REGULATION OF TETANUS TOXIN-INHIBITED TRANSMITTER RELEASE IN NEUROMUSCULAR JUNCTIONS IN A CALCIUM-FREE MEDIUM

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The intracellular system regulating the Ca^{++} concentration in the cytoplasm of synaptic endings plays an important role in transmitter secretion and, in particular, in modulating the level of secretion in the functioning synapse. These processes have previously been called "calcium-independent," in the sense that their intensity is determined by a much lesser degree than, for example, the electrosecretory process, by the calcium ion concentration in the external medium. "Calcium-independent" control mechanisms can conveniently be studied when transmitter release is inhibited by tetanus toxin (TT) [1, 7, 8], when the low level of residual transmitter secretion also is dependent only a little on the external Ca^{++} concentration, but the activating effects of repetitive stimulation and of other modulating influences remain sufficiently strong and can be estimated unambiguously [2, 3, 4].

With these considerations in mind, in the investigation described below the effectiveness of intracellular regulators of the free calcium concentration in the cytoplasm was estimated by comparing levels of transmitter secretion in intact and TT-poisoned synapses in normal-calcium and calcium-free media.

EXPERIMENTAL METHOD

August rats weighing 100-129 g were used. Isolated neuromuscular preparations (phrenic nerve — strip of diaphragm muscle) were placed in a chamber through which flowed Tyrode solution (temperature 34-35°C) of the following composition (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 12, NaH_2PO_4 1, glucose 11, pH 7.2-7.4, saturated with carbogen (95% O_2 + 5% CO_2). Transmitter secretion was studied by recording postsynaptic electrical responses, namely end-plate potentials (EPPs) and miniature EPPs (MEPPs) intracellularly by glass microelectrodes filled with 2.5 M KCl solution, whose resistance was 10-50 MΩ. The level of secretion was estimated from the frequency of MEPPs and the quantum composition of the EPPs; if necessary these parameters were aggregated per unit time or the contribution of these two types of response was analyzed separately. Considering that the frequencies of MEPPs along fibers are distributed logarithmically [2], the significance of differences was estimated by the t test with a level of significance $P = 0.05$. In the case of indirect stimulation of the muscle its contractions were inhibited as a result of electromechanical uncoupling in most muscle fibers through their detubulation. This was done by adding glycerol (400 mM) to the external solution and then (after 40 min) replacing this by the original solution. Purified TT (5×10^5 MLD/ml) was injected in a dose of 0.2 ml, under ether anesthesia, into the left cupola of the diaphragm 3-4 h before isolation of the preparation. Ionized calcium was removed from the external solution by addition of 0.1 mM EDTA to Tyrode solution not containing $CaCl_2$. Ouabain was used in a concentration of 0.1 mM. Indirect stimulation of the muscle fibers was effected by stimulating the nerve with square pulses 0.2 msec in duration.

EXPERIMENTAL RESULTS

The effect of ouabain on transmitter secretion (on the frequency of MEPPs) was investigated as a rule by changing the solutions in the following order: a short study in original Tyrode solution with normal calcium concentration ($+Ca^{++}$), in Tyrode solution without $CaCl_2$, a long period of study in calcium-free Tyrode solution with the addition of EGTA ($-Ca^{++}$), in

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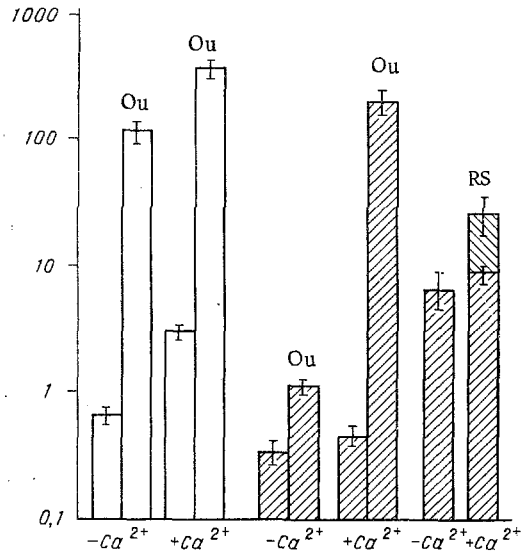

