EFFECT OF ARMIN ON THE ULTRASTRUCTURE OF THE NEUROMUSCULAR SYNAPSE

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Changes in the ultrastructure of motor endings under the influence of the cholinesterase inhibitor armin† ($5 \cdot 10^{-7}$ g/ml) at rest and during electrical stimulation of the nerve were studied in preparations of the rat phrenic nerve and diaphragm. At rest, armin was found to cause ultrastructural disturbances of the endings similar to those arising in the control during nerve stimulation. Electrical stimulation in the presence of armin caused no further changes in the ultrastructure of the neuromuscular junction. The results indicate the important role of disturbances of the function of the presynaptic apparatus in the mechanisms of the blocking action of armin on neuromuscular conduction.

KEY WORDS: neuromuscular synapses; cholinesterase inhibitors; ultrastructure.

In the modern view the blocking action of anticholinesterase drugs on the neuromuscular apparatus is based on acetylcholine accumulation in the region of the postsynaptic receptors because of inhibition of cholinesterase activity. Meanwhile armin and certain other cholinesterase inhibitors have a presynaptic action [1, 2, 4], although the mechanism of that action is not clear. Data in the literature on ultrastructural changes in neuromuscular synapses under the influence of cholinesterase inhibitors are relatively scanty. There are virtually no data on ultrastructural disturbances in experiments on isolated organs, which would allow the factors influencing the functional state of the synaptic apparatus to be strictly controlled.

With these facts in mind, in the investigation described below the ultrastructure of the neuromuscular junction was studied under the influence of armin in a resting state and during functional loading, namely electrical stimulation of the motor nerve *in vitro*.

EXPERIMENTAL METHOD

Preparations of the phrenic nerve and diaphragm of rats were used. The animals were killed by a sharp blow on the head. The neuromuscular preparations was isolated by Bülbring's method [5] and placed in a special bath containing aerated Liley's solution (pH 7.4, 37°C, 30 min). One half (experimental) of the phrenic nerve–diaphragm preparation was then subjected to indirect electrical stimulation with square pulses (5 Hz, 0.05 msec, 3 V, 10 min). Armin in concentration of $5 \cdot 10^{-7}$ g/ml (the minimal concentration completely inhibiting cholinesterase activity during incubation for 8 min) was added to the bath immediately before the beginning of stimulation. Preparations incubated under the same conditions (with and without stimulation), but without the addition of armin, served as the control.

For electron-microscopic investigation, pieces of tissue containing the largest number of neuromuscular junctions (regions of branching of the phrenic nerve) were quickly excised from the control and experimental preparations of diaphragm under the MBS-2 microscope and fixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, with the addition of 0.2 M sucrose for 1.5 h at 4°C. After dehydration, the material was embedded in Araldite. To identify regions with the largest number of neuromuscular junctions semi-thin sections were cut to a thickness of 1-2 μ, stained with toluidine blue, and examined under the light microscope. Ultrathin

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TABLE 1. Changes in Ultrastructure of Neuromuscular Synapse under the Influence of Armin ($5 \cdot 10^{-7}$ g/ml) at Rest and during Electrical Stimulation (M±m)

Feature	Conditions of testing	Control	Experiment (armin)
SV (per square micron)	At rest	91,0±8,1	57,5±5,4‡
	Electrical stimulation	51,0±5,3	35,8±3,7*
	P	<0,01	<0,01
Coated SV (per square micron)	At rest	0,82±0,12	1,88±0,25†
	Electrical stimulation	1,99±0,29	2,27±0,28
	P	<0,01	>0,05
Swelling of mitochondria	At rest	0,50±0,12	0,66±0,12
	Electrical stimulation	0,63±0,13	0,50±0,08
	P	>0,05	>0,05
Redistribution of mitochondria	At rest	0,56±0,09	1,81±0,17‡
	Electrical stimulation	0,86±0,10	1,80±0,12
	P	<0,05	>0,05
Membranous structures	At rest	0,69±0,16	1,35±0,16†
	Electrical stimulation	1,09±0,15	1,20±0,12
	P	>0,05	>0,05
Disturbance of integrity of presynaptic membrane	At rest	0,50±0,11	1,50±0,17‡
	Electrical stimulation	0,35±0,09	1,76±0,16
	P	>0,05	>0,05
Changes in postsynaptic region	At rest	0	1,22±0,18‡
	Electrical stimulation	0,18±0,12	0,87±0,28
	P	>0,05	>0,05
Detectability of neurofibrils	At rest	0,42±0,15	0,43±0,11
	Electrical stimulation	0,96±0,17	0,71±0,14
	P	<0,05	>0,05

