J. Physiol. (1964), **171**, *pp.* 504–513
With 1 *plate*
Printed in Great Britain

ACETYLCHOLINE AND CHOLINE ACETYLTRANSFERASE IN THE DIAPHRAGM OF THE RAT

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(*Received* 2 *January* 1964)

Although we know that skeletal muscle and many peripheral nerves contain acetylcholine (ACh) and exhibit choline acetyltransferase (ChAc) activity (acetyl-CoA: choline-*O*-acetyltransferase, EC 2.3.1.6), there is little evidence about how these components are distributed within a muscle. This is of some interest since it has been claimed that the spread of activity along the muscle fibres depends upon a cholinergic mechanism (Nachmansohn, 1959, 1963). If that is the case one might expect to find an even distribution of ACh and ChAc throughout the tissue without any great accumulation in the regions where motor nerve fibres and their endings are situated; particularly if neuromuscular transmission is not mediated by ACh, as is believed by the same author.

To investigate this problem, we have examined the gross distribution of ACh and ChAc activity in the rat diaphragm, comparing the zone of innervation with the remainder of the muscle. In addition, we have estimated the ACh content and ChAc activity in the phrenic nerve.

A preliminary account of these observations has been given at the 2nd International Pharmacological Meeting (Krnjević, 1964).

METHODS

The rats were of a Wistar strain, weighing between 100 and 300 g. In most experiments they were anaesthetized with ether and the tissues removed immediately after death in the following order: left phrenic nerve, left hemidiaphragm, right phrenic nerve and right hemidiaphragm. The hemidiaphragms were rinsed in cold Ringer-Locke's solution aerated with 5 % CO_2 in O_2, and then the zones of innervation were cut from the rest of the muscle with scissors.

Zone of nerve endings. This consists of a central strip of muscle extending about 1–2 mm on either side of the main intramuscular branch of the phrenic nerve (Pl. 1). Our belief that this region includes most of the motor nerve endings is based on the following evidence:

1. It contains practically all the fine terminal branches of the phrenic nerve seen with a microscope in a fresh preparation.

2. In microscopic sections of the diaphragm fixed and stained with osmic acid, terminal fibres were only found within 1–2 mm of the principal nerve branch.

3. Histochemical staining of end-plates in the diaphragm with acetylthiocholine (Cöers, 1953) shows that they are distributed almost exclusively in this strip (Pl. 1).

4. This is the only part of the diaphragm where typical junctional potentials (such as miniature end-plate potentials) can be recorded (Krnjević & Miledi, 1958).

In the right hemidiaphragm the intramuscular divisions of the nerve are spread over a larger area than on the left side; it was therefore necessary to cut out a wider strip. In most experiments, the average nerve ending sample came to about 40 % of the total weight of muscle tissue removed (see Tables 1 and 2).

Extraction of ACh from tissues. The procedure for the extraction of ACh from muscle was very similar to that followed in an earlier investigation of the rabbit plantaris muscle (Hebb, 1962). Pieces of tissue were dropped into a weighed beaker containing 2–4 ml. of 10 % trichloracetic acid (TCA). Usually each piece of muscle was minced in the TCA with fine scissors as soon as it had been added to the acid; but in some experiments it was not minced until after the beaker had been re-weighed. The recovery from muscle tissue was about the same in either case. Nerve tissue was only minced after the whole sample had been collected and usually after re-weighing the beaker since in this case the amounts of tissue (usually less than 100 mg) and of TCA (1 ml.) were so small that significant losses occurred if the mincing was not all done at one time.

The TCA was kept at 1–5° C during collection of the material and for 2–2½ hr afterwards. The samples of muscle were then centrifuged at 3300 rev/min for 6 min in 10 ml. conical tubes and the nerve samples filtered through sintered glass (No. 3). The precipitated material was washed again and resuspended in one-half of the volume of 10 % TCA used originally and centrifuged or filtered as before. Water (1–3 ml.) used to wash the surface of the precipitate and to rinse the beaker originally containing the sample was also added to the pooled samples of supernatant or filtrate.