Fig. 1. Effect of calcium ions on activation of secretion of quanta of transmitter due to ouabain and repetitive stimulation. Ordinate, level of secretion (in quanta/sec). Unshaded columns — intact terminals, shaded — poisoned with TT. Method of activation indicated above columns: Ou) ouabain, RS) repetitive stimulation with a frequency of 50 Hz for 1 sec. In the last case the short horizontal line separates the contribution of synchronous release (EPP). Presence or absence of Ca^{++} ions in external solution indicated below columns.

the same solution with the addition of ouabain ($-Ca^{++}$ + Ou) and, finally, in normal calcium solution containing ouabain ($+Ca^{++}$ + Ou). In each experiment the time course of the change in frequency of MEPPs after the change of solutions was estimated if all went well in the same fiber, but in most cases frequencies of MEPPs were recorded successively in about 10-30 synapses while the preparation remained in the given solution (Fig. 1). In addition, in all experiments for each procedure the mean level of secretion was estimated when it flattened out at a relatively stationary level.

The "calcium-independence" of TT-inhibited secretion, observed previously [3], also was found in this series of experiments: The decrease in frequency of MEPPs in the poisoned terminals due to removal of Ca^{++}, although rather more evident that in the previous experiments, was not significant, and on the whole we were unable to find any difference in the levels of secretion between intact and poisoned terminals in calcium-free medium compared with the toxin-inhibited terminal in normal calcium medium. Under the influence of ouabain, an inhibitor of the sodium pump, transmitter secretion by the intact terminal in calcium-free medium increased quite slowly to reach a high and sufficiently constant level, whereas activation of transmitter in the poisoned terminal, although it took place, was significantly less (Figs. 1 and 2). On restoration of the external Ca^{++} concentration to normal values (2 mM) the frequency of MEPPs rose in both cases, but particularly substantially in TT-poisoned synapses, reaching in absolute values almost the level of activation of the frequency of MEPPs induced by ouabain in intact terminals. This last fact is in general agreement with observations described previously [3]. The difference from them which was found — for example, the somewhat higher level of "ouabain" activation, especially in intact synapses — may be linked with keeping the preparations beforehand in calcium-free medium, where, because of inhibition of transmitter release, its operative reserves could increase.

In the course of repetitive stimulation of the poisoned neuromuscular junction in normal calcium medium synaptic activity increased on account of both MEPPs and EPPs; this situation, moreover, was repeated during subsequent testing in other fibers. The effect of high-frequency stimulation (50 Hz) was particularly marked, even during the first second (Figs. 1 and 3). In calcium-free medium also, secretion could be activated by high-frequency indirect stimula-

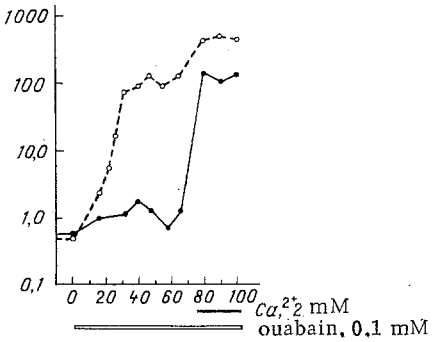

Fig. 2

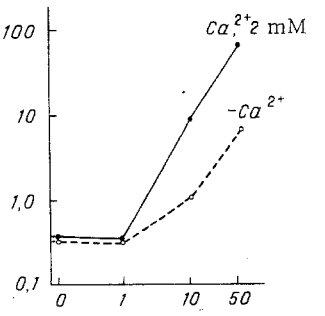

Fig. 3

Fig. 2. Changes in frequency of MEPPs in intact and TT-poisoned neuromuscular junctions under the influence of ouabain and depending on presence of Ca^{++} ions in external solution. Abscissa, time of experiment (in min); ordinate, frequency of MEPPs (in Hz) on logarithmic scale. Single line below graph denotes presence of Ca^{++} in external solution, double line — presence of ouabain. Empty circles — intact preparation, filled — poisoned with TT. Rat diaphragm. Temperature 35°C.

