ACETYLCHOLINE COMPARTMENTS IN MOUSE DIAPHRAGM

Comparison of the Effects of Black Widow Spider Venom, Electrical Stimulation, and High Concentrations of Potassium

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ABSTRACT

We have studied the effects of 25 mM potassium, electrical stimulation of the phrenic nerve, and crude black widow spider venom on the ultrastructure, electrophysiology, and acetylcholine (ACh) contents of mouse diaphragms.

About 65% of the ACh in diaphragms is contained in a depletable store in the nerve terminals. The rest of the ACh is contained in a nondepletable store that may correspond to the store that remains in denervated muscles and includes, in addition, ACh in the intramuscular branches of the phrenic nerve.

About 4% of the ACh released from the depletable store at rest is secreted as quanta and may come from the vesicles, while 96% is secreted in a nonquantized form and comes from an extravesicular pool. The size of the extravesicular pool is uncertain: it could be <10%, or as great as 50%, of the depletable store. K causes a highly (but perhaps not perfectly) selective increase in the rate of quantal secretion so that quanta account for about 50% of the total ACh released from K-treated diaphragms. K, or electrical stimulation of the phrenic nerve, depletes both the vesicular and extravesicular pools of ACh when hemicholinium no. 3 (HC-3) is present. However, most of the vesicles are retained under these conditions so that the diaphragms are able to increase slightly their rates of release of ACh when K is added.

Venom depletes the terminals of their vesicles and abolishes the release of quanta of ACh. It depletes the vesicular pool of ACh (since it depletes the vesicles), but may only partially deplete the extravesicular pool (since it reduces resting release only 10–40%). The rate of release of ACh from the residual extravesicular pool does not increase when 25 mM K is added.

Although we cannot exclude the possibility that stimulation may double the rate of release of ACh from the extravesicular pool, our results are compatible with the idea that the ACh released by stimulation comes mainly from the vesicles and that, when synthesis is inhibited by HC-3, ACh may be exchanged between the extravesicular pool and recycled vesicles.

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The acetylcholine (ACh) in vertebrate neuromuscular preparations is distributed among at least three different pools. About 20–33% of the ACh may be located in extraneural structures since this fraction of the total store remains in muscles after denervation (2, 17, 28). The remaining ACh is located in the axons and terminals of the intramuscular nerves where it seems to be distributed between vesicular and extravesicular pools. Considerable disagreement exists as to the functional significance of the extravesicular pool. Estimates of the fraction of ACh contained in this pool have ranged from small (16) to as great as 70% (22), and suggestions as to the function of the pool are equally disparate. Hebb (16) has suggested that the extravesicular pool is the site of ACh synthesis, and Marchbanks (27) has suggested that this pool is also the source of most of the ACh released from vigorously secreting nerve endings. On the other hand, Zimmerman and Denston (37) find that during intense secretion the synthesis of ACh occurs preferentially in, or very near, those vesicles that have most recently released their charge of ACh, and Dowdall (10) has suggested that ACh synthesis may not occur uniformly throughout the terminal cytoplasm but may occur mainly at, or near, the sites of ACh release.

Black widow spider venom eliminates the vesicular pool of ACh since it depletes nerve terminals of their vesicles (8). Therefore, we thought that a study of the effects of the venom on the storage of ACh in, and its release from, mouse diaphragms might yield information useful for determining the relative sizes of the vesicular and extravesicular pools. Use of an intact preparation provides an opportunity to correlate electrophysiological estimates of quantal release of ACh with biochemical estimates of total release; the difference between the two estimates should measure nonquantal release. In addition, the intact diaphragm allows us to determine whether the depletion of various pools is accompanied by changes in the ultrastructure of neuromuscular junctions.

In this report we describe the effects of crude black widow spider venom on the electrophysiology and morphology of mouse diaphragm and on the storage and release of ACh in this tissue. The effects of the venom on the storage and release of ACh are compared with the effects of high concentrations of potassium, electrical stimulation of the phrenic nerve, and treatment with hemicholinium no. 3 (HC-3).

MATERIALS AND METHODS

All experiments were performed with diaphragms from about 20-g male mice. The mice were decapitated and the phrenic nerves and diaphragms with the ribs and vertebral column attached were quickly removed and transferred to a dish containing oxygenated Krebs' solution at room temperature. The vertebral column was removed and the ribs were trimmed short.

Electrophysiology

A right hemidiaphragm and phrenic nerve were mounted in a Lucite chamber and perfused with bathing solution warmed to 35–37°C and equilibrated with 95% O_2 and 5% CO_2. The cut end of the nerve was drawn through a Vaseline seal into a small well of solution and was stimulated by platinum wires placed across the seal. After the hemidiaphragms had equilibrated for about an hour, the muscle fibers along the main intramuscular branches of the phrenic nerve were impaled with KCl-filled glass micropipettes (tip potential <10 mV, resistance 10–50 MΩ) and the electrical responses were recorded with conventional apparatus. When the preparations were perfused with the standard Krebs' solution, neuromuscular transmission and the electrical responses were maintained for at least 5 h (36).

When the effects of drugs or changes in the ionic composition of the Krebs' solution were studied, the chamber was perfused with the modified solutions. When the effects of the venom were studied, perfusion was stopped and a few milliliters of diluted venom were added directly to the chamber. Under these conditions, the temperature and oxygenation of the bath were maintained by a small heater and a bubbler built into the chamber. The volume of the chamber was about 3 ml.

The standard Krebs' solution contained (in mM): NaCl, 142; $NaHCO_3$, 8; $KHCO_3$, 4; KH_2PO_4, 1; glucose, 11; $MgCl_2$, 1; and $CaCl_2$, 2.

Carbachol Depolarization

Changes in the postsynaptic sensitivity of the endplate were monitored by measuring the depolarization of the muscle fibers produced by the application of carbachol in the bath. About 10 ml of carbachol solution (5–30 μg/ml) was run rapidly (about 20 s) into the chamber, and the chamber was then flushed immediately with 10 ml of standard Krebs' solution. The depolarization had usually reached its peak before the carbachol was flushed away. The response of any given muscle fiber to a given concentration of carbachol usually was not tested more than once, and each carbachol test was performed on a different fiber. The amplitudes and time-courses of the

depolarizations varied enormously from fiber to fiber, presumably depending on local diffusion conditions and on the location of the nerve terminals with respect to the surface of the diaphragm.

Collection of ACh for Bioassay

Usually two whole diaphragms were mounted in a chamber and perfused for an hour with Krebs' solution that contained eserine sulfate at a concentration of 2 μg/ml (about 6 μM eserine). Perfusion was then stopped, the bath was drained, and 2 ml of prewarmed, oxygenated eserinized solution was added. Every 15 min thereafter, the bathing solution was removed for assay and the chamber refilled with 2 ml of fresh solution. In some of these experiments the ribs were left attached to the diaphragms, and one hemidiaphragm was mounted so that its phrenic nerve could be stimulated. Although the ribs and intercostal muscles provided an additional source of ACh, these preparations were used because neuromuscular transmission could be monitored by observing the muscle twitch or measuring the amplitude of the compound action potential of the muscle fibers in response to indirect stimulation. The compound action potential was recorded through a blunt pipette (filled with 1 M NaCl in agar) dipped just below the surface of the bathing solution near the point where the phrenic nerve entered the muscle.

Extraction of ACh

Whole diaphragms were equilibrated for 1–2 h in Krebs' solution at 37°C, and then the experimental preparations were transferred to an appropriately modified Krebs' solution for an hour. Controls were left for an additional hour in Krebs'. The ribs were trimmed from some preparations before the equilibration was begun, and they were trimmed from other preparations just before homogenization. The muscles were ground in 0.4 ml of cold 10% TCA in glass homogenizers and then left for about 90 min in an ice bath. The homogenate and two TCA washes (0.2 ml each) were filtered, the filtrate was extracted four times with water-saturated ether and the aqueous residues dried under nitrogen. The dried extract was dissolved in 100 μl of 0.1 M formic acid, and 30 μl of the dissolved extract, together with 10 μl of a solution of tetraethylammonium chloride (TEA, 2–3 μg/ml), were applied to a strip of electrophoresis paper and run 1 h at 400 V (30). The strips were removed, exposed briefly to iodine vapor to stain the TEA bands, and dried overnight. The bands were then cut out and extracted with 1.5 ml of the standard Krebs' solution. With each experiment, we ran a blank and a standard containing 250 pmol of ACh (roughly the content of a normal diaphragm). Fresh buffer was used for each electrophoresis to keep the blank below 2 pmol. The recovery of the standard was 78 ± 13% (mean ± SD, n = 15). The values of the tissue content of ACh were corrected for the blank and for incomplete recovery.

A few of the diaphragms were weighed, but this was not done on a regular basis. The average wet weight of a hemidiaphragm without ribs was 22 mg (range 16–29); the average wet weight of a hemidiaphragm with ribs was 85 mg.