The acid extracts were then washed 4 times with ether in separating funnels. The volume of ether used for each muscle sample was equivalent to about 80 times, and for nerve samples 40 times, the original volume of TCA. After each extraction, and before removing the ether, the funnel was washed with 1–3 ml. of H_2O. This was run down the side of the funnel, which was rotated fairly rapidly, and was then collected from the tap and added to the extract. After the treatment with ether the volume of the extracts was between 2·5 and 6 ml. and the pH, tested on Narrow Range Indicator paper (British Drug Houses), was between 5·0 and 5·4. The extract was heated to 50–60° C and a stream of air or N_2 gas was passed over the surface to blow off residual ether.

After 2 drops of Universal Indicator (British Drug Houses) had been added to the samples they were made up to 9·0 ml. and divided into two equal portions: one, which served as a control, was made alkaline by adding 0·1 ml. N-NaOH, boiled briefly, neutralized with N-HCl and made up to 5 ml. by adding frog Ringer's solution; while the other, the test sample, was made up to 5 ml. with the addition of frog Ringer's solution only. The samples were then assayed on the eserinized frog rectus abdominis or on the eserinized dorsal muscle of the leech. When sufficient material was available, cross-assays were done on both of these or on one of them and on the guinea-pig ileum as well. On the average the results from the frog rectus were 10 % higher than results from the other test muscles; but the assays by the different methods gave reasonably consistent results. Leech muscle could not be used with some extracts, however, because the response to ACh was strongly depressed. On the other hand the frog rectus (from *Rana temporaria*) was sensitized to ACh by muscle extracts, as observed earlier (Hebb, 1962), and this made it easier to assay their activity.

A control run was included in each experiment; in this a known amount of ACh was added to about 1 g of rat muscle which had been minced, allowed to stand at room temperature for 1 hr (to destroy its own ACh) and then immersed in TCA (to destroy its cholinesterases). This sample was treated in the same way as the other muscle samples. The amounts of ACh found to be present in the control samples did not differ from the amounts added, within an error of ±15 %.

Estimation of choline acetyltransferase. The tissue samples were weighed and then homogenized in 0·3 M sucrose or cysteine-sucrose solution, giving concentrations of 25 mg (nerve tissue) or 100 mg (muscle)/ml. Cysteine-sucrose was freshly prepared by dissolving

L-cysteine HCl, neutralized with N-KOH, in a concentration of 6 mg/ml. in 0·3 M sucrose. The enzyme activity of cysteine-sucrose homogenates was somewhat higher than that of homogenates to which cysteine was not added. Treatment with ether (cf. Hebb & Smallman, 1956) did not increase the activity of the homogenates. Choline acetyltransferase was measured by incubating 0·1 or 0·2 ml. of homogenate for 1 hr at 39° C with 0·4 ml. of reaction mixture which itself had previously been incubated at 39° C for 10 min. One millilitre of this contained about 20-40 units of crude coenzyme A and 0·32 mg of crude phosphate acetyltransferase (EC 2.3.1.8) in 0·21 M-KCl, 0·015 M choline, 0·012 M acetyl-phosphate, 0·006 M-$MgCl_2$, 0·03 M cysteine, 0·016 M sodium phosphate buffer (pH 6·9) and 0·1 mM eserine sulphate. This incubation mixture differs in two respects from that described by Bull, Hebb & Ratković (1963); these authors used 0·17 M choline and 0·16 M sodium phosphate. Neither of these alterations has any material effect on the yield of ACh but lowering the choline concentration improves the assay. The amount of ACh formed at the end of 1 hr of incubation was estimated by assay either on frog rectus abdominis or on leech muscle. The results were expressed as the chloride of ACh.

Denervation experiments. In nineteen rats under ether anaesthesia, the left phrenic nerve was cut approximately 5 mm from the diaphragm, through an incision between the 7th and 8th ribs. The animals were killed at various times after the operation (see Table 5).

