EFFECT OF TETANUS TOXIN ON K^+ AND Na^+ CONCENTRATIONS IN SYNAPTOSOMES

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Tetanus toxin (TT) disturbs the coupling of depolarization with secretion in nerve endings [2, 4, 7, 11], uptake of mediators by synaptosomes [3, 4, 9], and osmotic sensitivity of terminals [6]. There is some evidence that it also alters membrane electrogenesis [10, 16].

Since the above processes directly or indirectly depend on the transmembrane Na^+ and K^+ gradient and since their disturbance may be the result of the action of TT on ion transport, it was decided to study the effect of TT on the Na^+ and K^+ concentrations in synaptosomes. The preliminary data were published previously [3].

EXPERIMENTAL METHOD

The cerebral cortex of albino rats weighing 180-200 g was homogenized in 9 volumes of 0.32 M sucrose (or 0.32 M sucrose, 1 mM EDTA, and 20 mM Tris-HCl), pH 7.4, at 20°C and synaptosomes were isolated as described previously [5, 12]. The synaptosomes (final concentration equivalent to 1-2 mg protein, determined by Lowry's method [13], in 1 ml medium) were incubated in medium of the following composition (in mM): NaCl - 139.5, KCl - 4.5, $CaCl_2$ - 1.8, $MgCl_2$ - 1.17, NaH_2PO_4 - 3.0, Na_2HPO_4 - 3.0, glucose 9.0, sucrose 16, Tris-HCl 18, pH 7.3 (37°C), with, in some experiments, 1.0 mM ouabain. The final volume of the samples was 2 ml.

In the experiments of series I polymer centrifuge jars containing the suspension of synaptosomes with TT (TT inactivated by boiling, TT + antitoxin, ouabain), were incubated for 15 min at 37°C. To study the effects of potassium depolarization, 10 µl of 2.5 M KCl solution was added to control and experimental samples and incubation continued for a further 3 min (the final K^+ concentration was increased to 17 mM). The synaptosomes were sedimented by centrifugation (K-24 centrifuge, East Germany) for 1 or 2 min at 9000g (18-20°C). The supernatant was removed and the residue carefully suspended by means of a pipet in 2 ml of 0.32 M sucrose. The synaptosomes were then sedimented by centrifugation for 1 or 2 min at 9000g, resuspended in 0.32 M sucrose, and sedimented again.

After the residue of synaptosomes had been rinsed, 2 ml of deionized water was added, the synaptosomes were resuspended, and a further 8 ml water added. The suspension was kept in the cold overnight. The total exposure of the synaptosomes to TT from the beginning of incubation until osmotic destruction was 50-60 min. The protein concentration was determined in the suspension of destroyed synaptosomes. Fragments of destroyed synaptosomes were sedimented by centrifugation and the Na^+ and K^+ concentrations in the supernatant were determined on a BIAN-140 flame photometer (model 803).

In the experiments of series II synaptosomes were incubated in glass tubes for 15 min or 1, 2, or 4 h with continuous shaking, and then separated from the incubation medium by ultrafiltration (filters from Schleicher und Schull, West Germany, pore diameter 0.45 µ). The residues of synaptosomes were washed with six portions, each of 2 ml, of 0.32 M sucrose. The total duration of washing was 1.5 min. The filters with residues of synaptosomes

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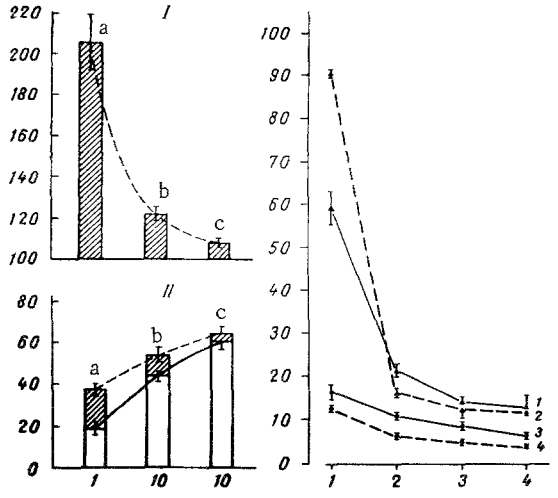