Bioassay of ACh with the Guinea Pig Ileum

The guinea pigs were of either sex and weighed from 300 to 1,000 g. They were stunned and bled, and about 2 feet of the lower ileum was quickly dissected out and placed in a large Petri dish containing warm (30°–35°C) bathing solution bubbled with 95% O_2 and 5% CO_2. The aboral end of a 6-cm length of ileum, devoid of Peyer's patches, was tied over the end of a plastic tube and gently massaged and perfused for about half an hour. The oral end of the ileum was tied closed and the preparation mounted in a glass tissue bath held at 35°C. The oral end of the ileum was tied to a high compliance spring (1 g/cm) connected to an isometric transducer, and the output of the transducer was amplified and displayed on a chart recorder. The volume of the tissue bath was 4 ml and it was connected through a glass condenser to a large reservoir of bathing solution that was gassed with 95% O_2 and 5% CO_2. The tissue bath was stirred and oxygenated by a stream of 95% O_2 and 5% CO_2 bubbled in through a fine hypodermic needle.

The solution bathing the ileum contained (in mM): NaCl, 113; $NaHCO_3$, 25; KCl, 4.7; KH_2PO_4, 1.2; glucose, 11; $MgSO_4$, 1.2; and $CaCl_2$, 2.5. The solution also contained the following drugs: morphine sulfate, 5 mg/ml; pyrilamine maleate, 0.5 mg/liter and eserine sulfate, 2 μg/liter.

Once an assay was begun, a fairly regular schedule of injections was followed. Usually 0.4 ml of a sample was injected into the bath and left until the contraction of the ileum had attained its peak (usually within 10 s or less). Then the bath was flushed with several volumes of fresh solution, and about 2 min later another sample was injected. The injection of each unknown sample was preceded by the injection of two bracketing standards of ACh. Each unknown sample was usually assayed twice in this manner, and the amount of ACh in the unknown was computed by linear interpolation from the average results of the two bracketing sets of standards. When this schedule was followed, a single piece of ileum could be used for 6–7 h.

The ACh standards, which usually contained 0, 12.5, 25, or 50 pmol of AChI per ml, were made up in the standard Krebs' solution for diaphragms and never contained drugs. The effects of the drugs on the ileum were tested separately, and we found that neither eserine nor HC-3 at the concentrations we used had any immediate effects on the response of the ileum to ACh.

Potassium-rich solutions acted directly on the ileum and caused it to contract. To minimize errors from this source, we injected only 0.2 ml of K-rich solutions onto the ileum. The injection of 0.2 ml of a solution of 25 mM K produced a contraction roughly equivalent to that produced by 2 pmol of ACh. In each of our experiments

with 25 mM K, we measured the contraction produced by 25 mM K and deducted its ACh equivalent from the ACh value determined for the unknown in 25 mM K.

When solutions that contained 0 mM Ca and 10 mM Mg were applied to the diaphragm, a complete set of ACh standards was prepared in these same solutions so that the ACh contents of the unknown could be determined from these standards without correction.

Venom had no immediate effects on the response of the ileum to a given dose of ACh. This was checked by comparing the responses of the ileum to ACh standards made up in normal Krebs' with the responses of the ileum to standards made up in Krebs' plus the usual dose of venom. However, the spontaneous activity of the ileum often increased after repeated brief exposures to the venom, and when the venom was applied for 30 s or more a contracture gradually developed which was only slowly reversed by repeated washing. To minimize these disturbances caused by the venom, the venom-containing samples were usually assayed last, the volumes injected were reduced to 0.2 ml, and each sample was tested only once.

The sensitivity of the ileum to ACh gradually increased during the early stages of an experiment. After several samples had been assayed, a steady state was reached and the injection of 5 pmol of ACh usually induced a contraction that developed about 0.5 g of tension (0.5 cm of shortening). The best ilei showed no spontaneous activity and responded clearly to 2 pmol of ACh, or less. The poorest ilei responded clearly to 5 pmol and showed spontaneous activity equivalent to about 2 pmol. Thus, the sensitivity and precision of the measurements were about 2 pmol, and the duplicate determinations of the ACh contents of unknowns usually agreed within this limit.

Three types of controls were performed to verify that the substance causing the ileum to contract was ACh: at the end of some experiments, atropine sulfate (1 μg/ml) was applied to the ileum and the solutions were retested; in some experiments, solutions were assayed both before and after they had been treated for 1 h at room temperature with 0.02 M NaOH, and, in one complete experiment with venom and 25 mM K, no eserine was used in the solutions applied to the diaphragms. The atropine always completely blocked the contractions of the ileum; the apparent concentration of ACh in a given sample was reduced over 90% by hydrolysis in NaOH, and, when no eserine was present in the solutions applied to the diaphragms, no specific contractions were observed when solutions collected from resting, K-stimulated or venom-treated preparations were applied to the ileum.

All results are reported as mean values ± the standard deviation (SD) with the number of measurements in parentheses.

Concentration of Eserine Required to Prevent Hydrolysis of ACh

We did a series of experiments to determine the effect of the concentration of eserine on the amount of ACh collected from resting diaphragms. In six of these experiments we sequentially applied eserine sulfate at doses of 2, 10, 30, and 2 μg/ml. In each experiment, each concentration was applied for at least an hour and, after a 15-min equilibration period, at least three 15-min samples were collected for each concentration tested. New steady states were established during the 15-min equilibration period. In four experiments we used preparations with ribs, and in the other experiments we used preparations without ribs. The normalized results are shown in Table I. The largest amounts of ACh were collected at an eserine sulfate concentration of 10 μg/ml. The apparent reduction obtained at 30 μg/ml was not due to an atropine-like action of the eserine on the ileum since the same concentration of eserine did not affect the response of the ileum to the ACh standards. The reduction may not be real since the application of this large concentration of eserine to the ileum usually caused contractions to develop after the initial response to ACh had partially subsided; therefore, these measurements may not be very reliable. However, there were no indications that more ACh was collected when the concentration of eserine was raised above 10 μg/ml, and it appears that the inhibition of the cholinesterase is complete at this concentration. However, repeated exposures of an ileum to this relatively high concentration of eserine often led to the buildup of excessive spontaneous activity, and therefore we used a concentration of 2 μg/ml in the collection experiments. This latter concentration could be repeatedly injected without obvious deleterious effects on the ileum. About 42% of the ACh released from diaphragms is hydrolyzed at this concentration of eserine sulfate.

We also incubated diaphragms in solutions that contained the two higher concentrations of eserine to determine whether the drug caused changes in the ACh content of the diaphragms. In one of these experiments, we also added 30 μM choline to the bathing medium. The average ACh contents of the muscles soaked for 2 h in eserine at concentrations of 0, 10, or 30 μg/ml were, respectively: 99 ± 30 (n = 10), 110 ± 32 (n = 6), and 91 ± 31 (n = 6) pmol per hemidiaphragm. Neither the eserine nor the added choline changed the ACh content of the diaphragms. When the results from all of the diaphragms used as controls in the course of this investi-

TABLE I

Effect of the Concentration of Eserine on the Amount of ACh Collected from Resting Diaphragms

Concentration of eserine sulfate	Amount of ACh collected in 15 min	No. of experiments
μg/ml	%	
2	100	7
10	173 ± 35	7
30	140 ± 22	6
2	92 ± 20	6

gation are averaged together, then the mean ACh content of a hemidiaphragm is 107 ± 28 pmol (n = 35)

Preparations of Venom

The cephalothoraxes of black widow spiders (obtained from Istituto Superiore di Sanità, Rome, Italy) *Latrodectus mactans tredecimguttatus* were kept frozen at −20°C and small quantities were thawed as needed. Usually four cephalothoraxes were thawed and the eight venom glands were removed and homogenized in 1 ml of cold 120 mM NaCl. The homogenate was then stored at about 2°C and remained fully active for at least 2 wk. To apply the venom, 100 μl of the homogenate was mixed with 10 ml of the bathing solution, the muscle bath was drained, and the diluted venom was added. A single venom gland contains about 50 μg total protein (13) and about 4 μg of the protein fraction that is active at vertebrate neuromuscular junctions. Hence the data reported here were obtained with solutions that contained about 5 μg total protein/ml or 0.4 μg active protein/ml. Solutions containing only 10% of this standard concentration raised miniature endplate potential (mepp) frequencies in the surface fibers of diaphragms. However, with these solutions the mepp frequencies tended to remain high for prolonged periods and neuromuscular transmission persisted for an hour; therefore the effects of these doses were not studied extensively.

Electron Microscopy

Muscles were fixed in the recording chamber. The bathing solutions were removed, the chamber was filled with cold 2% OsO_4 in 0.12 M phosphate buffer, pH 7.3, and the chamber was put into a refrigerator for 20 min. Small bits of muscle close to the course of the phrenic nerve were removed and the bits of muscle were transferred to fresh cold fixative for a total fixation time of 2 h. After fixation, some of the specimens were washed three times with 0.05 M veronal acetate buffer, pH 7.3. These specimens were then block-stained for 30 min at 4°C in 0.05 M veronal acetate buffer with 0.5% uranyl acetate, pH ~5. All specimens were dehydrated in alcohol and flat-embedded in Epon 812. Blocks were mounted in a Porter-Blum MT3 microtome (DuPont Instruments-Sorvall DuPont Co. (Wilmington, Del.), and semi-thin sections were cut, stained, and examined with a light microscope. When the semi-thin sections contained suspected neuromuscular junctions, thin sections were cut with a diamond knife, double-stained with uranyl acetate and lead citrate, and examined in a Philips EM 200 or EM 300 electron microscope.