RESULTS

Distribution of ChAc in diaphragms. The muscles were divided into two fractions, one containing the intramuscular portion of the phrenic nerve and most of the nerve endings (NM fraction) and the other the remainder of the muscle with only very few nerve endings (M fraction); by far the greater part of the ChAc activity of the muscle was found to be concentrated in the NM fraction. It can be seen from Table 1 that, in different experiments, between 81 and 95% (mean 87·8%) of the total ChAc activity was present in the NM fraction, even though this made up only about 40% of the total weight of muscle.

Distribution of ACh in diaphragms. Table 2 shows that the NM fraction also contained most of the ACh; the value in different experiments ranged from 82 to 96% of the total (mean 89·7%).

To ensure that as much of the innervated zone as possible was included in the NM fraction a relatively large strip of muscle was taken. It is probable that most of the ACh was present in a more limited area: when a strip much narrower than usual was cut out, as in Exp. 38, so that the NM component made up only 26% of the total weight, the ACh content was only slightly less than usual.

The rat diaphragm as a whole had a comparatively high concentration of ACh, ranging from 126 to 291 (mean 181·7) ng/g of muscle. This is several times more than has been found in some other skeletal muscle (Chang & Gaddum, 1933; Bhatnagar & MacIntosh, 1960; Hebb, 1962).

ChAc activity in phrenic nerve. The results are shown in Table 3. The activity is given for a unit weight of tissue, and also, in some cases, for a unit length. The last is a useful index for estimating the probable amount

TABLE 1. ChAc activity in the muscle fraction containing the phrenic nerve and most of the motor nerve endings (NM), and in the remainder of the muscle (M). In each experiment samples from two or more muscles were pooled for the estimation

Expt.	No. of hemi-diaphragms	Mean weight of hemidiaphragm (mg)	Mean weight of NM as proportion of total weight of muscles (%)	ChAc activity (μgACh/hr/hemidiaphragm) NM	ChAc activity (μgACh/hr/hemidiaphragm) M	ChAc activity in NM as proportion of total activity in muscles (%)
38	6	237	38·2	29·3	6·7	81·5
41	4	197	48·6	31·0	1·8	94·5
43*a*	2	300	40·0	28·8	4·7	86·0
43*b*	2	299	36·8	16·5	1·3	92·7
	Over-all mean values	243	41·5	27·9	4·3	87·8

TABLE 2. ACh in NM and M fractions. In each experiment samples from several muscles were pooled together

Expt.	No. of hemi-diaphragms	Mean weight of hemidiaphragm (mg)	Mean weight of NM as proportion of total weight of muscles (%)	ACh content (ng/hemidiaphragm) NM	ACh content (ng/hemidiaphragm) M	ACh content of NM as proportion of total in muscle (%)
38	6	239	26·1	31·7	6·7	82·5
39	8	265	43·5	31·5	1·8	94·6
40	8	172	47·4	42·6–52·5	2·5	94·5–95·6
45	6	282	40·9	47·0–58·0	8·3	85·5–87·9
46	6	294	38·0	37·5	6·7	85·0
	Over-all mean values	247	39·9	40·1	4·8	89·7

of ACh associated with the intramuscular branches of the phrenic nerve. It should also help to eliminate apparent variations in concentration caused by different amounts of adjacent tissue removed with the phrenic nerve or by drying. The ChAc activity of these nerves expressed per unit weight is only about one third to one quarter of that previously found in mammalian ventral spinal roots (Hebb & Silver, 1956).

TABLE 3. ChAc activity in phrenic nerve. Several nerves were pooled in each experiment

Expt.	No. of nerves	Total weight (mg)	Total length (cm)	ChAc activity (μgACh/hr) per unit weight (g^{-1})	ChAc activity (μgACh/hr) per unit length (cm^{-1})
38	5	50·6	15·8	1560	5·0
41	4	40·4	14·4	2260	6·3
43*a*	2	27·0	—	900	—
43*b*	2	17·0	—	1340	—
	Mean values			1610	5·6

TABLE 4. ACh content of phrenic nerve

Expt.	No. of nerves	Total weight (mg)	Total length (cm)	ACh content (μg/g)	ACh content (ng/cm)
48	12	158·4	54·3	2·2	6·4
39	8	79·2	29·5	2·4	6·45
40	8	78·8	27·6	2·3	6·5
31	12	121·0	—	2·2	—
	Mean values			2·25	6·44