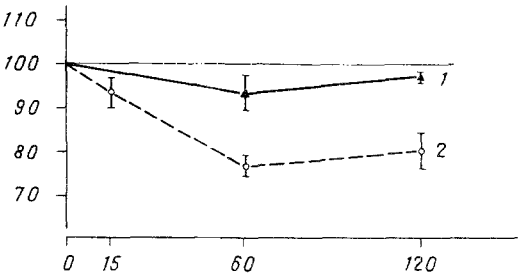

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Effect of TT on sedimentation rate of synaptosomes: I) protein concentration in residue of synaptosomes (% of control), II) difference (shaded part of columns) in protein concentration in residue of synaptosomes between control (unshaded columns) and experiment (in % of protein concentration in original samples). Conditions of centrifugation of synaptosomes: a) 10 ml, 2 min; b) 2 ml, 1 min; c) 2 ml, 2 min. Number of experiments given in parentheses.

Fig. 2. Effect of number of washings with sucrose on Na^+ (1, 2) and K^+ (3, 4) concentrations in TT-poisoned (2, 4) and control (1, 3) synaptosomes. Results of one experiment. Abscissa, number of washings; ordinate, concentration of cations (in μeq/100 mg protein).

Fig. 3. Time course of K^+ concentration in synaptosomes incubated with TT. 1) TT inactivated by boiling, 2) TT. Abscissa, time (in min); ordinate, K^+ concentration (in % of control).

were placed in polyethylene flasks and extracted with 2 ml of 1 N HNO_3 overnight. To each flask 8 ml of water was added, the filters were removed, and the concentrations of Na^+ and K^+ in the extracts were measured on the PFM 30 MZ flame photometer. The results were expressed in microequivalents Na^+ and K^+ per 100 mg protein of the suspension applied to the filter, or in millimoles, taking the osmotically sensitive volume of the synaptosomes to be 2.9 μl/mg protein [14] and to be unchanged by TT [16].

The TT used was from batches 22 and 23 from the Leningrad Institute of Vaccines and Sera, purified by gel-filtration [8]. The titer of purified TT was 0.8×10^4-2.0×10^4 MLD (for rats) per milligram protein. Since the TT was obtained either in 0.1 M phosphate buffer or in 0.02 M Tris-HCl, the equivalent amounts of the corresponding buffer were added to tubes not containing TT. The TT was inactivated by boiling in a waterbath for 15 min or by the addition of "Diaferm-3" antitetanus serum. The residual toxicity of the mixture of TT (1000 MLD) and antitoxin was under 0.5 MLD. To assess the significance of differences between the samples, Student's test (P_t) or Wilcoxon's nonparametric test (P_T) was used.

EXPERIMENTAL RESULTS

In the experiments of series I synaptosomes incubated for 15 min were investigated. After two washings with sucrose, each for 1 min, the residues of synaptosomes incubated in the presence of TT were found to contain 20% more protein than residues of the control synaptosomes (Fig. 1b). The difference with respect to protein was evidently due to the faster sedimentation rate of the "tetanus" synaptosomes as a result, for example, of changes in their electrokinetic potential after binding of TT with the membrane gangliosides (binding of TT may itself exaggerate the protein concentration in residues of synaptosomes but only by a few per cent, provided that all TT added to the sample is sedimented). In conformity with this hypothesis, the difference with respect to protein was practically completely

TABLE 1. Effect of TT and Ouabain on K^+ and Na^+ Concentrations in Synaptosomes (M ± m)

K^+ in medium, mM	Experimental conditions	Number of experiment	K^+		Na^+	
			μeq/100 mg protein	mM	μeq /100 mg protein	mM
4,5	Control	21	11,99±0,49	41,34±1,69	17,55±1,25	60,52±4,31
	Toxin, 500-800 MLD	21	8,01±0,36*	27,62±2,24	17,77±1,26	61,28±4,34
	Ouabain, 1 mM	5	4,64±0,47*	16,00±1,62	21,45±2,20†	73,97±7,59
17	Control	13	11,99±0,82	41,34±2,83	17,80±1,29	61,38±4,45
	Toxin, 500-800 MLD	13	9,73±0,86‡	33,55±2,96	18,54±1,42	63,93±4,90

*$P < 0.001$ compared with control.
†$P_T < 0.05$ compared with control.
‡$P_t < 0.05$ compared with K^+ concentration in synaptosomes poisoned with TT, in the presence of 4.5 mM K^+.