Determining the Number of Muscle Fibers in a Mouse Diaphragm

Three diaphragms from 20- to 23-g male mice were dissected with ribs attached, pinned to a piece of balsa wood anchored in the bottom of a Petri dish, and fixed for 2 h in cold 2% O_8O_4 in 0.12 M phosphate buffer, pH 7.3. The diaphragms were dehydrated, the balsa wood was removed, and the whole diaphragms were embedded in Epon 812 in another Petri dish. Three or four pieces of tissue were cut from each block, some from the thinner portions of the diaphragms and others from the thicker portions. Semi-thin sections were cut from the mid portions of each piece with a Porter-Blum MT3 microtome, the sections were photographed, and the fibers counted. The stained semi-thin sections permitted the unambiguous identification of muscle fibers. The total length of the sections counted in each of the three diaphragms ranged from 4.3 to 10.0 mm, and the estimates of the total number of fibers in individual diaphragms ranged from 10 to 16 × 10^3. The average number of fibers, weighted according to the length of muscle counted in each diaphragm, was 14 × 10^3 or 7 × 10^3 per hemidiaphragm.

RESULTS

Effects of 25 mM K

These data will be presented first to describe the behavior of normal mouse diaphragms.

ELECTROPHYSIOLOGY: 90 muscle fibers were impaled in 10 diaphragms. The average amplitude of the mepp's was 1.1 ± 0.5 mV, their average frequency of occurrence was 5.3 ± 4.9/s and the average membrane potential of the muscle fibers was −70 ± 9 mV (Fig. 1). When 25 mM K was applied, the muscle fibers depolarized to −44 ± 6 mV (n = 103); the mepp frequencies rose to levels well over 100/s in 80% of the fibers (Fig. 1) and rose to levels from 10 to 100/s in the remainder of the fibers. The average mepp frequency was about 300/s and was sustained for periods up to an hour. The mepp amplitudes ranged from 0.2 to 0.5 mV in fibers with low mepp frequencies; the amplitudes could not be measured in fibers with high mepp frequencies. The mepp frequency did not increase when 25 mM K was applied in a solution with no Ca and 10 mM Mg.

When 25 mM K was applied in the presence of eserine, the mepp frequencies rose as usual and then the mepp amplitudes declined rapidly so that after a few minutes the oscilloscope showed a noisy trace with no obvious mepp's. This profound decrease in mepp amplitude did not occur when the rise in mepp frequency was prevented by applying K in an eserinized solution with 0 Ca and 10 mM Mg. The decline in amplitude was not prevented by adding 100 μm choline to the bath, and, since eserine does not prevent the synthesis of ACh, it seems likely that the decline in ampli-

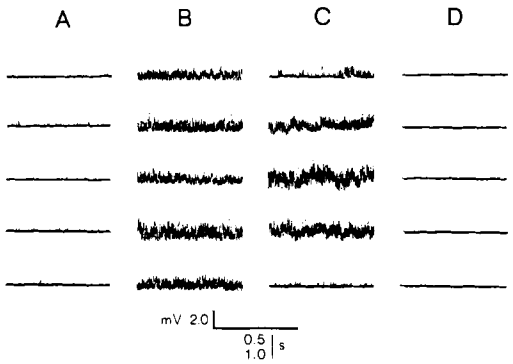


FIGURE 1 Effect of 25 mM K and black widow spider venom on mepp frequencies of endplates in mouse diaphragms. Column A shows records obtained from five junctions from a diaphragm bathed in standard Krebs'. Column B shows records from five junctions from a different diaphragm bathed in Krebs' with 25 mM K. Column C shows a sequence of records obtained from a single junction in the first diaphragm after black widow spider venom (10 μl/ml) had been applied. The first four records in this column were obtained at roughly 1-min intervals after the venom had been applied, and the fifth record was obtained ~10 min after the venom had been applied. The venom was left on the diaphragm for an hour and then washed away with standard Krebs'. 10 fibers were impaled and no mepp's were recorded. Krebs' with 25 mM K was applied, and ~5 min later the records in column D were obtained. No mepp's are evident. Time calibration: 1.0 s, columns A and D; 0.5 s, columns B and C.

tude was due to desensitization caused by the accumulation of ACh in the synaptic clefts (33). The accumulated ACh may also have increased the electrical noise by causing the random opening and closing of conductance channels in the postsynaptic membrane (21).

RELEASE OF ACH: 25 mM K increases the rate of secretion of ACh from mouse diaphragms by about fourfold on the average, and the high rate of secretion can be sustained for at least an hour (Fig. 2). When the extra K is removed, the rate of secretion falls to normal levels and increases again when the extra K is restored. During an hour of soaking in 25 mM K, the ACh contents of hemidiaphragms declined from control levels of 107 ± 28 pmol (n = 35) to 62 ± 10 pmol (n = 6). The total amount of ACh secreted during the hour is 2.5 times as great as the amount lost from the tissue, so it is clear that, even in the absence of added choline, mouse diaphragms can synthesize ACh rapidly enough to sustain high rates of release.

Qualitatively, the effect of 25 mM K on total ACh release resembles its effects on quantal release as reflected in the mepp frequencies. Quantitatively, however, there is an order of magnitude discrepancy between the fractional increase in mepp frequency (about 60-fold) and the fractional increase in total ACh release (about

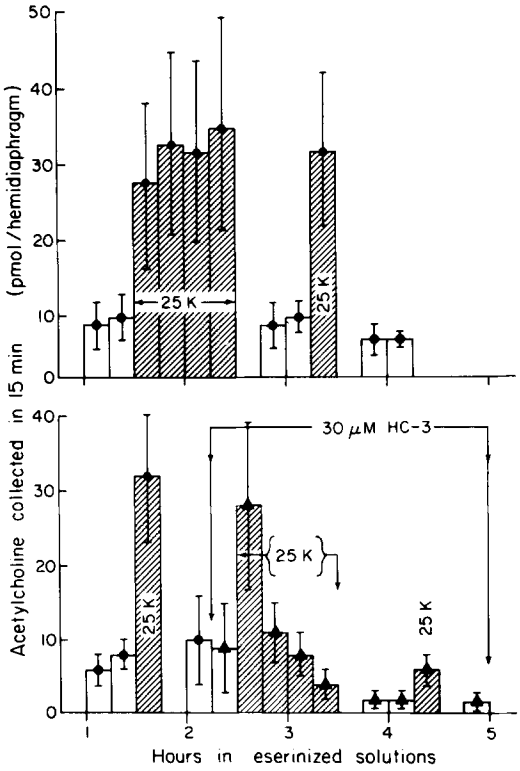


FIGURE 2 Effect of K on the rate of release of ACh from mouse diaphragms. Each graph presents the mean results of five experiments ± SD. All preparations without ribs. Upper graph: controls. After two collections from resting preparations, a solution with 25 mM K was applied for an hour. The preparations were then washed for 15 min, two resting samples were collected, and 25 mM K was applied again for 15 min. Lower graph: experiments with 30 μM HC-3. After two collections from resting preparations in standard Krebs', a solution with 25 mM K was applied for 15 min. Then the preparations were washed for 15 min, and a collection was made in normal Krebs'. 30 μM HC-3 in normal Krebs' was then applied, and one resting sample was obtained. 25 mM K in HC-3 was then applied for an hour, and then the preparations were washed for 15 min in Krebs' with HC-3. After two resting collections in HC-3, the 25 mM K in HC-3 was applied again. Data not corrected for hydrolysis. ●, Solutions without HC-3; ▲, solutions with 30 μM HC-3.

TABLE II

Effects of Various Treatments on the Rate of Release of ACh from Resting Diaphragms

Treatment	ACh collected in 15 min			
	Before	After	A-B	A/B
	pmol/hemidiaphragm			%
A. Preparations with ribs				
Stimulate nerve, 10/s	9.3 ± 2.8 (6)	7.8 ± 2.6 (6)	−1.5 ± 3.6 (6)	84
Stimulate nerve, 10/s + HC-3	12.6 ± 3.7 (5)	3.8 ± 4.1 (5)	−8.5 ± 4.7* (4)	30
Venom	14.9 ± 6.1 (7)	9.2 ± 6.0 (7)	−5.7 ± 1.9* (7)	62
Venom + HC-3	11.9 ± 2.3 (4)	7.3 ± 2.5 (4)	−4.7 ± 2.7* (4)	61
B. Preparations without ribs				
25 K	6.9 ± 2.3 (5)	6.5 ± 1.9 (5)	−0.4 ± 3.0 (5)	94
25 K + HC-3	7.2 ± 2.9 (5)	1.8 ± 0.6 (5)	−5.4 ± 2.7* (5)	25
Venom	6.3 ± 0.9 (8)	5.7 ± 2.4 (8)	−0.6 ± 2.8 (8)	90
Venom + HC-3	5.3 ± 1.5 (5)	2.9 ± 1.6 (5)	−2.4 ± 1.7* (5)	55

Part A, grand mean (before) 12.2 ± 5.0 (22). Part B, grand mean (before) 6.4 ± 1.9 (23). Mean values ± SD No. of experiments in parentheses.