TABLE 5. Weight, ACh content and ChAc activity of denervated hemidiaphragms expressed as percentage of control values (from right muscles in same rats)

Expt.	No. of muscles	Period after denervation (days)	Weight (%)	NM fraction ACh content (%)	M fraction ACh content (%)
45	6	20	94	17·2–21·4	20
44	3	43	57	9·1	—
46	6	44	75	4·4	0 (< 25)
				ChAc activity (%)	
43*a*	2	41	90	6·2	3·2
43*b*	2	41	93	2·7	3·7

ACh content of phrenic nerve. This was also estimated for a unit weight and in some cases unit length of nerve, as indicated in Table 4. These results show a relatively high degree of consistency between different experiments. They agree very well with a recent estimate for the rabbit's sciatic nerve (Carlini & Green, 1963).

Changes in ACh content and ChAc activity of the diaphragm after denervation. Both ACh and ChAc were much reduced in denervated muscle. The change was sufficiently great to make the detection of ACh in some samples

difficult or even impossible, especially in M fractions which normally contain only a small amount. From the data given in Table 5, it is evident that after some 6 weeks the ACh content and the ChAc activity had diminished to a comparable extent. The changes in ChAc were similar in the NM and M fractions.

DISCUSSION

These results give no support to claims that a cholinergic mechanism is responsible for the conduction of impulses along muscle fibres (Nachmansohn, 1959, 1963) and that the muscle fibres themselves release ACh (Hayes & Riker, 1963). The uneven distribution of ChAc activity within muscle and its almost total disappearance after denervation are particularly significant, since the ability to synthesize ACh must be essential to any such mechanism.

There is an especially glaring contrast between nerve, with an acetylating capacity of about 1600 μg ACh/g/hr, and the M fraction of denervated muscle which can be considered almost 'pure' muscle and which can only synthesize about 1 μg ACh/g/hr. Although some activity still remains after denervation, this may not have a strictly muscular origin. The fact that ACh and ChAc are reduced in a similar way in the NM and M regions suggests that the normal content in the M fraction is probably derived from outlying branches of the phrenic nerve. It has been suggested that Schwann cells may preserve some of the ChAc activity of motor nerves after their degeneration (Birks, Katz & Miledi, 1960). Schwann cells, or some other remnant of the phrenic nerve, may account for the activity in the denervated diaphragm. This residual activity (3–6 % of normal), which is about 10-fold greater than in degenerated rabbit nerves after a similar interval (Hebb, 1962), is more than sufficient to supply ACh for the spontaneous release seen after denervation (Mitchell & Silver, 1963).

As there is such a high concentration of ACh and ChAc in the phrenic nerve, it is necessary to consider what proportion of the total content in the diaphragm is present in the intramuscular part of the phrenic nerve and its myelinated terminal branches, and is therefore not immediately available for release or synthesis at the nerve endings.

Nerve fibres were counted in phrenic-diaphragm preparations treated with osmic acid. The phrenic nerves contained 400 myelinated fibres, mostly having a diameter of 5–6 μ. The branches supplying the anterior half of the hemidiaphragm had some 180 fibres and this number remained roughly constant to within a mm of the end of the intramuscular course of the nerve. The posterior half (excluding the crus) was supplied by about 150 fibres but there was a gradual reduction in numbers to some 50 at 2 cm from the point of nerve entry; the mean count was estimated to be 120. From these figures the main nerve branches in the anterior and posterior portions of the hemidiaphragm might be expected to contain approximately 45 and 30 % of the ACh and ChAc in an equal length of phrenic nerve. However, it is necessary to take into account a progressive increase in the proportion of

small fibres (3–4 μ), no doubt as a result of branching. A further complication is that in the middle portion of both the anterior and posterior halves of the muscle, the diameter of the nerve fibres is mostly between 10 and 12 μ or double that seen in the phrenic nerve. Although these two factors tend to cancel each other out, the mean fibre *volume* is probably only about three-quarters of that in the phrenic nerve.

