INCIDENTAL STRUCTURAL AND FUNCTIONAL EFFECTS OF EXPERIMENTAL PROCEDURES USED TO STUDY TRANSMITTER RELEASE MECHANISMS

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The discovery of structural-functional correlations at the cellular level is one of the most fruitful methods in cell physiology and has contributed much to our understanding of the fine mechanisms of secretion of synaptic transmitters and, in particular, to the elucidation of the quantum-vesicular nature of its exocytosis. It will be clear that this situation calls for a particularly careful evaluation of the effect of the experimental procedures on the results. Detubulation of a muscle, which disturbs electrical-mechanical coupling, facilities intracellular recording of electrical responses to its direct stimulation, and it is therefore widely used to study synaptic processes. The statistical characteristics of transmitter secretion after this procedure remain substantially unchanged, but some anomalies of shape of postsynaptic potentials in neuromuscular junctions of detubulated preparations [4] can be explained by disturbance of their ultrastructure, similar to what is observed with an increase in the osmotic gradient [3]. As regards the use of such a popular fixative as formaldehyde, it is to be expected that if one source of extracellular calcium is blocked by detubulation, its familiar functional effect will also be changed [1].

The aim of this investigation was to study incidental effects of experimental procedures used to abolish mechanical displacement of the muscle (detubulation) and its prefixation (formaldehyde) for electron microscopic study.

EXPERIMENTAL METHOD

Experiments were carried out on August rats weighing 100-120 g. An isolated preparation of the diaphragm (phrenic nerve + a strip of diaphragm muscle) was placed in a chamber through which flowed carbogenized Tyrode solution (20°C). This was later replaced by Tyrode solution with glycerol (400 mM) for 40 min, after which the preparation was rinsed for a sufficiently long time with the original Tyrode solution. Material for ultrastructural investigation was prefixed in the chamber by addition of neutral formalin to give a final formaldehyde concentration in the rinsing solution of 4%. The material was then postfixed in 1% buffered OsO_4 solution and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate by Reynolds' method and examined in the JEM-7A electron microscope. The preparation was fixed at different stages of the detubulation procedure: after 35 min in medium with glycerol, at the beginning of rinsing of the preparation with glycerol-free Tyrode solution (40 min), and 80 and 170 min after the beginning of the procedure. Strips of the other half of the cupola of the diaphragm muscle, kept in the original solution for as long as in the corresponding experiment, served as the controls. The effect of the various procedures (addition of glycerol and of formaldehyde) on transmitter secretion was judged by recording intracellular miniatrue end-plate potentials (MEPPs) with a glass electrode (2 M KCl) by the usual method. For morphometric analysis of the electron micrographs, a semiautomatic "Leitz-ASM" image analysis system (West Germany) was used. The mean values of the following parameters were determined: area of axon terminal, area occupied by vesicles relative to free area of terminal, volume of synaptic vesicles, and number of vesicles in a cross section of an axon terminal.

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TABLE 1. Morphometric Parameters of Neuromuscular Synapse during and after Detubulation of Muscle (M ± m)

Time after beginning of procedure, min	Area of axon terminal, $nm^2 \cdot 10^6$	Volume of synaptic vesicles, $nm^3 \cdot 10^8$	Concn. of vesicles, %	Mean number of vesicles in cross-sect. of terminal
Control	1,0±0,15	22,7±1,1	17,0±1,85	217
35	2,6±0,26	23,8±0,9	7,3±0,96	150
40	2,0±0,12	22,6±0,9	9,1±1,06	140
80	1,7±0,18	28,7±1,0	9,8±0,49	128
170	1,1±0,23	20,7±0,9	23,0±3,57	231