TABLE 2. Effect of Inactivated TT on K^+ and Na^+ Concentrations in Synaptosomes (M ± m)

Experimental conditions	Number of experiments	K^+, % of control	Number of experiments	Na^+, % of control
TT	21	66,81±1,72	20	100,52±3,35
TT inactivated by boiling	4	90,38±1,81*	3	96,34±16,21
Toxin + antitoxin	4	97,73±8,89*	3	99,29±1,11

*$P_t < 0.001$ compared with native TT.

abolished when the duration of centrifugation was increased to 2 min (Fig. 1c) or it increased if the dilution factor was reduced (Fig. 1a).

Extracts of synaptosomes incubated with TT contained more Na^+ and less K^+ than the control. A decrease in the K^+ concentration in extracts of "tetanus" synaptosomes (Table 1) was found in the population of synaptosomes sedimented by centrifugation for both 1 min and 2 min, and the decrease did not depend, moreover, on the number of washings (Fig. 2). Conversely, the washing procedure affected the difference in Na^+ concentration in residues of synaptosomes. It was observed after the first washing (Fig. 2) but disappeared after the second (Table 1). Since washings removed mainly extracellular Na^+, it can be concluded that there were no significant changes in the Na^+ concentration in the synaptosomes under the influence of TT under the experimental conditions used. Ouabain caused loss of K^+ from synaptosomes and entry of Na^+ into them (Table 1), in agreement with data in the literature [15].

The decrease in the K^+ concentration in extracts of synaptosomes cannot be explained by incomplete extraction, for the same result was obtained by extraction of the synaptosomes with an aqueous solution of the detergent Triton X-100, and also for synaptosomes subjected to freezing and thawing followed by extraction, or finally, when 1 N HNO_3 was used as the extractant.

The results given below are evidence of the specificity of the effect of TT observed. The TT preparation did not cause hemolysis of sheep's red blood cells, i.e., it was not contaminated with tetanus hemolysin. At the same time, inactivation of the TT by boiling or by the addition of antitetanus antitoxin almost completely abolished the ability of TT to reduce the K^+ concentration in the synaptosomes (Table 2; Fig. 3).

The decrease in the K^+ concentration in the synaptosomes depended on the dose of TT in the incubation medium (Fig. 4). However, an increase in the dose was accompanied by an increase in the effect but only up to a certain limit, i.e., the action of TT was characterized

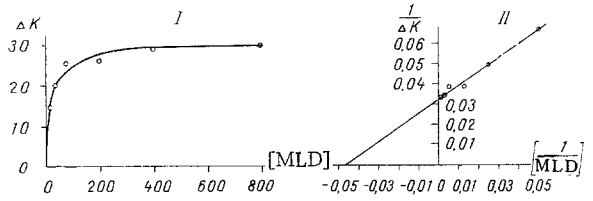

Fig. 4. Effect of different doses of TT on K^+ concentration in synaptosomes. I: Abscissa, dose of TT (MLD in 1 ml); ordinate, difference (in %) between K^+ concentration in control and TT-poisoned synaptosomes (ΔK). II: the same, on Lineweaver–Burk plot.

by saturation. In agreement with other data [1] on the much greater sensitivity of central synapses to TT, the value for the effect constant obtained by a Lineweaver–Burk plot (about 22 MLD, see Fig. 4, II) was 3 orders of magnitude smaller than doses causing rapid disturbance of neuromuscular transmission [1, 6, 7].