If we take experiment 39 (Table 2) as an example, the intramuscular nerves had a mean length of 4·4 cm and the NM fractions contained an average of 31·5 ng of ACh, while the corresponding phrenic nerves held 6·45 ng ACh/cm (Table 4). If the last figure is corrected to allow for the reduction in fibre counts and fibre volume in the muscle (see above), the ACh present in the main intramuscular branches would come to 8 ng, or about a quarter of the total. A further allowance must be made for ACh in the myelinated part of the motor terminals. With a diameter of 3 μ and a length of about 1 mm (Edds, 1950), 10,000 terminal branches in the hemidiaphragm (Krnjević & Mitchell, 1961) would contain 4 ng of ACh. So in this experiment, of the total ACh content of 31·5 ng, some 12 ng (or 38 %) were probably in the intramuscular nerve branches.

From these and similar calculations, it appears that, *if the nerve fibres within the diaphragm have the same composition as in the phrenic nerve*, they would contain no more than between one quarter and one half of the ACh and ChAc found in the muscle. This suggests that there is a substantial accumulation of ACh and ChAc in the nerve endings. A similar conclusion was reached by Bhatnagar & MacIntosh (1960) from their studies of the action of hemicholinium on the ACh content of a cat muscle, but Thies (1962), using the same approach, found considerable variation in the apparent accumulation in different muscles of the rat.

Since the volume of a motor nerve ending in the rat diaphragm is probably 200–400 μ^3 (R. Thies, personal communication) the total volume of endings in the hemidiaphragm should be $2\text{–}4 \times 10^{-6}$ cm^3. This can be compared with a volume of 180×10^{-6} cm^3 for a 1 cm length of phrenic nerve (estimated from the cross-section), of which about 40 % (72×10^{-6} cm^3) would be in the fibres (Krnjević, 1955). Hence the concentrations of ACh and ChAc in the nerve endings must be approximately 100 times greater than in the phrenic nerve to account for the observed excess of ACh and ChAc activity. The excess of ACh (20–40 ng or 100–200 pmoles for the hemidiaphragm) would be sufficient to allow the transmission of 1000–2000 impulses at the neuromuscular junction, since the transmission of one impulse in the diaphragm is associated with a mean release of 0·12 pmole of ACh (Krnjević & Mitchell, 1961).

However, another possibility must be considered and this is that the nerve fibres within the diaphragm do *not* have the same composition as in the phrenic nerve. In cut nerves, the ChAc activity increases by about 100 % in a region 2–3 cm long just above the point of section (Hebb & Waites, 1956; Hebb & Silver, 1961; Hebb, 1962). It has been suggested that the severed axons undergo changes which make them comparable to presynaptic terminals (Hebb & Silver, 1961). If the concentration is

raised in a similar way within 2–3 cm of the endings of normal nerves, this might be sufficient to account for the apparent excess in the diaphragm, without postulating any substantial store of ACh in the endings themselves. The latter would contain only about 360–720 pg (or 2–4 pmole) of ACh, or enough for some 20–40 impulses; but if ChAc activity is as efficient *in vivo* as it is *in vitro*, the activity in the endings would be sufficient to synthesize ACh at the rate of about 150 pg/sec, which is 3 times the maximal rate of release of ACh observed from the phrenic nerve during prolonged stimulation (Barnes & Duff, 1954; Straughan, 1960; Cheymol, Bourillet & Ogura, 1962).

The second scheme seems no less likely than does a hypothetical store of ACh in the endings. It is consistent with the well-known fact that the phrenic nerve cannot maintain a maximal release of ACh if it is excited more often than about once every 10 sec (Lundberg & Quilisch, 1953; Krnjević & Mitchell, 1961; Brooks & Thies, 1962); the quantity actually released falls off very sharply with a rise in frequency, so that the total output in a given time reaches a maximum at about 25/sec (Straughan, 1960). It is difficult to explain this satisfactorily if the nerve endings contain a store of ACh sufficient for several thousands of impulses.

