DISTURBANCE OF NEUROSECRETION IN MYONEURAL JUNCTIONS OF MUSCLE POISONED WITH TETANUS TOXIN

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UDC 612.816.018.014.46: 576.851.097.29

During prolonged recording of electrical activity of the rat diaphragm, a fall in amplitude of the respiratory volley was observed after injection of $2 \cdot 10^5$ MLD tetanus toxin into the muscle. Intracellular recording of synaptic electrical activity in nerve-muscle preparations of the diaphragm isolated 3-3.5 h after injection of the toxin revealed a decrease in spontaneous secretion of mediator, as shown by a shift of the maximum of distribution of myoneural synapses with respect to mean frequencies of miniature end-plate potentials, from 3-7 to 0.1-0.4 spikes/sec. Electron-microscopic investigations showed no change in the cellular structure of the myoneural junction under these circumstances, but the number of synaptic vesicles in the axon terminals was significantly increased. It is concluded that the secretion of mediator is disturbed by the action of the toxin.

The paroxysmal syndrome in tetanus is due to the central action of tetanus toxin [3-6, 10, 12, 14]. The toxin has been known for a long time to act on the peripheral myoneural apparatus [15, 16] and, in particular, features of a denervation syndrome have been observed in muscle due to the action of tetanus toxin [16, 19]. The writers have described the development of a block to neuromuscular transmission under the influence of the toxin [4-8], but the mechanisms of this disturbance have not been explained. In the investigation described below the state of the myoneural synapses in the rat diaphragm, poisoned locally with tetanus toxin, was investigated by microelectrode and electron-microscopic methods.

EXPERIMENTAL METHOD

Experiments were carried out on male August rats weighing 100-200 g. In long-term experiments the respiratory electrical activity of the diaphragm was recorded with surface electrodes [1]. The electromyogram (EMG) of the diaphragm and its integral characteristics also were recorded by means of special units of the Disa electromyograph.

Synaptic electrical activity was recorded intracellularly in nerve-muscle preparations isolated from the rat diaphragm and placed in a thermostatically controlled (36-37°C) chamber through which Tyrode solution saturated with carbogen (95-96% O_2 + 4-5% CO_2) was passed. The transmembrane potential difference (TMD) of the muscle fibers in the region of the synaptic endings of the phrenic nerve ventrally to its entry into the muscle was detected with glass microelectrodes filled with 2.5 M KCl solution and recorded by means of a matching system [11] on a Disa-Indicator oscilloscope. Details of the method were described previously [9].

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Laboratory of Pathophysiology of Toxico-Infections and Group of Electron Microscopy, Institute of Normal and Pathological Physiology, Academy of Medical Sciences of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 72, No. 12, pp. 27-31, December, 1971. Original article submitted May 28, 1971.

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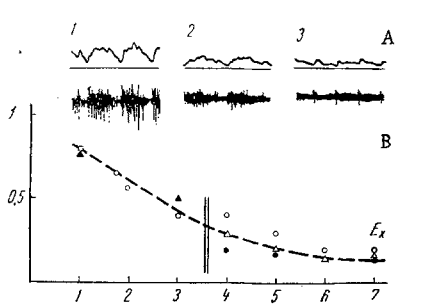

Fig. 1

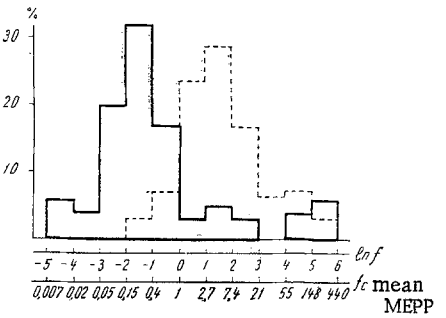

Fig. 2

Fig. 1. Changes in natural electrical activity of the diaphragm after injection of tetanus toxin. A: EMG (bottom record) and its integrated characteristics (top record) before injection (1) and 3.5 h (2) and 7 h (3) after injection of toxin ($2 \cdot 10^5$ MLD) in cupola of diaphragm; B: decrease in maximal amplitude of respiratory volley after injection of toxin. Abscissa, time after injection (in h); ordinate, amplitude of volley (in MV). Results of three experiments in which initial amplitudes were closely similar in absolute magnitude are illustrated.

Fig. 2. Changes in level of spontaneous secretion of mediator in myoneural junctions of muscle poisoned with tetanus toxin. Continuous line shows distribution of synapses by mean frequency of MEPP in nerve-muscle preparation isolated 3-3.5 h after injection of $2 \cdot 10^5$ MLD tetanus toxin into diaphragm; broken line shows analogous distribution for normal preparation. Ordinate, frequency of occurrence (in %), abscissa, discharge intervals on logarithmic scale; bottom scale gives absolute values of mean MEPP frequencies (spikes/sec), top scale gives values of their natural logarithms. Temperature of medium 36-37°C. Time of observation 1-2 h. Each histogram plotted from results for not less than 200 synapses.