* Differences significant at the 95% level (Student's *t* test).

fourfold) (29). This indicates that there is an appreciable secretion of ACh in a nonquantized form, particularly at rest (22, 32). Recent estimates of the number of ACh molecules in a quantum range from 6 to 10 × 10^3 (12, 25). If we assume a value of 8 × 10^3, then the rate of secretion of ACh due to quanta is 0.4 pmol/15 min for a resting hemidiaphragm (7,000 junctions, mean mepp frequency of 5/s). The average measured rate of ACh release is 6.4 pmol/15 min (Table II), or 11 pmol/15 min when corrected for the hydrolysis that occurred at the eserine concentration of 2 μg/ml. Thus, quantal release accounts for <4% of the total amount of ACh released from resting diaphragms and, therefore, 96% of the ACh released is not quantized and must come from an extravesicular pool.

In K-stimulated preparations, the situation is very different. The average mepp frequency is about 300/s, which is equivalent to a rate of release of ACh of 25 pmol/15 min. The total measured rate of release, corrected for hydrolysis, is 51 pmol/15 min. Thus, quanta account for about 50% of the total amount of ACh released from K-stimulated diaphragms and for about 63% of the increment in total ACh release which occurs when K is applied. The apparent increment in nonquantal release, 15 pmol/15 min, suggests that, in addition to stimulating quantal release about 60-fold, K may also increase about 2.3-fold the rate of release of ACh from the extravesicular stores. However, this latter conclusion is not firm since it is critically dependent on the value assumed for the number of ACh molecules in a quantum. If we assume that all of the extra ACh released by K is secreted as quanta, then we calculate the number of ACh molecules in a quantum to be 13 × 10^3, a figure only 30% greater than the upper limit estimated by Kuffler and Yoshikami (25).[1]

When 25 mM K is applied in the presence of HC-3, the rate of release of ACh increases, but the increase is not sustained and the rate even-

[1] Fletcher and Forrester (12) have estimated the quantal size in rat diaphragms to be only 6 × 10^3 molecules. Their calculation assumed an epp to be composed of 400 quanta. A more reasonable figure would be 200–300 (19). If the latter figures are used, the number of molecules in a quantum would lie between 9 and 12 × 10^3.

tually falls below the original resting rate (Fig. 2). When K is removed and reapplied after 45-min rest in HC-3, an additional small increase in ACh release occurred. Since HC-3 inhibits ACh synthesis (3), the decline in the release was presumably caused by the depletion of the releasable stores of ACh. During the hour of soaking in K + HC-3, the ACh contents of a hemidiaphragm declined to 38 ± 6 pmol (n = 6), a decrease of 69 pmol from the control level. Since K + HC-3 reduces the total output of ACh to levels below the original resting level, it must deplete ACh from both the vesicular and the extravesicular pools. We estimated above that quanta accounted for about half of the total ACh released from K-treated diaphragms. If this same ratio applies in the presence of HC-3, then the extravesicular pool would account for about half of the ACh released, or 35 pmol/hemidiaphragm. This quantity should provide an upper limit for the size of the extravesicular pool. K + HC-3 has similar effects on slices of rat brain cortex (31).

When HC-3 was applied to resting hemidiaphragms for 1 h, the ACh contents were unchanged: 106 ± 20 pmol (n = 5). Furthermore, the rate of release from resting diaphragms did not change markedly during this time. If HC-3 had completely inhibited ACh synthesis in resting diaphragms, there should have been a loss of 44 pmol in an hour. Apparently, HC-3 has little effect on ACh synthesis in resting diaphragms. Similar observations have been made previously with cat superior cervical ganglion (3).

MORPHOLOGY: Neuromuscular junctions in diaphragms that had been soaked for 4 h in eserinized Krebs' appeared normal. Fig. 3 shows a neuromuscular junction in a preparation that had been fixed 15 min after dissection, and Fig. 4 shows a junction from a muscle that had been fixed after it had been perfused for 4 h in eserinized Krebs' solution. The ultrastructure of this latter junction is properly preserved, showing that prolonged soaking in eserine does not affect the morphology of the nerve endings. This finding corroborates previous observations (35).

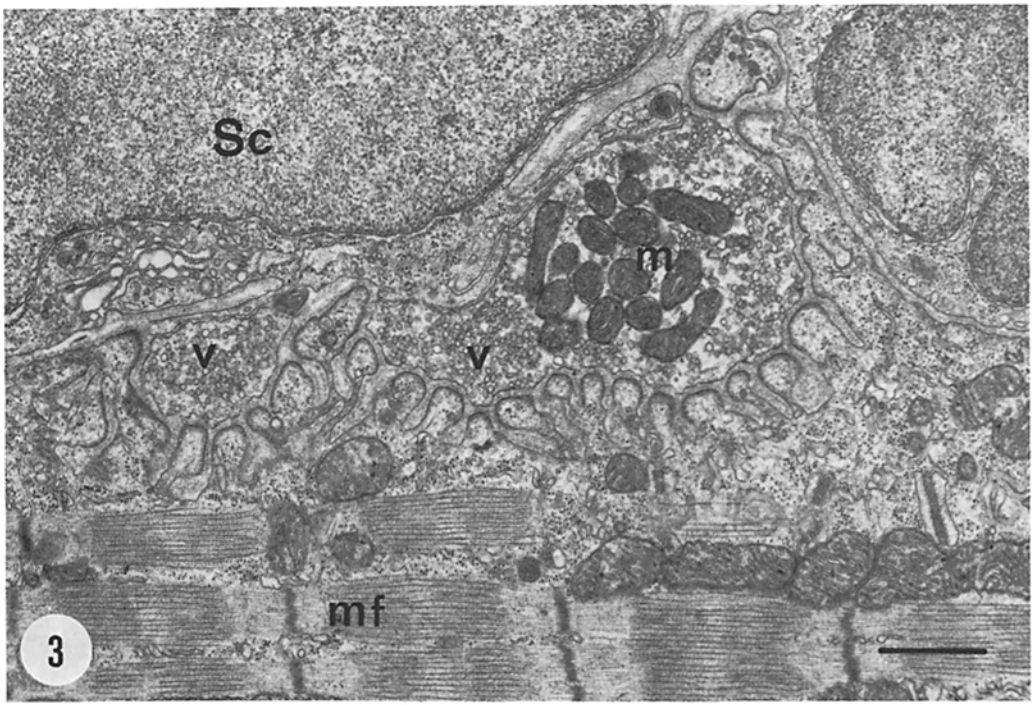
Terminals that have been depleted of transmitter by soaking for 1 h in 25 mM K, eserine, and HC-3 and then rested for 45 min in eserine and HC-3 contain the normal complement of synaptic vesicles (Fig. 5). This result confirms previous work showing that nerve terminals in rat diaphragms and frog muscles can retain most of their synaptic vesicles after they have been depleted of transmitter by stimulation in HC-3 (5, 20). It seems likely that the ability of these terminals to respond slightly to added K (Fig. 2) may be due to the fact that they have retained their vesicles. The quantal release mechanism, which accounts for most of the ACh released by 25 mM K, is intact, and the response is small only because the depletable ACh stores are almost exhausted.

Since the nerve terminals in these diaphragms had retained their vesicles, it could be argued that the residual store of ACh was located in the vesicles and that the vesicular pool of ACh was not part of the depletable store. However, we will show below that a similar residual store remains in venom-treated diaphragms whose nerve terminals contain no vesicles. Therefore, it seems likely that the residual store of ACh that remains in diaphragms treated with K + HC-3 is not located in the vesicles and that the vesicles in these preparations are almost completely empty of transmitter. This residual store, about 35% of the total ACh in a control diaphragm, may correspond to the store that exists in denervated muscle (2, 24, 28, 29).

In summary, K + HC-3 appears to deplete both the vesicular store of ACh responsible for quantal release and the extravesicular store responsible for nonquantal release. The vesicles are retained and may account for the ability of the depleted terminals to secrete a small amount of ACh in response to added K.

Effect of the Venom

ELECTROPHYSIOLOGY: When the surface fibers of untreated diaphragms were impaled along the course of the main intramuscular branches of the phrenic nerve, mepp's were recorded in 90 of 91 penetrations. When venom was added, the mepp frequencies rose to uncountable levels (>300/s) within a few minutes and then began to subside (Fig. 1) (23, 26). Neuromuscular transmission was totally blocked within 20 min. When the same regions of the diaphragms were reimpaled an hour after the venom had been added, mepp's were recorded in only 14 of 123 penetrations. The amplitudes of these mepp's ranged from 0.3 to 0.9 mV and their frequencies ranged from 3 to 100/s. The resting potentials of the muscle fibers had decreased to −59 ± 10 mV. The decrease in resting potential was not due to the summation of mepp's, since mepp's were not recorded at most junctions. It may be due to a direct effect of the venom on the muscle fibers.



