ULTRASTRUCTURAL CHANGES IN THE NEUROSECRETORY APPARATUS OF THE MYONEURAL JUNCTION ASSOCIATED WITH FUNCTION

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In experiments on albino rats the diaphragm muscle was frozen in a resting state and also during brief (1, 10 sec) repetitive (50 Hz) indirect stimulation, after which it was fixed in formaldehyde and osmium tetroxide for electron-microscopic study of the myoneural junctions. The technique used gave reasonably adequate presentation of the structural elements of the myoneural junction and it revealed some distinctive features of the localization of the synaptic vesicles: an even distribution along the axon terminal in a resting state and a decrease in the number and redistribution of the vesicles during stimulation. The most significant difference was the discovery of vesicles in the synaptic space.

It is only in recent years that investigations of the ultrastructure of the myoneural junction have shown that the changes arising in the neurosecretory apparatus of the axon terminal in various functional states, induced by stimulation of the nerve or by factors acting on the presynaptic membrane (a direct current, the ion concentration, and so on) are concerned predominantly with the number, distribution, and size of the synaptic vesicles [2, 5-7]. At the same time, the changes observed by no means always correlated with the physiological assessment of the state of the presynaptic structures [4]. One reason for this disagreement could be the inadequacy of the usual methods of fixing material in functional and morphological investigations of synapses. For example, electrophysiological investigation of fixation of the myoneural junction with formaldehyde has shown that under these circumstances there is a powerful (several thousand quanta) and asynchronous discharge of mediator [1]. In the investigation described below, the method of preliminary feeding of the tissue was used to study ultrastructural changes in the neurosecretory apparatus of the myoneural junction during its function.

EXPERIMENTAL METHOD

Myoneural junctions in the diaphragm of August rats weighing 100-120 g were anesthetized with ether and part of the diaphragm in the synaptic zone was frozen in situ through an incision in the abdominal wall by applying a large copper cylinder with one end shaped into a truncated cone, previously cooled in liquid nitrogen. The frozen piece of tissue was quickly excised and placed in formol-sucrose solution, cooled to between 0 and −2°C, and then fixed with osmium tetroxide and embedded in araldite. Sections were examined in the JEM-7A electron microscope. The diaphragm was frozen a short time (20 min) after unilateral division of the phrenic nerve in the neck and also 1 and 10 sec after the beginning of stimulation of the peripheral segment of the divided nerve at a frequency of 50 Hz.

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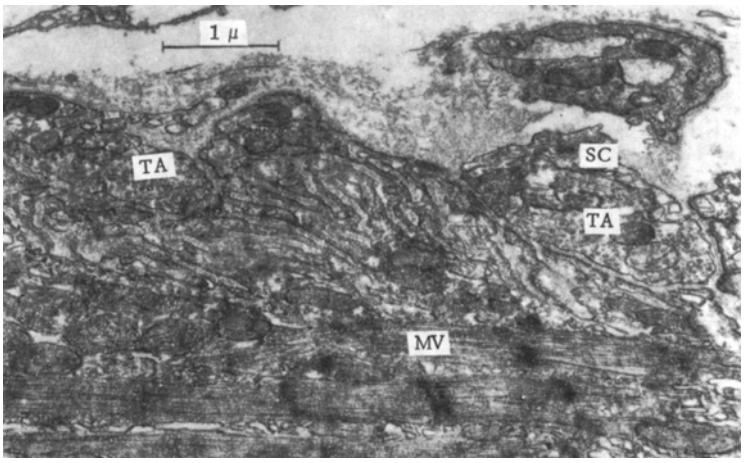

Fig. 1. Myoneural junction from rat diaphragm frozen during indirect repetitive stimulation. TA) axon terminal; SC) process of Schwann cell; MF) muscle fiber.

EXPERIMENTAL RESULTS

The method of fixation used preserves the main features of structure of the myoneural junction described when ordinary methods are used (Fig. 1). No significant damage was found to its ultrastructural elements, and destructive changes were observed only in some of the axoplasmic mitochondria.

Synaptic vesicles at rest were distributed all along the axon terminal uniformly, especially between the mitochondria (Fig. 2a).