Material for electron-microscopic investigation was fixed in situ or in the chamber with buffered formol–sucrose solution, and fixation was completed in cold formol–sucrose and then in osmium tetroxide. The material was embedded in Araldite. Sections were shadow-cast with uranyl acetate and lead citrate and examined in the JEM 7A electron microscope.

Dried toxin (batch 9, from native toxin of batch 255, strain 471 MIVS), containing 10^4 MLD for mice/mg, was diluted (100 mg/ml) in phosphate buffer (0.005 M, pH 7.2). Laparotomy was performed on a rat under ether anesthesia, the toxin was injected in a small volume (0.2 ml equals $2 \cdot 10^5$ MLD) into the diaphragm dorsally to the point of entry of the nerve, and the incision was then closed. Control experiments were carried out on healthy animals or on animals injected with inactivated toxin.

EXPERIMENTAL RESULTS

Respiratory EMG of the Diaphragm. After injection of the toxin ($2 \cdot 10^5$ MLD) the EMG showed a progressive decrease in amplitude of the respiratory volley in the poisoned diaphragm (Fig. 1). No changes were observed in the character of the respiratory electrical activity itself. The animals usually died 7-9 h later with signs of paralysis of the respiratory musculature.

Synaptic activity in the myoneural junctions of the diaphragm was investigated 3-3.5 h after injection of the toxin, when the amplitude of the respiratory volleys was significantly reduced by comparison with that in the control animals at the same time.

Spontaneous Synaptic Activity in Muscle Fibers. The state of neurosecretory system in the muscle synapses was judged from the spontaneous synaptic activity characteristic of an active myoneural junction. The level of spontaneous activity of the single synapse was evaluated from the mean frequency of miniature

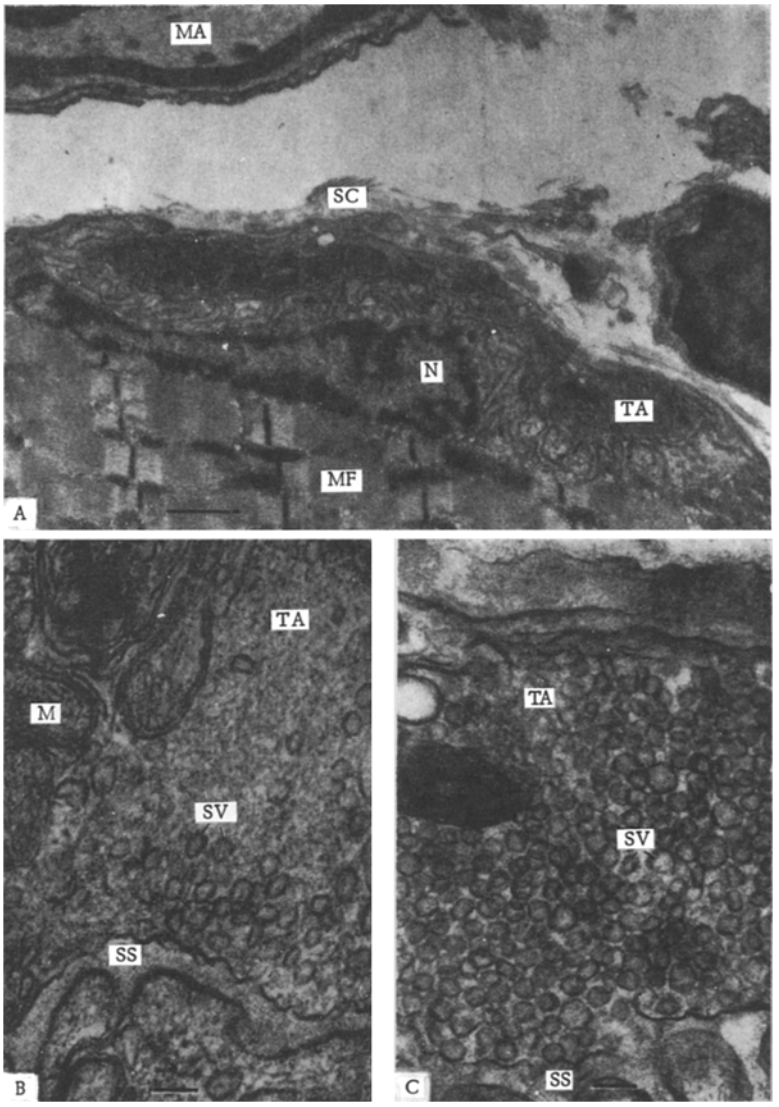

Fig. 3. Ultrastructure of myoneural junction in rat diaphragm. Prefixation with formol–sucrose in situ. Fixation with osmium tetroxide. Shadow-casting with uranyl acetate and lead citrate. A) General appearance of synapse in poisoned muscle ($2 \cdot 10^5$ MLD toxin 3 h before fixation): MA myelinated axon; TA axon terminal; SC Schwann cell; N fundamental nucleus; MF myofibrils. State of vesicular apparatus of terminal axon in normal muscle (B) and in myoneural junction poisoned with tetanus toxin (C), marked increase in number of synaptic vesicles in poisoned synapse: TA axon terminal; SS synaptic space; M mitochondria; SV synaptic vesicles. Straight line corresponds to 1 μ for A and 0.1 μ for B and C.