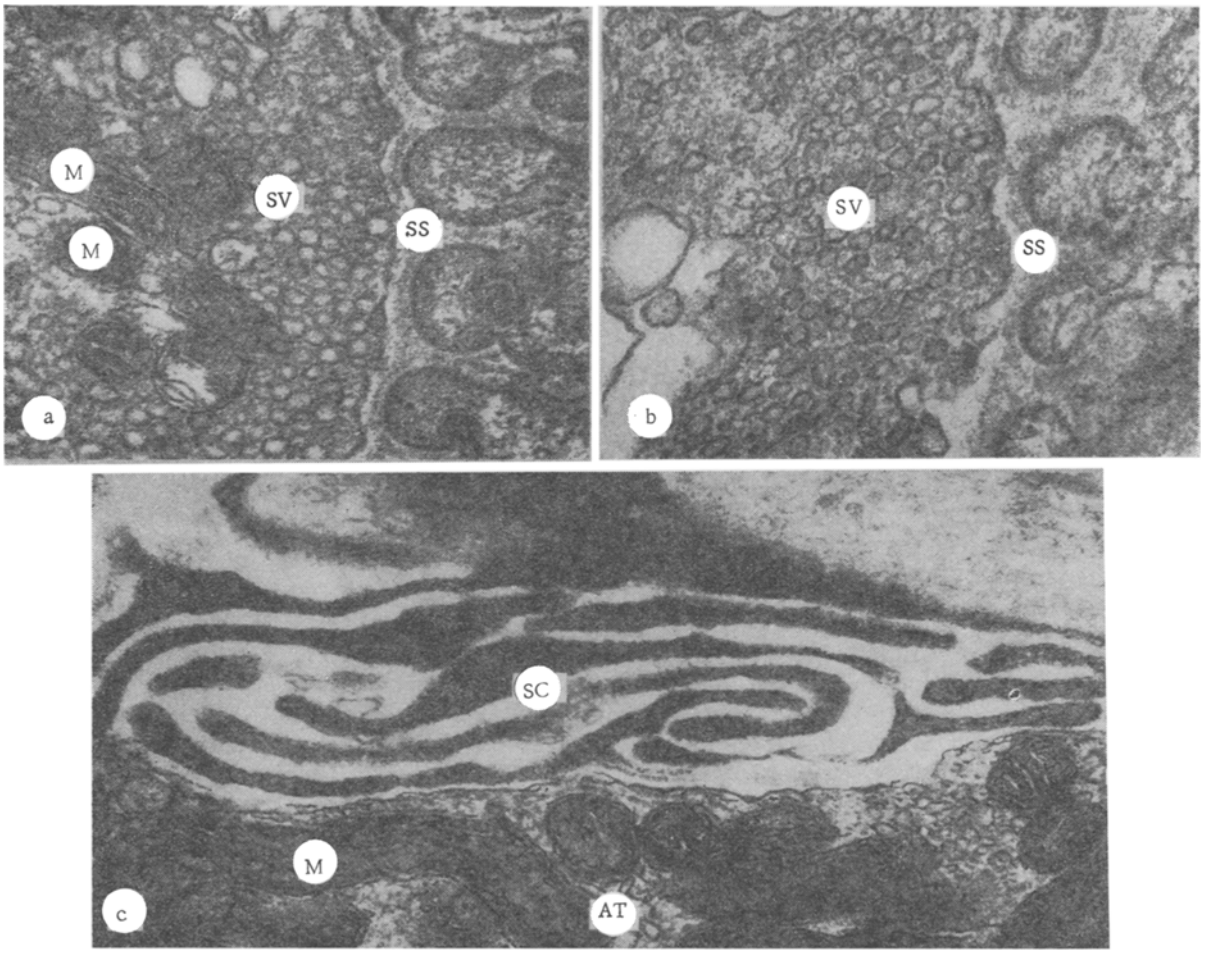

Fig. 1. Electron micrographs of neuromuscular junction at different stages of the experiment (80,000×): a) control; b) end of "rinsing" (80 min after beginning of experiment); c) 170 min after beginning of experiment. M) Mitochondrion; SV) synaptic vesicles; SS) synaptic space; AT) axon terminal, SC) Schwann cell.

EXPERIMENTAL RESULTS

A study of control neuromuscular synapses showed no significant changes in their ultrastructure at all times of their stay in the chamber. Axon terminals were uniformly packed with synaptic vesicles, vacuoles of different sizes and shapes were found in them, and the structure of the mitochondria was normal.

The structure of the neuromuscular synapse was basically preserved during and after detubulation, although on fixation in solution containing glycerol the pre- and postsynaptic membranes in some areas were blurred and interrupted, so that the synaptic space was not always clearly defined (Fig. 1). Another complication of synaptic organization was a change

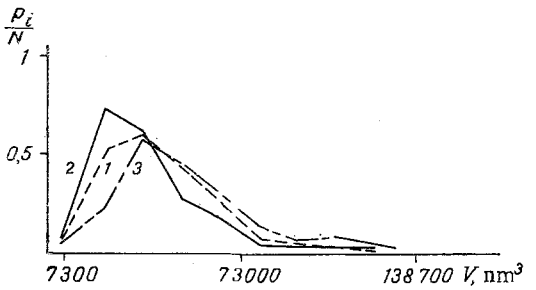

Fig. 2. Distribution of synaptic vesicles by volume. Abscissa, volume of synaptic vesicles (in nm^3); ordinate, relative number found. 1) Control; 2) 170 min after beginning of experiment; 3) 80 min after beginning of experiment.