During stimulation of the nerve the number of synaptic vesicles in some of the terminals was reduced, in some cases considerably. Redistribution of the vesicles also was found: the remaining vesicles were arranged along the presynaptic membranes (Fig. 2b). It is interesting to note that terminals with different numbers of vesicles could be observed even in the same motor end-plate.

A constant difference between the terminals of the working and resting muscle was a considerable change in the number of complex vesicles. After stimulation their number was considerably increased, especially in terminals in which the number of synaptic vesicles was reduced (Fig. 2b).

The most significant feature distinguishing myoneural junctions fixed by the preliminary freezing method was that vesicular structures corresponding in size to vesicles in the terminals were found in the synaptic space both of the resting and of the stimulated synapses (Fig. 3). Some of them had normal electron density and a distinct three-layered membrane. At the same time other vesicles were found whose membrane was only partially preserved or was very indistinct. Furthermore, circular structures in which no membrane could be distinguished were found in the synaptic space. As a rule vesicles with a clearly defined membrane lay near to the presynaptic membrane. The patterns of interaction between vesicles and presynaptic membrane observed in these cases (Fig. 3) can be interpreted as a process of extrusion of the vesicles into the synaptic space. Indistinct vesicular structures were found close to the postsynaptic membrane, sometimes in a position suggesting interaction with that structure (Fig. 3).

Vesicles in the synaptic space were found in approximately equal numbers in different sections through the same terminal, from which it could be concluded that they are more or less uniformly distributed there. The number of distinct vesicles in the synaptic space varied from 1 to 8 per axon terminal outlined. Comparison of the number of vesicles in the synaptic space of the myoneural junctions frozen in a resting state and during stimulation of the nerve revealed no significant difference. An attempt was made to find a difference between the number of vesicles interacting with the presynaptic membranes. However, in this case also counting gave inconstant results in the different terminals and no obvious difference could be found.

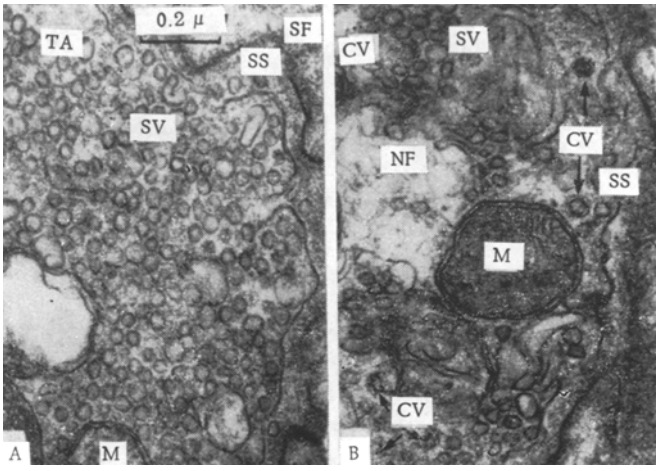

Fig. 2. State of neurosecretory structures at rest (A) and during stimulation of phrenic nerve (B). TA) axon terminals; SV) synaptic vesicles; CV) complex vesicles; M) mitochondria; SS) synaptic space; NF) neurofibrils; SF) synaptic folds.

The method of preliminary freezing thus revealed fresh details of the ultrastructural state of the myoneural junction associated with its function. First of all, the results indicate that different functional states of the synapse are characterized by different localization of the synaptic vesicles in the axoplasm of the terminal.

As has already been mentioned, one new result of great fundamental importance obtained by the method of preliminary freezing was the discovery of vesicles in the synaptic space. It is particularly difficult to explain their presence in the synaptic space of resting synapses. If the synaptic vesicles immediately join the receptor membrane during spontaneous secretion from the terminal, the probability of their discovery in an ultrathin section when the frequency of spontaneous secretion was 4-10 pulses/sec would be extremely small. Their presence in the synaptic space in relatively large numbers suggests that in the resting synapse vesicles do not all join the postsynaptic membrane, nor do they do so immediately, and it is therefore necessary to make a fresh evaluation of the role of the synaptic space in synaptic processes. Usually in descriptions of the conduction of excitation from nerve to muscle the liberation of mediator by some means or other is considered to be practically equivalent to its binding with the subsynaptic membrane. Yet, so far as the myoneural junction is concerned, even if the binding takes place on the other side of the space, the mediator must cover a distance equal to twice the diameter of the synaptic vesicles. If cholinergic reception takes place in the synaptic folds, this distance is increased many times over.