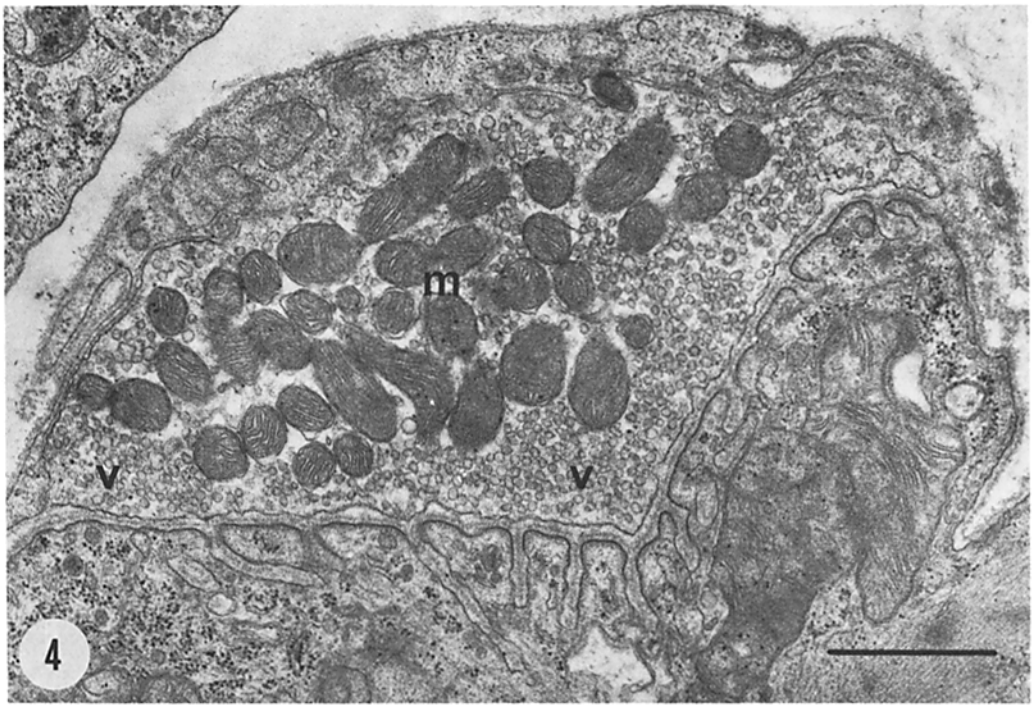


FIGURE 3 Electron micrograph of a neuromuscular junction in a mouse diaphragm fixed after 15 min of perfusion with Krebs' solution. The axon terminal shows a normal distribution of synaptic vesicles (v) and a cluster of mitochondria (m). The axon terminal is surrounded by the Schwann cell. *Sc*, Schwann cell nucleus; *mf*, myofibrils. This specimen was block stained. Bar, 1 μm.

FIGURE 4 Electron micrograph of a portion of a neuromuscular junction from a mouse diaphragm fixed after 4 h of soaking in Krebs' solution with eserine sulfate, 2 μg/ml. The subcellular organization of this terminal appears normal. The terminal contains many synaptic vesicles (v) and mitochondria (m) and is completely surrounded by a Schwann cell process. Bar, 1 μm.

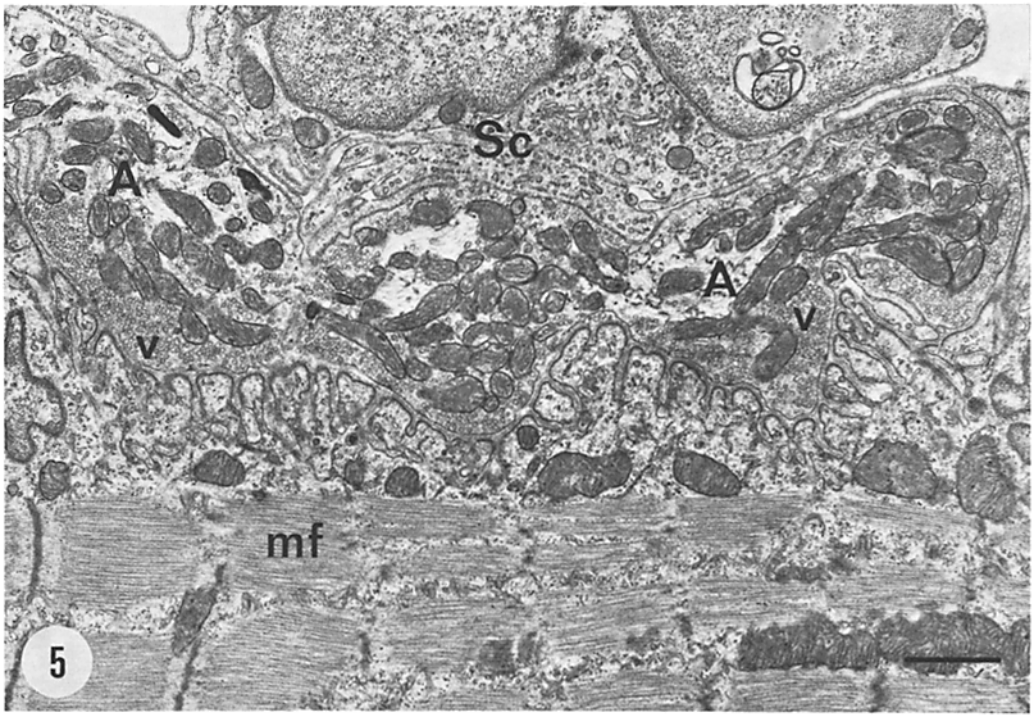
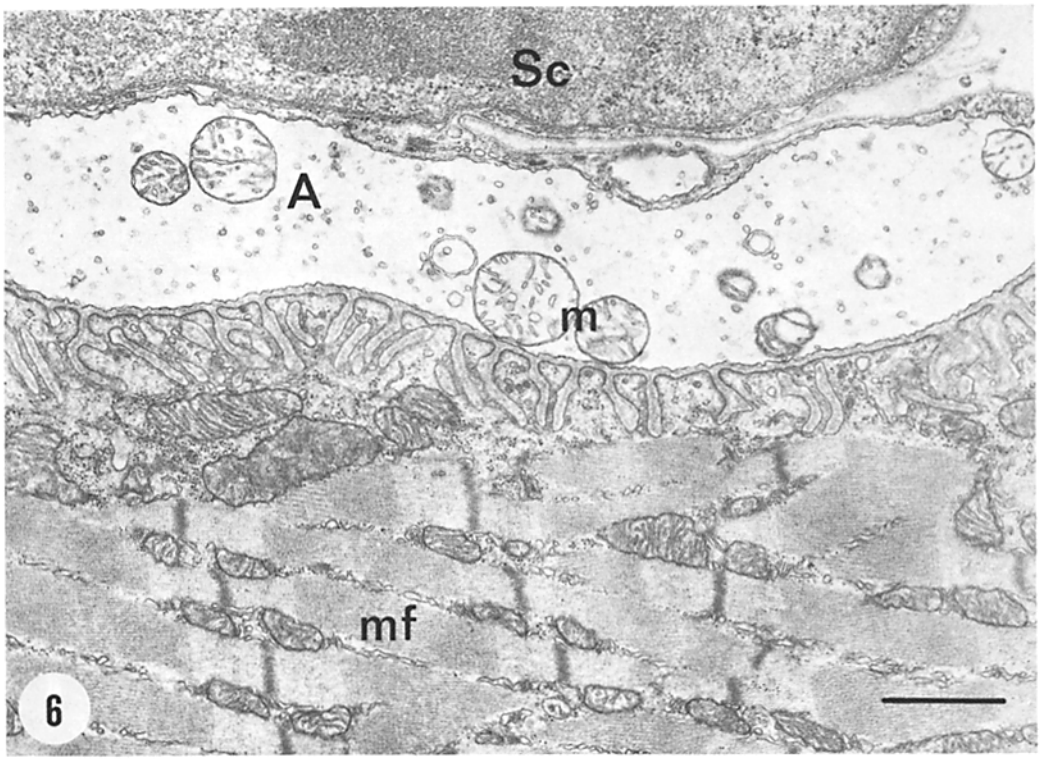


FIGURE 5 Low power electron micrograph of a portion of a neuromuscular junction from a preparation that had been treated with the same sequence of solutions as the preparations used to obtain Fig. 2, lower. The diaphragm was first perfused for 1 h with eserinized Krebs' (2 μg eserine sulfate/ml) and then for 15 min with Krebs' + eserine + 30 μM HC-3. The K concentration was then raised to 25 mM for an hour, then the diaphragm was washed for 45 min in Krebs' + eserine + HC-3, and finally it was fixed. A vast expanse of the nerve ending (*A*) is seen in this micrograph. The subcellular organization of the terminal appears normal, and it contains a normal complement of synaptic vesicles (*v*). The terminal is completely surrounded by a Schwann cell (*Sc*). *mf*, Myofibrils. Bar, 1 μm.

When venom was washed away, the muscle fibers gradually repolarized during an hour of recovery but the mepp frequencies remained unchanged. If 25 mM K was applied to venom-treated diaphragms, the muscle fibers depolarized to -46 ± 4 mV but mepp's were recorded in only a few penetrations. When mepp's were recorded, their frequencies were in the range of 10–100/s. Similar results were obtained when the venom was applied in a solution with 0 Ca, 10 mM Mg, and 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate (EGTA) or in a solution with eserine. We observed, however, that when venom was applied in eserine the mepp amplitude declined rapidly after the mepp frequency had increased. The decline was not prevented by adding 100 μM choline to the bath. As appeared to be the case with 25 mM K, the decline in mepp amplitude seems to be due to desensitization.