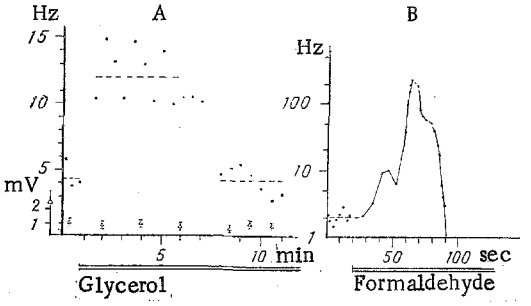

Fig. 3. Time course of changes in spontaneous transmitter secretion in rat neuromuscular junction during various experimental procedures. A) Frequency-amplitude characteristics of MEPPs in one fiber on addition of glycerol (400 mM): abscissa, time after beginning of experiment (in min); ordinate, amplitude of MEPP (in mV) and their frequency (in Hz). Triangles denote mean amplitudes of MEPPs for about 1 min, with error of means; circles denote frequency of MEPPs (broken line shows mean frequencies of MEPPs); B) change in frequency of MEPPs on addition of formaldehyde (final concentration 4%). Ordinate, frequency of MEPPs (in Hz) on logarithmic scale. Broken line represents mean frequency of MEPPs for many fibers before addition of formaldehyde; continuous line connects points belonging to one fiber.

in structural relations between axon terminals and Schwann cells, which was found at the beginning of "rinsing" and continued until the end of the experiment. Just as in the control, besides synaptic vesicles and mitochondria, which preserved their usual structure, vacuoles were found in the axon terminals.

A morphometric study of the vesicular apparatus of the axon terminals at different stages of the experiment revealed a definite time course of the changes (Table 1). During the detubulation procedure the mean area occupied by synaptic vesicles relative to the free area of the axon terminal (concentration of vesicles) decreased initially: it was least in medium with glycerol. This fact may also be linked to some extent with the observation that the zone near the presynaptic membrane, except in one or two areas, was virtually free from synaptic vesicles. The concentration of vesicles later increased a little during "rinsing" and was more than doubled in the final stage of the experiment, when it was somewhat higher than initially.

The mean area of the axon terminal, on the other hand, was increased: its values were higher than the control at all stages of the procedure. However, this did not compensate the reduction in concentration of vesicles, and the total number of vesicles per area of section through the terminal at these times was always less than in the control.

In the course of detubulation a tendency was found for the synaptic vesicle to enlarge. Not until the 170th minute was the mean diameter of the vesicles virtually the same as in the control. The character of distribution of the vesicles by volume changed more substantially (Fig. 2), although the difference between the distribution curves was significant only between the 80th minute (end of rinsing) and the 170th minute. At the end of rinsing considerable heterogeneity of the synaptic vesicle population was observed, not only in size, but also in electron density of the contents.

In the early stages and after the end of detubulation of the muscle, the ultrastructure of the neuromuscular junction thus remained virtually intact. Meanwhile changes in ultrastructure and, in particular, in the morphometric parameters, possibly due to the osmotic effects of glycerol, showed a definite time course which did not end with completion of the detubulation process (40 min after the beginning of rinsing out of glycerol).

Assessment of the functional effects of addition of glycerol revealed quite prolonged but temporary activation of transmitter secretion, sometimes reaching considerable levels (Fig. 3A). With rapid change of the solution "unusual" local potentials, at times leading to action potentials (AP) and fibrillation of the muscle, also appeared. However, as a rule toward the end of the period in Tyrode solution with glycerol the situation stabilized and the frequency of MEPPs returned to its initial level. Accurate evaluation of the amplitudes of MEPPs was difficult because of the fall of membrane potential observed in many fibers in the course of detubulation.

On fixation of the nerve–muscle preparation with formalin after the end of detubulation, a very considerable increase in the frequency of MEPPs was observed (Fig. 3B); usually it developed in two peaks and terminated by a fairly rapid fall to zero. The fall was gradual only in a few cases (to 10 min).

Structural and functional changes thus discovered can be linked with an increase in tonicity of the Tyrode solution due to addition of glycerol which, despite its ability to penetrate through the membrane, evidently induces a short-term osmotic effect, namely an increase of secretion and certain structural changes. The activating effect of glycerol, by contrast with that of sucrose [2], which penetrates with difficulty through the membrane, was transient in character (Fig. 3A). Possibly, however, it may be responsible for the fall in the number of vesicles and, in particular, the reduction in their concentration in the juxtamembranous zone.

As regards the action of formaldehyde, it did not differ significantly from that in intact preparations [1, 5], evidence of the absence of any noticeable changes in the secretory structures of the neuromuscular junction due to the detubulation procedure.

Certain structural changes affecting the vesicular apparatus and caused by glycerol thus persist, although admittedly for only a short time, after the end of detubulation and may probably be revealed by more delicate physiological tests. Whatever the case, these changes do not prevent the effects of formaldehyde on function.

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