Fig. 3. Effectiveness of reactivation of spontaneous secretion by indirect repetitive stimulation of fibers under influence of TT in normal calcium and calcium-free medium. Abscissa, frequency of stimulation (in Hz) on logarithmic scale; ordinate, frequency of MEPPs (in Hz) on logarithmic scale. Filled circles and continuous line represent medium with normal calcium concentration, empty circles and broken line — calcium-free medium. Rat diaphragm. Temperature 35°C.

tion of the poisoned fibers. In this case, however, it was weaker (Figs. 1 and 3) and developed only on account of spontaneous activity (MEPPs) and was usually observed only in the first of the fibers tested. With an increase in stimulation time the frequency of MEPPs sometimes increased to higher values, in which case the increase persisted after the end of stimulation. Subsequent testing of other fibers in calcium-free medium was as a rule almost ineffective.

If modern views on calcium ions as an essential and irreplaceable cofactor in exocytosis of quanta of transmitter from the terminal are accepted, a number of important conclusions can be drawn from evaluation of the level of release of quanta of transmitter when the external calcium supply is interrupted. First, the almost total preservation of the powerful activating influences of ouabain in the calcium-free medium, leading to inflow of sodium into the cytoplasm and displacement of calcium by it from its intracellular reservoirs, is evidence of their considerable size in the intact terminal. Inhibition of "ouabain" activation by TT in calcium-free medium may reflect the depletion of these reservoirs because of blocking of their calcium uptake pathways, disturbance of calcium release by these reservoirs and, finally, the presence of several sources of calcium variously sensitive to the action of the toxin. In connection with this last possibility attention must be drawn to the opposite direction of the regulatory functions of intracellular calcium accumulators, which can be reduced on the one hand to "ridding" of the cytoplasm of free calcium (to maintain a high calcium gradient between the external and internal media) and its storage, and on the other hand, and no less important, the supplying of quantities of calcium that are needed to modulate secretion to the sites where this takes place, i.e., ultimately to participate in the actual process of exocytosis. This may thus be a question of intracytoplasmic transport of Ca^{++} ions, the stages and steps of which perform the above-mentioned functions to various degrees. Several candidates have now been put forward for the role of these stages [5, 6, 9]: first, there are the mitochondria, smooth endoplasmic reticulum, and even vesicles, i.e., reservoirs of a particular capacity, the "scavengers" of the cytoplasm, capable at the same time of migrating

toward the contacting membrane and of releasing calcium on depolarization; second, there are the calcium-binding membrane structures (proteins and gangliosides), which are evidently an important factor regulating electrosecretory coupling, whereas their capacity on the "scavenging" plane is probably small.

Replenishment of the first type of calcium depots is affected by TT mainly indirectly, evidently through blocking of the calcium transport system located on the outer contacting membrane (it is sensitive also to ruthenium red, a blocker of sialic groups). As a result, accumulation of calcium by reservoirs located close by, for example, the smooth endoplasmic reticulum, or even the vesicles themselves, is limited. It is also possible that it is these highly labile [5] calcium transporters that are responsible for its rapid shifting toward active zones for regular release of the transmitter, and even for the organization of these zones themselves. Their functioning is connected with the second type of calcium depots, of low capacity, which include sialate-containing structures located on the inner side of the contacting plasma membrane. They also bind TT [9] as well as ruthenium red and, since they determine organized exocytosis of quanta of transmitter by active zones, they are evidently the principal target of the toxin.

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