Junctions that exhibited no mepp's responded in an essentially normal manner to bath-applied carbachol. The transient application of carbachol at doses ranging from 5 to 30 μg/ml caused transient depolarizations that ranged from 3 to 25 mV both at normal junctions and at venom-treated junctions that did not exhibit mepp's. The average depolarizations produced by a given concentration of carbachol were the same at the two types of junction. Because of the great spread in the results, we cannot exclude the possibility that venom may have reduced postsynpatic sensitivity at most by 50%. However, even such a reduction in sensitivity could not account for our failure to record mepp's at over 80% of the junctions: mepp's were not recorded because quanta of ACh were not released, presumably because vesicles had been depleted (Figs. 6, 7, and 8).

MORPHOLOGY: Fig. 6 shows a portion of a neuromuscular junction from a diaphragm that



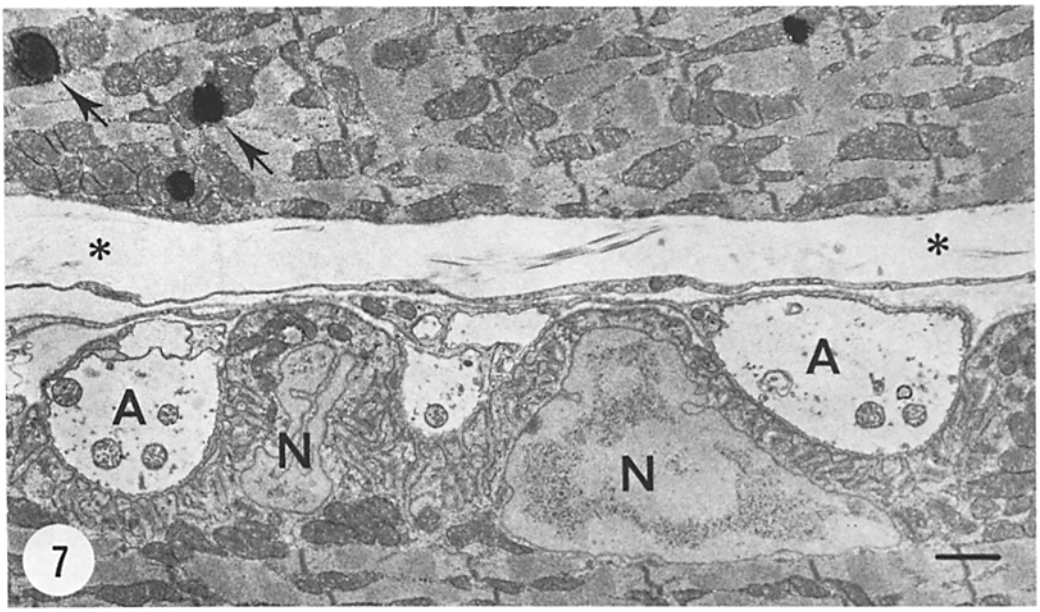


FIGURE 6 Electron micrograph of a portion of a neuromuscular junction from a diaphragm that had been perfused for an hour with standard Krebs' solution and then treated for an hour with venom, 10 μl/ml. The nerve ending (*A*) is moderately swollen and contains only a few residual vesicular structures resembling synaptic vesicles. The mitochondria (*m*) appear swollen. Note that the muscle mitochondria are normal. *mf*, Myofibrils; *Sc*, Schwann cell nucleus. Bar, 1 μm.

FIGURE 7 Low power electron micrograph of a portion of a neuromuscular junction from a preparation that had been perfused for 2 h with Krebs' containing eserine sulfate, 2 μg/ml, and then treated for 1 h with venom, 10 μl/ml, in eserinized Krebs'. Three portions of a single axon terminal (*A*) are seen and all are depleted of synaptic vesicles and are moderately swollen. The asterisks indicate the extracellular space between adjacent muscle fibers. *N*, muscle fiber nucleus; arrows, lipid droplets. Bar, 1 μm.

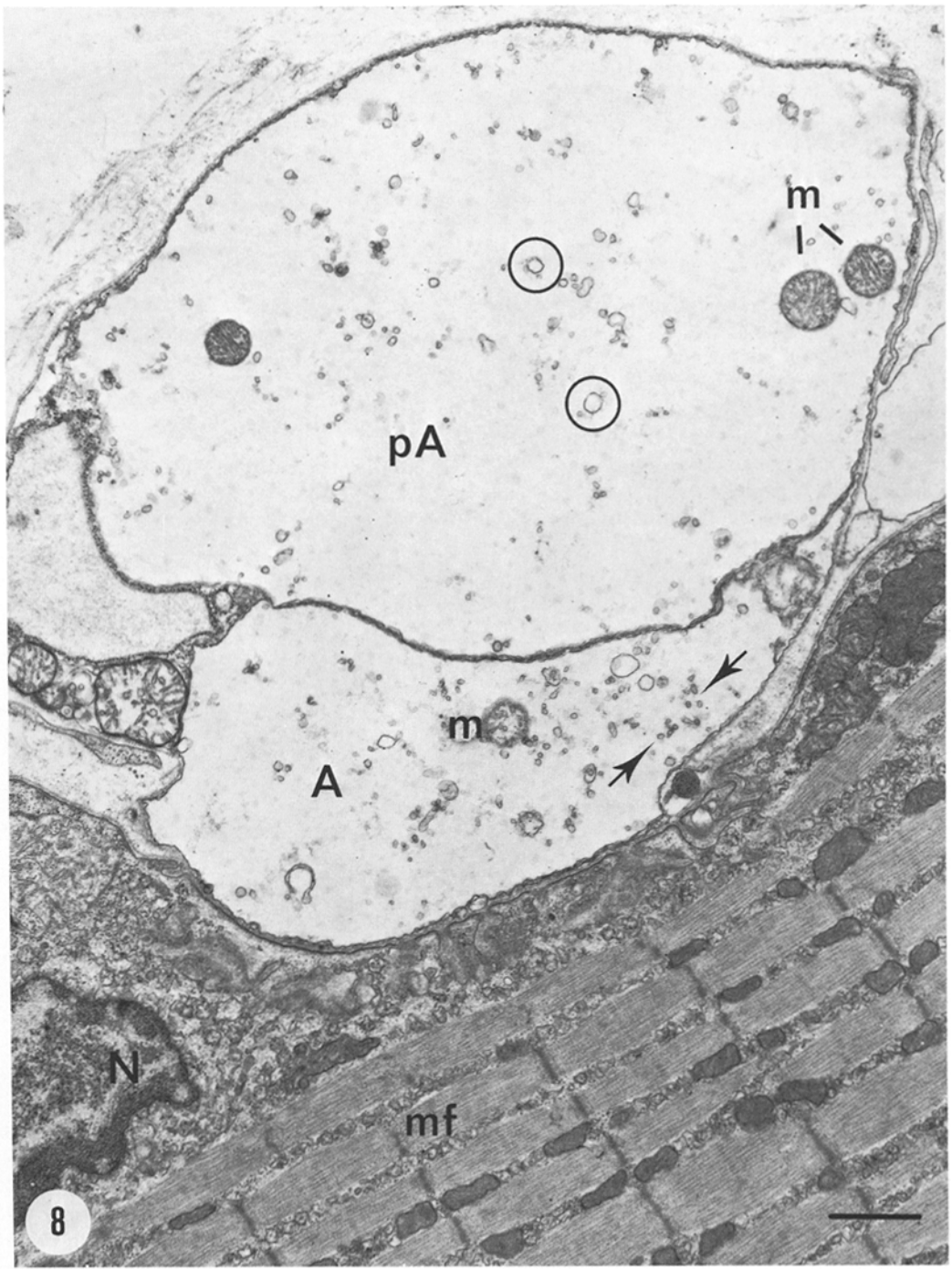


FIGURE 8 Electron micrograph of a portion of a neuromuscular junction from another mouse diaphragm that had been perfused for 2 h with eserinized Krebs' (2 μg eserine sulfate/ml) and treated for 1 h with venom, 10 μl/ml, in eserinized Krebs'. The terminal (*A*) appears swollen and only a few vesicular structures resembling synaptic vesicles remain (arrows). The overlying preterminal axon (*pA*) is grossly swollen. Both the terminal and the preterminal axon contain some large vesicular structures (circles). The Schwann cell processes that normally surround the nerve terminal are not seen in this micrograph; presumably they have been displaced by the swollen ending. The mitochondria (*m*) are moderately swollen. *N*, muscle fiber nucleus; *mf*, myofibrils. Bar, 1 μm.

had been perfused for an hour in Krebs' solution and then treated for an hour with venom. The terminal is moderately swollen, but its axolemma appears to be intact and it contains a few vesicular structures resembling synaptic vesicles scattered throughout the axoplasm. The mitochondria are also swollen. The underlying muscle fiber and its organelles, the postjunctional infoldings and the surrounding Schwann cell all appear normal.

Fig. 7 is a low power micrograph of a neuromuscular junction from a diaphragm bathed for 2 h in eserinized Krebs' solution and then treated for 1 h with venom in eserinized Krebs' solution. The three regions of the terminal seem moderately swollen, appear to be depleted to synaptic vesicles, and contain swollen mitochondria.