*P<0.05 compared with control.
†P<0.01.
‡P<0.001.

sections were stained with uranyl and lead salts and examined in the JEM-100B electron microscope. The number of synaptic vesicles (SV) on the whole area of the ultrathin section through the nerve ending was counted on photographic prints and the number of SV per unit area calculated [15]. In addition, the state of the organelles both of the presynaptic region and of the region of the muscle fiber immediately adjacent to the postsynaptic membranes was assessed on these same photographs. The assessment was made in accordance with a 5-point system: 0) absence of the given feature, 0.5 point) feature indistinctly present, 1 point) clearly visible, 2 points) prominent, and 3 points) very prominent. The numerical data thus obtained formed a variance series of the particular feature for each experimental condition. At least 20 neuromuscular synapses from at least three preparations were studied with respect to each feature. Statistical analysis was carried out by Student's method.

EXPERIMENTAL RESULTS

The statistical analysis yielded the results shown in Table 1. They show that electrical stimulation of the phrenic nerve of the control preparations caused a statistically significant decrease in the number of SV compared with the control without stimulation and an increase in the number of so-called coated vesicles, redistribution of the mitochondria (their displacement from the center of the terminal nearer to the presynaptic membrane), and an increase in the detectability of the neurofibrils (Table 1, Fig. 1).

Incubation of the neuromuscular preparations in armin solution without electrical stimulation of the nerve also led to a significant decrease in the number of SV, an increase in the number of coated SV, and redistribution of mitochondria. These changes were statistically significant (Table 1). However, by contrast with stimulation in the control, armin caused a significant increase in the number of membranous structures of varied profile observable in the cytoplasm of the nerve endings and an increase in the area of presynaptic membrane with its integrity disturbed. On the electron micrograph this was shown by an irregular interrputed line; the detectability of the neurofibrils remained at the control level (Table 1, Fig. 2A). Ultrastructural changes also were found in the postsynaptic region of the muscle fiber, in the form of swelling of the mitochondria or the appearance of myelin figures and lipid droplets connected with the mitochondria, and also on vacuoles in the region of the sarcoplasmic reticulum, and disorganization of myofibrils (Fig. 2B).

During electrical stimulation in the presence of armin, the number of SV was significant-

