of the mean, in 27 estimations.

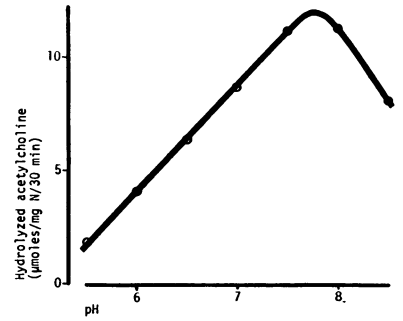

FIGURE 5 Effect of pH on cholinesterase activity of the isolated human muscle membrane.

DISCUSSION

Several studies have been reported on measurement of cholinesterase activity of motor end plates of experimental animals (Table III). In all but our previous study on the motor end plate of rat intercostal muscle (1), measurements were made on muscle segments which contained cholinesterase of both motor end plates and non-end plate components. Correction for cholinesterase of non-end plate components was made only by Buckley and Nowell (10) and Buckley and Heaton (11), but their method was later found to have yielded low activity (15).

In the indirect measurement, the difference of cholinesterase activity between the muscle segments containing motor end plates and the segments without motor end plates is considered to be the cholinesterase activity of motor end plates. This method cannot be employed in human skeletal muscle, because the high cholinesterase activity of the segment without motor end plates renders the difference small and within the experimental error. Only about 2% of the cholinesterase activity of human intercostal muscle was estimated to be in the motor end plates, compared with 20% in rat tibialis anterior muscle (1).

Direct measurements of cholinesterase activity of motor end plates in human skeletal muscle have been found to be inaccurate, for the same reason, when carried out in muscle segments containing cholinesterase-

TABLE III

Cholinesterase Activity of Motor End Plate

Cholinesterase activity*	Muscle	Substrate	Method	Reference
1.21×10^8	Human intercostal	Acetylcholine	Direct	Present study
2.69×10^8	Rat intercostal	Acetylcholine	Direct	(1)
1.45×10^7	Rat tibialis anterior	Acetylcholine	Indirect	(1)
3.52×10^7	Rat rectus abdominus	Acetylcholine	Direct	(9)
1.79×10^7	Rat diaphragm	Acetylthiocholine	Direct	(10)
	Rat extraocular muscle	Acetylthiocholine	Direct	(11)
$0.35–6.46 \times 10^7$	Focal end plate			
$0.09–0.59 \times 10^7$	Fine end plate			
	Guinea pig extraocular muscle	Acetylthiocholine	Direct	(11)
$0.54–7.31 \times 10^7$	Focal end plate			
$0.11–2.84 \times 10^7$	Fine end plate			
3.70×10^8	Rabbit gastrocnemius	Acetylcholine	Direct	(12)
2.90×10^7	Mouse diaphragm	Acetylcholine	Direct	(13)
5.99×10^7	Mouse gastrocnemius	Acetylcholine	Direct	(13)
1.6×10^9	Frog sartorius	Acetylcholine	Indirect	(14)

* Molecules of substrate hydrolyzed per millisecond per end plate.

active non–end plate components. The isolated muscle membrane is much more suitable for studies on the activity and properties of cholinesterase activity in motor end plates.

The function of non–end plate cholinesterase, which is localized mainly in the sarcoplasmic reticulum (16), is not known, but, from the comparative studies in muscle from man and from the rat, probably plays a very minor role, if any, in neuromuscular transmission. Motor end plates of humans appear to be more sensitive to acetylcholine than end plates of rats. In order to reduce the amplitude of muscle action potentials evoked by supramaximal nerve stimulation by 50%, only about 2.5 mg of acetylcholine was required when injected into the brachial artery of humans which supplies tissue weighing about 1000 g (17, 18), whereas approximately 1.0 mg of acetylcholine was required when injected into the common iliac artery of rats which supplies less than 10 g of tissue (19). Since the cholinesterase activity per unit synaptic area of end plate was approximately the same in man and rat, other factors may influence acetylcholine sensitivity.

Using cholinesterase activity of human motor end plate of 1.21×10^8 (acetylcholine molecules hydrolyzed per millisecond per motor end plate), we are able to make some quantitative estimations of the metabolism of acetylcholine at the end plate, although this value and other values obtained from the literature are obtained in experiments in vitro and may deviate from the actual values in vivo.

In human intercostal muscle, 4.04×10^{10} molecules of acetylcholine have been reported to be stored in each motor end plate (20), and a mean of 218,000 quanta of acetylcholine has been reported to be releasable (21). If all the acetylcholine molecules stored in the motor end plate are releasable, the entire acetylcholine store would be hydrolyzed in 335 msec, and 650 quanta of acetylcholine can be hydrolyzed in 1 msec. Actually the releasable acetylcholine is only a fraction of the total acetylcholine store, and therefore acetylcholine greater than 650 quanta can be hydrolyzed in 1 msec.

The number of cholinesterase active sites in a motor end plate has been measured only in mice (Table IV). If one utilizes these numbers for the motor end plate of human intercostal muscle, the number of acetylcholine molecules hydrolyzed by one active site per minute (turnover number) ranges from 2.07×10^5 to 18.3×10^5 molecules, and the time required for one enzyme site to hydrolyze one acetylcholine molecule (turnover time) is 33–289 μsec.

The number of acetylcholine molecules released from the nerve ending of a motor end plate per impulse has not been reported in humans. In experimental animals, assessment of this number has been made by measuring acetylcholine recovered from muscle after stimulation of the motor nerve, and by determining the number of acetylcholine molecules required to stimulate a motor end plate when applied by micropipette (Table IV). These studies indicate that about 10^7 molecules of acetylcholine per impulse are released from a motor end plate in experimental animals. If one utilizes this number for human intercostal muscle, the time required to hydrolyze acetylcholine which is released by one impulse is 83 μsec.

The number of possible acetylcholine receptor sites in an end plate was reported to be 4×10^6 in mouse diaphragm as measured by binding of curare compounds (22).

TABLE IV

Number of Cholinesterase-Active Sites, Acetylcholine Released per Impulse, and Acetylcholine Required for Stimulation, Per End Plate

	Muscle	Reference
Cholinesterase-active sites*		
$2.0–2.4 \times 10^7$	Mouse diaphragm	(22)
$0.9–1.2 \times 10^7$	Mouse diaphragm	(23)
$2.5–3.5 \times 10^7$	Mouse sternomastoid	(23)
	Mouse extraocular muscle	(23)
2.0×10^5	Focal end plate	
0.4×10^7	Fine end plate	
Acetylcholine recovered‡		
4.64×10^5	Mouse diaphragm	(24)
1.2×10^5	Cat tibialis anterior	(25)
1.44×10^6	Guinea pig diaphragm	(26)
6.64×10^6	Rat diaphragm	(27)
Acetylcholine required‡		
3.3×10^8	Frog	(28)
6.0×10^8	Frog sartorius	(29)
9.05×10^6	Rat diaphragm	(30)

* Sites per end plate, measured by the number of bound DPF molecules.

‡ Molecules of acetylcholine per impulse per end plate.

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