An increase in the K^+ concentration in the medium from 4.5 to 17 mM caused no change in the Na^+ and K^+ levels in the control synaptosomes, but the K^+ concentration in the "tetanus" synaptosomes was increased (Table 1, $P_t < 0.05$). Since the calculated value of the K^+ concentration in the "tetanus" synaptosomes was higher than 17 mM, entry of K^+ against the concentration gradient was most probably due to intensification of its active transport.

The kinetics of the decrease in the K^+ concentration in the synaptosomes was investigated by a rapid filtration technique (Fig. 3). The K^+ concentration in the "tetanus" synaptosomes began to fall after only 15 min of incubation, after 1 h the difference between the control and experiment reached a maximum (in the previous series of experiments the total duration of exposure of the synaptosomes to TT reached 1 h), and after incubation for 2-4 h the difference disappeared because of worsening of the state of the control synaptosomes.

The decrease in the K^+ concentration observed in the synaptosomes and also the fall in resistance [10] and hyperpolarization of the membranes [10, 16] under the influence of TT can be explained by increased permeability of the synaptosomes for K^+. The entry of K^+ into "tetanus" synaptosomes when they were in a state of weak potassium depolarization was evidently because of intensified active K^+ transport. The difference in the secretory responses of TT-poisoned and intact nerve endings to potassium depolarization is manifested most clearly within the concentration range from 15 to 30 mM [4, 6, 16], which is optimal for Na^+,K^+-ATPase. Assuming that the cationic pump in poisoned nerve endings is activated within this K^+ concentration range, a decrease in the effectiveness of potassium depolarization must be the regular result of the hyperpolarizing effect of Na^+,K^+-ATPase activation.

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EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON DENERVATION-LIKE CHANGES IN FROG MUSCLE FIBER MEMBRANES INDUCED BY AXOPLASMIC TRANSPORT BLOCKADE BY COLCHICINE

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Blocking neurotrophic influences on muscle by denervation alters the electrophysiological properties of the muscle fiber membrane: The resting membrane potential (RMP) is reduced, input resistance (R_0) and the time constant (τ) of the muscle membrane are increased, and extrasynaptic sensitivity to acetylcholine (ACh) develops [2, 6, 8, 13]. Administration of inhibitors of protein synthesis (actinomycin D or cycloheximide) to experimental animals at the same time as the nerve is divided inhibits the development of postdenervation changes in mammals. It has accordingly been postulated that neurotrophic control is exerted through the muscle fiber gene [9].

Blockade of axoplasmic transport (AT) by colchicine, although not disturbing the conduction of excitation along nerve fibers and not interrupting neuromuscular transmission, is known to induce denervation-like changes in muscle fibers: a decrease in RMP, an increase in R_0 and τ, and the appearance of extrasynaptic sensitivity to ACh [1, 2, 7, 10]. On the basis of these investigations it has been suggested that substances carried to the muscle by AT take part in neurotrophic control of skeletal muscle fibers.

However, the question of whether the effect of AT blockade by colchicine is realized through the muscle fiber gene, as has been demonstrated for surgical denervation [4, 8, 9, 11], remains uncertain. The answer to this question is important from the point of view of possible comparison of mechanisms leading to the appearance of extrasynaptic acetylcholine sensitivity after denervation of a muscle and after AT blockade by colchicine solution. The investigation described below was devoted to this problem.

EXPERIMENTAL METHOD

Experiments were carried out on the sartorius muscle of *Rana temporaria* in winter, by a standard microelectrode technique. RMP was recorded and R_0 and τ of the membrane measured by the membrane voltage drop method, and the character of sensitivity of the postsynaptic membrane to ACh was studied by application of the mediator from a micropipet [11]. During the experiment the muscle was kept in a bath with circulating Ringer's solution of the following composition (in mM): NaCl 115, KCl 2.5, $CaCl_2$ 1.8 in phosphate buffer, pH 7.25, at 20 ± 0.05°C. Treatment of the nerve supplying the sartorius muscle with 10 mM colchicine solution (from Merck, West Germany) was carried out by the method described in [2]. Actinomycin D (from Reanal, Hungary) and puromycin (from Serva, West Germany) were injected intraperitoneally. The frogs were kept in a terrarium with circulating water at room temperature.

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