Although it is often thought that large amounts of ACh are bound in synaptic vesicles inside the nerve endings, from a recent estimate of the ACh content of a brain fraction consisting almost entirely of such vesicles, it appears that only a very small quantity of ACh is likely to be associated with individual vesicles (V. P. Whittaker, personal communication).

These observations, which are in agreement with our interpretation, raise doubts about the hypothesis that the release of ACh from individual synaptic vesicles is directly responsible for the quantal activity observed at the muscle end-plate (del Castillo & Katz, 1956). For an explanation of this phenomenon, it may be profitable to re-examine the original suggestion that it is generated by subunits of the presynaptic expansion of the nerve ending (Fatt & Katz, 1952).

SUMMARY

1. Hemidiaphragms from rats were divided into a neural fraction containing the intramuscular branches of the phrenic nerve and most of the motor endings, and another fraction consisting of the largely nerve-free remainder.

2. Most of the ACh content (mean 89·7 %) and ChAc activity (mean 87·8 %) of the muscle were present in the neural fraction, even though this accounted for only 40 % of the total weight.

3. After chronic denervation the ChAc content everywhere diminished

to about 4% of the normal values, while ACh became practically undetectable in the nerve-free portion of muscle.

4. On average the phrenic nerve trunk contained 2·25 μg/g of ACh and had a ChAc activity of 1610 μg/g/hr of ACh.

5. If it is assumed that the concentration of ACh and ChAc remains identical at all levels in the phrenic nerve fibres, the intramuscular branches of the phrenic nerve probably would not account for more than one quarter to one half of the total ACh and ChAc content in the muscle.

6. The possibility that the excess of ACh and ChAc in the diaphragm is due to a moderate increase in concentration throughout the intramuscular nerve branches seems more compatible with other evidence than the hypothesis of a substantial store of ACh and ChAc in the prejunctional nerve endings.

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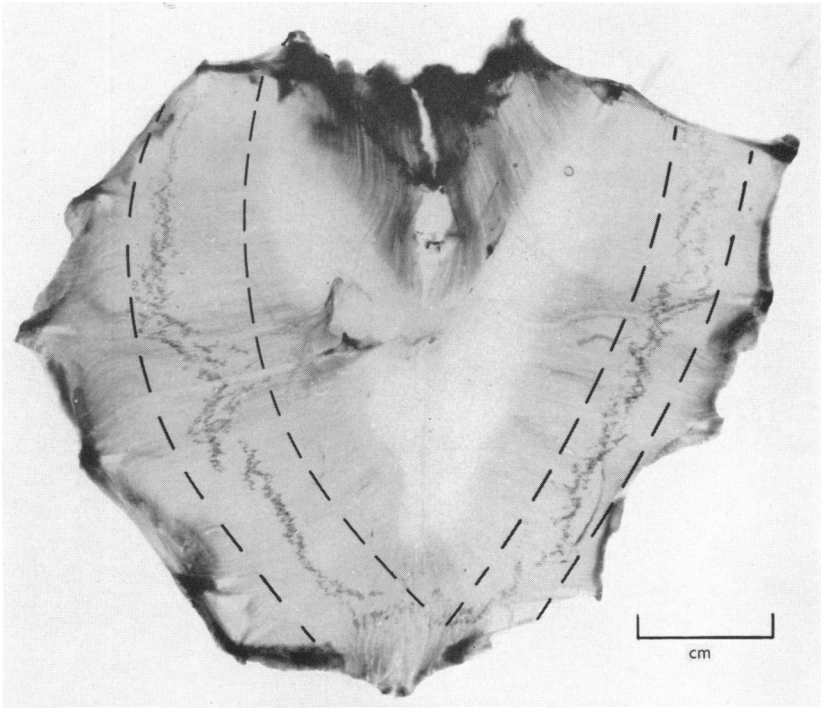
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EXPLANATION OF PLATE

The distribution of motor nerve endings in the rat diaphragm, as shown by staining for cholinesterases (see Silver, 1963). The dotted lines indicate approximately where the nerve-muscle (NM) fractions were separated from the rest of the diaphragm.