The reserves of mediator, estimated from functional tests, can be divided into two fractions: "readily accessible" (the small fraction), which is replenished from the much larger "not easily accessible" fraction. It has been suggested that the "easily accessible" mediator is contained in the synaptic vesicles in direct contact with the presynaptic membrane in the axon terminal [3]. However, counting the vesicles in this zone has shown that factors which increase the "readily accessible" reserves reduce the number of vesicles in this zone, while factors acting in the opposite manner increased the number of vesicles [4].

It may be assumed that vesicles lying in the synaptic space also contain this "easily accessible" supply of mediator, for on the basis of these experimental results even the roughest calculation shows that they must number several hundreds per terminal and 1000-2000 per motor end-plate. It can also be assumed that the number of vesicles in the synaptic space is continually being replenished by secretion through the presynaptic membrane, and that collision between these vesicles and the postsynaptic membrane leads to the spontaneous generation of miniature end-plate potentials. The arrival of a spike in a terminal leads to the appearance of ionic currents and to a change in the ionic situation on both sides of the presynaptic membrane. The electric field generated in the synaptic space synchronizes the movements of the vesicles or parts of them lying in it toward the postsynaptic membrane. The possibility likewise cannot

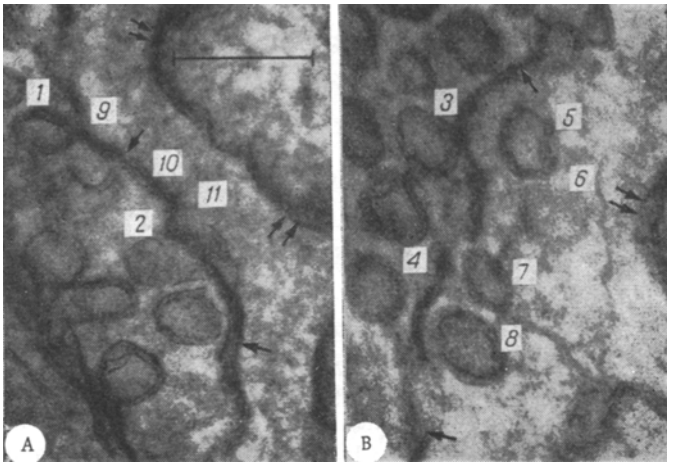

Fig. 3. Different localization of vesicular structures (1-11) in the region of synaptic contact: 1-4) interaction with presynaptic membrane; 5-8) in synaptic space; 9-11) hypothetical interaction with postsynaptic membrane. Arrow indicates presynaptic membrane, two arrows indicate postsynaptic membrane.

be ruled out that under these circumstances the state of the substance in the space, a polysaccharide gel, also is charged. Interaction between the mediator and the postsynaptic membrane and the ionic currents generated stop the movements of the vesicles in this area. The spike arriving along the nerve must also activate the release of synaptic vesicles into the synaptic space, as is shown by a decrease in their number in some terminals of the stimulated synapses. This last phenomenon shows that the terminals entering a motor end-plate may liberate mediator alternately, although the mechanism of organization of this alternation is not clear, especially if it is considered that the spike reaches all terminals forming a given end-plate at the same time.

This hypothesis explains the discovery of vesicles in the synaptic space after neurosecretion has been rapidly blocked by preliminary freezing of the tissue (although the possibility cannot be completely ruled out that the method used may itself cause displacement of the vesicles.) It also to some extent eliminates, as has already been mentioned, the contradictory findings obtained in experiments conducted with the ordinary methods of fixation, which evidently activate interaction between vesicles in the synaptic space and the postsynaptic membrane. In that case the decrease in the number of synaptic vesicles in the zone of the axon terminal near the presynaptic membrane, after the action of factors which are shown by function tests to increase the "easily accessible" reserve [4], can be explained by the extrusion of some of these vesicles from the terminal into the synaptic space. Conversely, accumulation of vesicles in the presynaptic zone as a result of procedures reducing this reserve can be induced by delaying the expulsion of synaptic vesicles from the axon terminal.

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