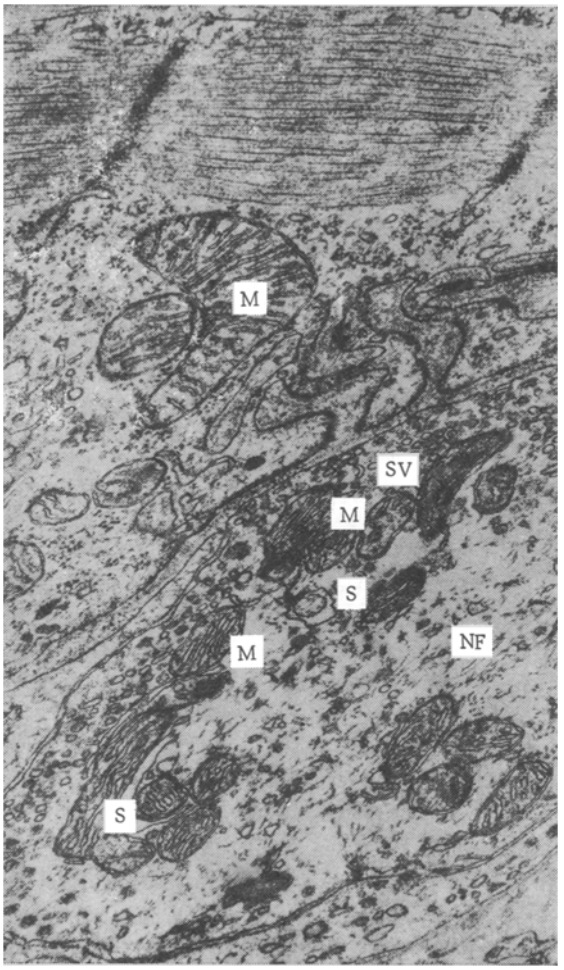

Fig. 1. Neuromuscular synapse of control phrenic nerve–diaphragm preparation 10 min after electrical stimulation of nerve. Number of SV reduced; mitochondria (M) located near synaptic space; numerous neurofibrils (NF); S) membranous structures. 27,000×.

ly reduced compared with the action of armin at rest or stimulation in the control. According to the remaining features the electrical stimulation in the presence of armin caused no additional significant changes in synapse ultrastructure (Table 1, Fig. 2B).

The decrease in the number of SV and simultaneous increase in the number of coated SV after electrical stimulation of the motor nerve were observed previously by other workers in experiments on neuromuscular preparations [6, 9, 16]. Such changes are nowadays regarded as being "functionally dependent" [16], and due to liberation of the transmitter from SV by exocytosis, followed by repair of the membrane of SV from the plasma membrane through the intervention of coated SV, which are the connecting link in this mechanism of the membrane cycle [6]. Displacement of the mitochondria nearer to the presynaptic membranes is evidently connected with the supplying of energy for this process.

Some workers have noted swelling of the mitochondria and disturbance of the integrity of the presynaptic membrane (indistinctness, blurring, and apparent interruption of its contours) during electrical stimulation [6]. In the present experiments no significant differences were found with respect to these features between the control at rest and during electrical stimulation (Table 1).

The results of the present investigation showed that incubation of unstimulated preparations in armin solution and electrical stimulation in the control led to similar changes in the number of ordinary and coated SV. However, unlike stimulation, armin caused an increase