Fig. 8 shows another neuromuscular junction from a preparation treated with venom and eserine. This micrograph shows both a greatly swollen terminal and its swollen preterminal axon. In this case, the swelling was so extensive that it seems to have displaced the Schwann cell that normally overlies the junctional region. A similar finding was made previously when high concentrations of the purified toxin were applied to frog muscle (13). Both the nerve ending and the preterminal axon are almost completely devoid of vesicles. The effects of the venom on the morphology of the neuromuscular junctions are not influenced by eserine, and the changes seen in mouse diaphragms are similar to those observed previously in frog muscles (4, 5, 8). Thus, electrophysiology and morphology show that venom, in the presence of eserine, abolishes the release of quanta of ACh and depletes the terminals of their synaptic vesicles.

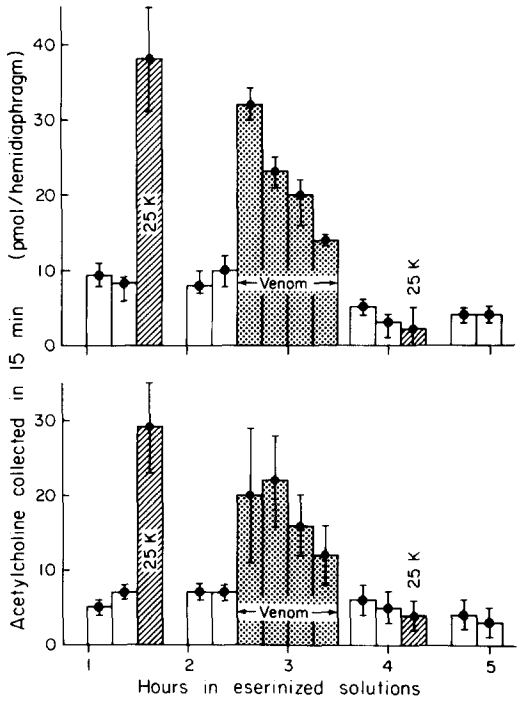


FIGURE 9 Effect of black widow spider venom on the rate of release of ACh from mouse diaphragm. Ordinates and abscissae as in Fig. 2. Upper graph: preparations with ribs, mean results of three experiments, bars indicate spread of results. Lower graph: preparations without ribs, mean results of five experiments ± SD. After two collections at rest, 25 mM K was applied for 15 min, and then the preparations were washed for 15 min with standard Krebs'. After two additional resting collections, venom (10 μl/ml Krebs') was applied for 1 h and then the preparations were washed with the standard Krebs' for ~10 min. After two more resting collections, 25 mM K was applied again for 15 min. Data not corrected for hydrolysis.

RELEASE OF ACH: The effect of venom on the release of ACh from mouse diaphragms is illustrated in Fig. 9. The results obtained from preparations with ribs were qualitatively similar to the results obtained from preparations without ribs, but the amounts of ACh collected from the former preparations were greater. In both types of preparation, venom caused an increase in the rate of release of ACh that reached a maximum after 15–30 min and then began to subside. Venom was washed away after an hour, and by this time the rate of release of ACh had fallen nearly to the original resting level. Similar results were obtained in two experiments in which venom was added in a modified Krebs' solution with no Ca and 10 mM Mg. These results are similar to those obtained when purified toxin is applied to slices of mouse brain cortex (34). When 25 mM K was applied after venom had been washed away it had no effect on the rate of release of ACh from the diaphragms, whereas when 25 mM K was applied before the venom, it increased the rate of release of ACh by about fourfold on the average. The ACh contents of venom-treated hemidiaphragms were 45 ± 15 pmol (n = 9), a level slightly higher than the level achieved in K + HC-3.

Thus, venom causes a loss of ACh from diaphragms almost equal to the loss caused by K + HC-3 and it abolishes the ability of the diaphragms to respond to added K. This last observation suggests that the pool of ACh depleted by

venom is the same as that depleted by K + HC-3, and this suggestion was confirmed in two experiments in which venom was applied to diaphragms that had been previously treated for an hour with K + HC-3. Venom increased the rate of release of ACh from these diaphragms by <5 pmol/15 min, about the same increase observed when 25 mM K was reapplied to such preparations (Fig. 2).

Although venom and K + HC-3 seem to deplete virtually the same pools of ACh, it is clear that venom and K + HC-3 have different effects on the rate of release of ACh from resting diaphragms. Venom reduces the resting rate of release of ACh by about 10% in diaphragms without ribs and by about 40% in preparations with ribs, whereas K + HC-3 reduces the resting rate of release of ACh from diaphragms without ribs by about 75% (Table II). Since venom-treated diaphragms contain virtually no vesicles, the ACh released from these diaphragms must come from an extravesicular pool. Thus, venom-treated diaphragms retain some of the extravesicular pool that is depleted by K + HC-3. The size of this extravesicular pool in venom-treated diaphragms should be given by the difference between the ACh contents of these diaphragms and the ACh contents of diaphragms treated with K + HC-3. The difference is small, 7 ± 17 pmol (about 10% of the depletable store), and not significant; therefore, it appears that venom-treated preparations contain a small extravesicular pool for ACh that turns over rapidly.

It is not clear whether normal diaphragms contain an equally small extravesicular pool. The fact that venom has little effect on resting release suggests that it may also have little effect on the size of the extravesicular pool. However, it is difficult to be sure of this, since venom may affect the permeability of the nerve terminal membrane to cations (11, 14, and footnote 2), and it is conceivable that venom partially depletes the extravesicular pool of ACh and simultaneously increases the permeability of the nerve terminals to ACh, or reduces their membrane potential, so that the flux of ACh from the pool remains constant.

K-treated diaphragms are able to sustain rates of release of ACh greater than those induced by venom (Fig. 2). The fact that the rate of secretion declines in the presence of venom implies that venom interferes with the synthesis or storage of ACh. It seems possible that the apparent effects of venom on synthesis are not direct but instead are consequences of the depletion of vesicles. One indication that venom does not directly inhibit ACh synthesis is the finding that ACh is released from the extravesicular pool of venom-treated diaphragms at rates significantly greater than the residual rates characteristic of diaphragms treated with K + HC-3. This suggests that, in contrast to the effects of K + HC-3, venom does not interfere with synthesis in the extravesicular pool, and that its apparent effects on the synthesis of quanta occur as a consequence of the depletion of vesicles.

As a check on whether some synthesis of ACh occurs during venom action, we did some experiments in which venom was applied in the presence of HC-3. The rate of secretion of ACh tended to fall off more rapidly when venom was applied in HC-3 and there was a significant reduction in the total amount of ACh released during an hour. In four experiments in which diaphragms with ribs were used, the reduction in the amount of ACh released was 44 ± 31 pmol/hemidiaphragm (35%), and in four experiments in which diaphragms without ribs were used, the reduction was 30 ± 19 pmol/hemidiaphragm (44%). Some synthesis of ACh seems to occur during venom action, suggesting that venom does not block synthesis immediately. Hence, it is possible that the HC-3-like effects of venom on ACh content and release arise primarily as a result of the loss of vesicles and are not due to a direct inhibitory effect of venom on ACh synthesis.

Baba et al. (1) have reported that venom is a powerful inhibitor of high-affinity choline transport in synaptosomes from rat brain, and this suggests that venom may have direct HC-3-like effects on ACh metabolism. However, they observed complete inhibition of choline transport only after synaptosomes had been treated with venom for 10 min, whereas ACh release was stimulated within 20 s and was 90% complete after 15 min. Hence, their results also are compatible with the view that the apparent inhibitory effects of venom on ACh metabolism develop after, and are consequences of, the loss of vesicles.

Our results indicate that venom-treated diaphragms are depleted of vesicles, secrete few

[2] Gorio, A., and A. Mauro. 1978. Reversibility and mode of action of black widow spider venom on vertebrate neuromuscular junction. Manuscript submitted for publication.

quanta of ACh and cannot increase their rates of ACh release when K is applied. However, ACh continues to be released from an extravesicular pool at 62–90% of the normal rate. Because venom and K applied in the bath have access to all structures in the diaphragm, the location of the extravescular pool is unclear. We thought that information on the location of the extravesicular pool might be furnished by experiments in which ACh release was evoked by electrical stimulation of the phrenic nerve.

Effects of Electrical Stimulation of the Phrenic Nerve

ELECTROPHYSIOLOGY: The amplitude of the compound action potentials of muscles bathed in eserinized Krebs' decreased by over 90% during the first few minutes of stimulation at 10/s. Asynchronous twitching of the fibers continued for about half an hour and then the muscle became quiet. When stimulation was stopped after an hour, the compound action potential recovered in a few minutes. When HC-3 was present, the compound action potential fell quickly and all twitching ceased within 10 min. When stimulation was stopped after an hour, no recovery occurred during 15 min of rest.