end-plate potentials (MEPP) of the muscle fiber. An important criterion of the state of the synaptic apparatus of the whole muscle as a cellular organization is the law of distribution of myoneural synapses by the level of their spontaneous activity, as reflected by the histogram of the muscle fibers with respect to the mean MEPP frequency. Comparison of such histograms for the intact isolated preparation and for the preparation isolated 3-3.5 h after injection of the toxin showed (Fig. 2) that the level of spontaneous synaptic activity was sharply reduced in the poisoned muscles: the principal maximum of the histogram was displaced into the region of lower frequencies (the modal discharge interval in the experimental series was 0.1-0.4 spike/sec and in the control series 3-7 spikes/sec). No significant changes in shape of the recorded

MEPP or magnitude of the TMP at rest were found. The control tests showed that injection of inactivated toxin does not give rise to disturbances of neurosecretion of the type observed.

Ultrastructure of the Myoneural Junction. The results of the electron-microscopic investigation showed (Fig. 3) that the myoneural synapse in the poisoned muscles was unchanged in structure. Changes were discovered only in the structures of the axon terminal. In some cases the mitochondria were swollen, their matrix translucent, and occasionally their cristae destroyed. In some sections the presynaptic membrane was ill-defined in places. The most significant change was an increase in the number of synaptic vesicles, which in some cases filled all the free space of the axon terminal. No changes were found in subsynaptic structures.

The facts observed call for comment.

1. Absence of EMG changes characteristic of the central effects of the toxin ("spontaneous" or afteractivity) observed in certain muscles of many species of animals [5] and, in particular, in the diaphragm [17], and also results indicating movement of the toxin along nerve fibers [4-6] suggest that the peripheral effects of the toxin were evaluated at the chosen times (3-3.5 h after injection).

2. The changes in character of the synaptic processes observed immediately after injection of the toxin (depression of mediator secretion) were appreciably intensified during the stay of the preparation in the chamber (for 2 h). The histogram of mean MEPP frequencies was therefore plotted from estimates of the unsteady state of the preparation from this point of view. Besides the developing effect of the toxin, other factors connected with isolation of the preparation may also be relevant: 1) disturbance of communication between the neurosecretory apparatus of the axon terminal and the trophic center of the cell (the neuron body); 2) the absence of nervous impulses, with their evident substantial effect on the level of spontaneous secretion, to the synaptic endings; 3) the nonidentity of the surrounding medium (Tyrode solution) with the internal medium of the organism. The intact preparation is reasonably resistant to these factors, but they evidently can potentiate the effects of developing injury.

3. Some of the changes discovered in synapse ultrastructure, such as swelling of the mitochondria, are evidently nonspecific for the effect of the toxin for they are observed during the action of a wide variety of factors and even in normal preparations; they are perhaps the result of histological treatment. The following interesting fact was observed with respect to the vesicular apparatus: in the poisoned myoneural junction the addition of formalin (fixation in the chamber) led to the secretion of only a few quanta of mediator, whereas under normal conditions this situation evokes the discharge of several thousand of quanta (judging from the sudden increase in frequency of the MEPP) [2]. It is therefore difficult to decide whether the electron-microscopic picture reflects a preceding change (accumulation of mediator in the synaptic vesicles before fixation) or whether it is connected with the fact that the action of tetanus toxin on the presynaptic membrane prevents formaldehyde from exhibiting its pharmacological effect.

The changes discovered in the synaptic apparatus in the muscle poisoned with toxin affect presynaptic structures. The combination of structural and functional changes in the injured myoneural junction suggests that inhibition of neurosecretion is the result of interference with the liberation of mediator from the terminals and not of its exhaustion. From this standpoint it is interesting to compare the effects of tetanus toxin and of α-bungarotoxin, which also blocks neuromuscular transmission by its action on presynaptic structures. During the action of α-bungarotoxin, when the marked activation of liberation of the mediator is quickly followed by its exhaustion, the electron-microscopic picture reflects exhaustion of reserves of the mediator: the number of synaptic vesicles in the terminals is reduced or they may disappear completely [13].

When conduction in the inhibitory synapses of the central nervous system is blocked by tetanus toxin, it is the presynaptic component which is affected also [14]. Results indicating specific binding of the toxin by the brain tissue fraction containing terminal membranes [18] may point to localization of the injury in this process in the membranes. Central and peripheral synaptic effects of the toxin can be assumed to be similar in type: they are associated with disturbance of the liberation of mediator through the presynaptic membrane, and the chemical nature of the mediator evidently does not play a decisive role in this process.

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