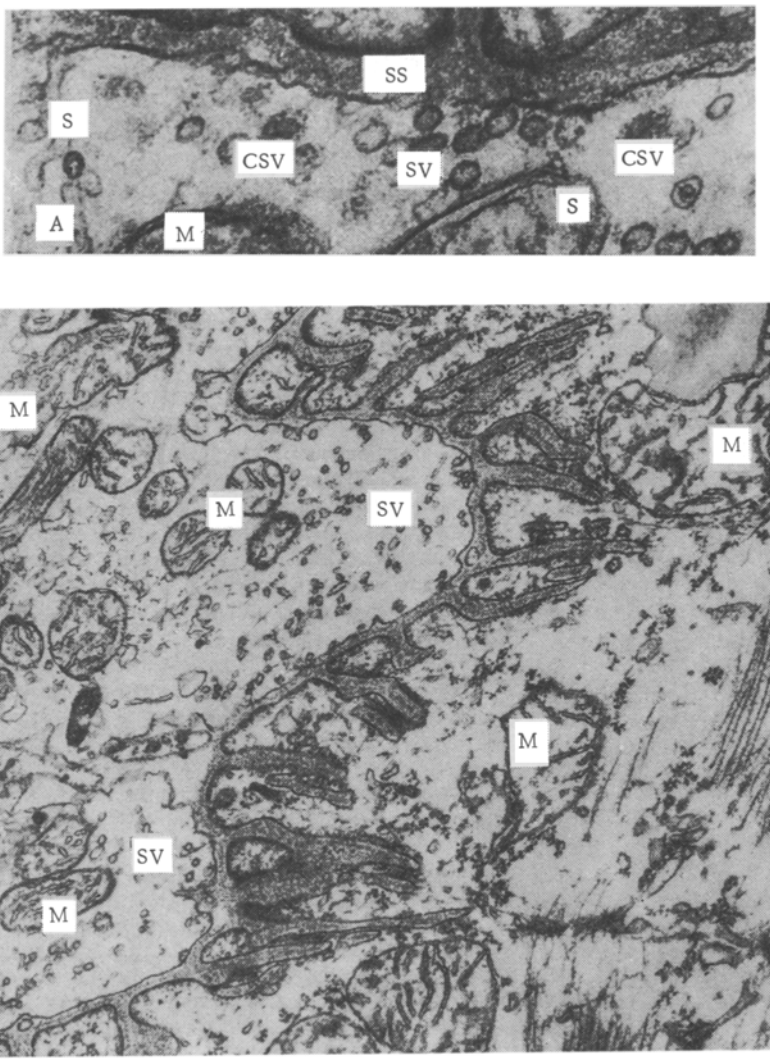

Fig. 2. Neuromuscular synapse of diaphragm preparation. A) Incubation in armin solution at rest. Discontinuity and irregularity of contour of presynaptic membrane; CSV) coated synaptic vesicles; SS) synaptic space (85,700×); B) incubation in armin solution during electrical stimulation. Few SV present; M of muscle fiber swollen; disorganization and partial lysis of myofibrils (MF). Remainder of legend as in Fig. 1 (25,700×).

in the number of membranous structures of varied profile in the cytoplasm of the nerve ending, an increase in the area of presynaptic membrane with disturbance of the integrity, and also changes in the ultrastructure of the postsynaptic region. According to many invesigators, these membranous structures together with coated SV are intermediate links in the mechanism of the membrane cycle [6]. Some workers interpret the appearance of both thse structures as a sign of an increased choline demand for the intra-axonal synthesis of acetylcholine [16].

The increase in the area of changes in the electron-microscopic picture of the presynaptic membrane (distrubance of integrity) is perhaps connected with a disturbance of phospholipid metabolism under the conditions of choline deficiency due to inhibition of acetylcholine hydrolysis by cholinesterase. Damage to the organelles of the nerve ending under the conditions of choline deficiency has been observed in experiments *in vitro* on the superior cervical ganglion [13].

Changes in the ultrastructure of the postsynaptic region similar to those now observed were described previously in the motor end plates of the rat diaphragm after intraperitoneal injection of organophosphorus compounds into the animals [10-12, 14]. The authors cited connect these disturbances with changes taking place in these terminals.

Incubation of phrenic nerve–diaphragm preparations in armin thus leads to changes in the ultrastructure of the nerve endings basically similar to those found during electrical stimulation in the control. Since under normal conditions the only cause of an increase in functional activity of an ending during electrical stimulation of the nerve is depolarization of its membrane by spreading action potentials, the changes in the ultrastructure of the terminal under the influence of armin at rest may be evidence of depolarization of the membrane of the nerve ending in the course of cholinesterase inhibition: The accumulating acetylcholine may depolarize the terminals and so block conduction of action potentials [7].

Another factor leading to depolarization of terminals is an increase in the extracellular potassium concentration during activation of muscle [3] and glial [18] cells by stabilized acetylcholine.

The fact that electrical stimulation in the presence of armin caused no additional changes in synapse ultrastructure, i.e., stimulation in its presence was ineffective (except for a small decrease in the number of SV), may perhaps indicate that armin depresses conduction of action potentials in motor nerve terminals, probably through the mechanism of depolarization.

The writers showed previously [1] that secretion of mediator induced by electrical stimulation of a nerve is depressed by armin. This fact is supported by electrophysiological evidence of depression of the quantum composition of the endplate potential under the influence of armin [2] and other organophosphorus cholinesterase inhibitors [10, 17].

Disturbances of the ultrastructure of the postsynaptic region under the influence of armin at rest may be connected with nonfunctional leakage of mediator from the cytoplasm of the endings [1, 8].

The results are evidence of the important role of presynaptic disturbances in the mechanism of the blocking action of armin on neuromuscular conduction.

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