RELEASE OF ACH: When the phrenic nerve was stimulated, the rate of release of ACh was about doubled and was maintained constant for at least an hour (Fig. 10). When venom was applied after 15 min of rest, the usual release of ACh was obtained. When the stimulation was carried out in a solution with 30 μM HC-3, the rate of release of ACh first increased and then declined so that after an hour the rate was less than the original resting rate (Table II). When venom was applied after 15 min of rest in HC-3, there was only a small additional release of ACh. Much of the residual ACh that was released by venom in these experiments could have come from unstimulated nerve terminals in the intercostal muscles.

These results strongly suggest that stimulation of the nerve in the presence of HC-3 depletes a large part of the pool of ACh normally depleted by venom and also depletes the extravesicular pool responsible for resting release. Hence, the extravesicular pool seems to be located in the nerve terminals.

The average ACh contents of hemidiaphragms stimulated in 30 μM HC-3 for 1 h at 10/s was 54 ± 30 pmol (n = 7), which is slightly more than

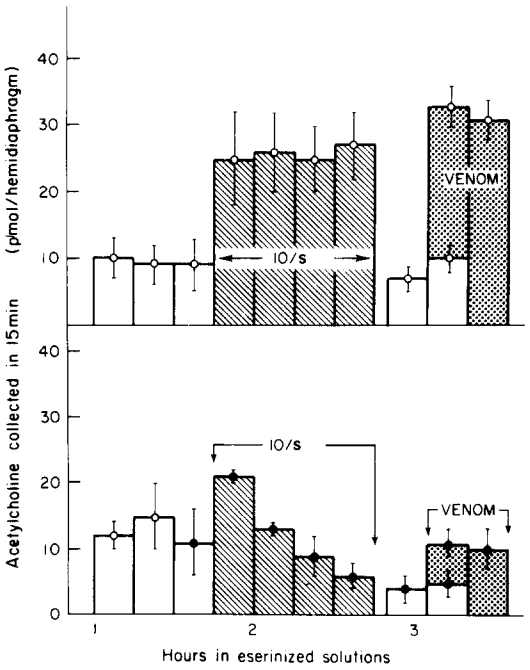


FIGURE 10 Effect of stimulation of the phrenic nerve on the venom-induced secretion of ACh. Ordinates and abscissae as in Fig. 9. All preparations with ribs. Mean results of five experiments ± SD. Upper graph: control experiments (○). After three collections from resting preparations in standard Krebs', the phrenic nerve was stimulated for an hour at 10/s. Then the preparations were washed for ~5 min, and one resting sample was collected. Venom (10 μl/ml) then was applied to three of the preparations. Lower graph: results with 30 μM HC-3 (●). After two resting collections in normal Krebs', HC-3 was added and one resting collection was obtained. Then the phrenic nerve was stimulated for an hour at 10/s. After stimulation was stopped, the preparations were washed with HC-3 for 5 min, and a resting collection in HC-3 was obtained. Venom in HC-3 was then applied to three of the preparations. Data not corrected for hydrolysis.

the ACh contents of diaphragms treated with venom, or with K and HC-3. The great variability of the ACh contents of these stimulated diaphragms precludes any detailed comparison with the ACh contents of diaphragms treated with venom or K and HC-3.

RELEASE OF ACH FROM RESTING DIAPHRAGMS: Table II summarizes the effects of various means of stimulation on the release of ACh from resting diaphragms. This table includes data from all the diaphragms used in this investigation and includes more preparations than were

used to prepare the figures. Electrical stimulation of the phrenic nerve, or treatment of the diaphragms with 25 mM K, caused significant reductions in the resting rate of release of ACh only when HC-3 was present, and, then the rate was reduced by 70–75%. When venom was applied in the absence of HC-3 the final resting rate of release of ACh was reduced by 10–40%, and when venom was applied in the presence of HC-3 the final resting rate of release was reduced by 39–45%.

BALANCE SHEET: If HC-3 completely inhibits ACh synthesis in stimulated diaphragms, then the amounts of ACh released to the bath from these diaphragms should equal the decrease in the ACh content of the tissue. Since the total amount of ACh released to the bath may include a small contribution from extra-neural compartments, we corrected our release data for the residual rates of release (Table II) that occurred from these preparations. The corrected quantities were equal for venom-treated, K-treated, and electrically stimulated preparations, and the average amount collected was 35 ± 13 pmol/hemidiaphragm (n = 16). When this figure is corrected for the hydrolysis that occurred at the concentration of eserine we used (Table I), then the average amount of ACh secreted to the bath is 61 ± 23 pmol/hemidiaphragm. This is 88% of the change in tissue content. The difference, 8 ± 31 pmol (n = 15), is not statistically significant, so that an approximate balance is obtained indicating that our measurements are not in serious error.

DISCUSSION

Our results show that there are two major stores of ACh in mouse diaphragm: one that is depleted by venom or by stimulation in HC-3, and another that is not depleted by these procedures. The depletable store contains about 65% of the ACh in the diaphragm, and release from this store accounts for 70–75% of the ACh released at rest. This store is located mainly in the nerve terminals, since it can be depleted by indirect electrical stimulation in HC-3, and it contains the ACh that participates directly in neuromuscular transmission.

The nondepletable store contains about 35% of the ACh in the diaphragm, and release from this store may account for 25–30% of the total resting release. We don't know the location of this store. Much of it may correspond to the store of ACh that remains in denervated muscle (2, 17, 24, 28) and it may include, in addition, residual ACh in the depleted terminals plus ACh in the axons and Schwann cells of the intramuscular branches of the phrenic nerve.

Most of the ACh released from the depletable store at rest is not secreted as quanta and is derived from an extravesicular pool. Our estimates of the size of this pool range from <10%, to about 50%, of the depletable store. Most of the ACh released from stimulated diaphragms is secreted as quanta, and the vesicles seem to be required for this secretion. The initial high rates of ACh release induced by venom subside as the vesicles are depleted, and vesicle-depleted terminals do not release quanta of ACh and do not respond to added K. Vesicle-depleted diaphragms continue to secrete extravesicular ACh, however, and they presumably synthesize the ACh required for this release. These considerations support the ideas (*a*) that quanta account for all of the increment in the rate of ACh release induced by stimulation, and (*b*) that the quanta are released from the vesicles after they have fused with the axolemma. However, we cannot exclude the possibility that stimulation may also approximately double the rate of release of extravesicular ACh. Table III summarizes our findings on the sizes of the various ACh compartments in mouse dia-

TABLE III

Release of ACh from Various Compartments in Mouse Diaphragms

Conditions	Compartment			
	Total	Vesicular	Extravesicular	Nondepletable*
	Content (pmol/hemidiaphragm)			
Normal	107	62–35	7–35	38
After K + HC-3	38	0	0	38
After venom	45	0	7	38
	Rate of Release (pmol/hemidiaphragm × 15 min)			
Rest	11	0.4	8	3
During K	51	40–25	8–23	3
After K + HC-3	3	0	0	3
After venom	9	0	6	3

* These figures are the data from diaphragms treated with K + HC-3. They may overestimate the size of the non-depletable pool, and the flux from this pool, since it is possible that K + HC-3 did not totally exhaust the depletable stores of ACh.

phragms and on the rates of release of ACh from these compartments under various conditions.

There are two unexplained features of our data: (*a*) why HC-3 seems to have little effect on ACh synthesis in resting diaphragms (even though there is an appreciable turnover of ACh), whereas it has powerful inhibitory effects on stimulated diaphragms; (*b*) why stimulation in HC-3 causes the depletion of extravesicular ACh if stimulation does not increase directly the release of ACh from the extravesicular pool.

Our data indicate that ACh turnover in resting nerve terminals occurs mainly in the extravesicular pool. The synthesis of ACh for this pool presumably occurs throughout the cytoplasm, and the choline required for this synthesis may come from internal stores since the turnover of ACh seems not to require choline in the bath. HC-3 may inhibit ACh synthesis by blocking choline transport from the extracellular space. Hence, ACh synthesis at rest may not be inhibited by HC-3 either because HC-3 does not have access to the sites of synthesis or because the synthesis is not dependent on extracellular choline. In stimulated diaphragms, however, newly synthesized ACh is preferentially released (9), perhaps because it is used to reload recycled vesicles (6, 7, 18, 37). This synthesis may occur only in highly restricted regions of the terminal immediately adjacent to the sites of ACh release (10) and may be critically dependent on choline supplied by a high-affinity transport system (15). Thus, stimulated preparations may be more sensitive to extracellular HC-3 either because the drug has access to the sites of synthesis or because the synthesis is more dependent on the high-affinity choline transport system.

ACh could be depleted from the extravesicular pool, without being released directly from this pool, if ACh could be exchanged between the extravesicular pool and the vesicles. Numerous biochemical experiments indicate that in stimulated preparations the exchange of ACh between the two pools is slow relative to the rates of synthesis and release (9, 10, 37), so that the two pools appear to turn over independently. However, in the intact tissues, a slow exchange may occur which is revealed only when ACh synthesis is inhibited and the stores are drained to exhaustion, as in our experiments.

In conclusion, our results indicate that most of the ACh released from resting nerve terminals comes from an extravesicular pool and is not quantal in nature. The increase in the rate of release of ACh that occurs upon stimulation by the various procedures may be due entirely to the release of quanta derived from the vesicles. When ACh metabolism is inhibited by HC-3, the extravesicular pool is depleted because ACh is redistributed between the two pools and released from the vesicles.

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