STATE OF RAT ERYTHROCYTE MEMBRANE Na,K-ATPase ACTIVITY IN BOTULINUM TOXIN POISONING

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KEY WORDS: botulinum toxin poisoning; Na,K-ATPase and Mg-ATPase.

Experimental studies have shown indirectly that active ion transport can be disturbed under the influence of botulinum toxin (BT) in cells of organs and tissues that differ in their morphological and functional features [3-5]. Meanwhile there is virtually no direct evidence on mechanisms of the disturbance of active ion transport and, in particular, activity of transport ATPase of biological membranes has not been studied in the course of botulinum toxin poisoning (BTP).

It was accordingly decided to study activity of Na,K-ATPase in the erythrocyte membranes in experimental BTP. The choice of erythrocytes as test object was not by chance. First, the structure of erythrocyte membranes reflects the particular features of the structural biological membranes of various tissues [6]. Second, although the direct target for the action of BT is the nervous system or, more precisely, nerve endings of synaptic structures, it was interesting to investigate the possible secondary pathways of disturbance of the functions of erythrocyte membranes in BTP.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred albino rats weighing about 250 g. BTP was produced by intraperitoneal injection of type C BT in a dose of 0.025 mg/kg body weight (1 MLD = 0.0005 mg of the dry toxin). Experiments were carried out 2 h after injection of BT, i.e., in the preclinical, latent period of poisoning, and also about 1 day after the injection, when a syndrome of paralysis and asphyxia had developed. Parallel experiments were carried out *in vitro* with preliminary preincubation of erythrocyte membranes with BT in a concentration corresponding to its mean calculated concentration in experiments *in vivo*. Erythrocyte membranes were isolated and ATPase activity determined by the usual methods [1, 2]. The protein concentration in the membranes was determined by Lowry's method [7].

EXPERIMENTAL RESULTS

In the preclinical period of poisoning, characterized by intensive desorption of the toxin from the zone of its injection, by its hematogenous spread, and fixation in different tissues, marked changes in activity of the transport Na,K-ATPase of the erythrocyte membranes already appeared. Table 1 shows clearly that Na,K-ATPase activity during this period was reduced ($P < 0.05$), whereas Mg-ATPase activity was increased. Na,K-ATPase activity 1 day after intraperitoneal injection of the toxin, i.e., when a paralytic syndrome had developed, complicated by respiratory insufficiency, was appreciably lowered, whereas Mg-ATPase activity was back to normal.

The stable fall in erythrocyte membrane Na,K-ATPase activity in the course of poisoning was in all probability the result of the specific effect of the toxin. A conclusive argument in support of this conclusion was given by the results of experiments *in vitro* in which active BT or BT inactivated by boiling was added to the preparation of erythrocyte membranes. Just as in the experiments *in vivo*, BT caused sharp depression of Na,K-ATPase after preincubation of the toxin for only 7 min with erythrocyte membranes. As the results of the subsequent experiments showed, preincubation of erythrocyte membranes with BT in concentrations corresponding to the mean values calculated in the experiments *in vivo*, was accompanied by inhibition of Na,K-ATPase ($P < 0.001$). Meanwhile preincubation of erythrocyte membranes

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TABLE 1. Effect of BT on Activity of ATPases (in μmoles P_i/mg protein/h) of Erythrocyte Membranes (M ± m)

Series of experiments	Experimental conditions	Mg-ATPase	Na,K-ATPase
Experiments in vivo	Control	0,39±0,04 (15)	0,21±0,02 (15)
	Preclinical period of poisoning	0,54±0,04* (16)	0,13±0,01* (16)
	Preclinical period of poisoning, after injection of unithiol	0,70±0,03* (16)	0,27±0,03† (16)
	Preclinical period of poisoning after injection of glutathione	0,57±0,03* (12)	0,16±0,03 (12)
	Paralytic stage of generalized BTP	0,40±0,03 (14)	0,13±0,01* (14)
Experiments in vitro	Control (membranes treated with boiled toxin)	0,49±0,07 (14)	0,20±0,02 (14)
	Preincubation of membranes (1 mg protein/ml) with native toxin	0,49±0,05 (14)	0,10±0,01* (14)
	Preincubation of membranes with native toxin followed by addition of of unithiol to incubation medium	0,37±0,05 (14)	0,18±0,02† (14)
	Preincubation of membranes with native toxin followed by addition of glutathione to incubation medium	0,45±0,04 (14)	0,17±0,02† (14)

Legend. *P < 0.05 relative to corresponding controls, † P < 0.05 relative to corresponding stage of poisoning without injection of preparation *in vivo* or to series of experiments *in vitro* with preincubation of membranes with native toxin. Number of animals given in parentheses.

with boiled BT caused no clear inhibition of Na,K-ATPase activity. In all modifications of the experiments in which membranes were preincubated with boiled and active BT, Mg-ATPase activity remained virtually unchanged.

To study the degree of injury caused by the toxin to erythrocyte membrane enzyme proteins and the possibility of reactivation of Na,K-ATPase, series of experiments were carried out *in vitro* in which donors of SH-groups were added to the incubation medium: unithiol and glutathione in a concentration of 1 mM. As the experiments in this modification showed, both compounds inhibited the inhibition of Na,K-ATPase by BT. Parallel experiments *in vivo*

with intraperitoneal injection of glutathione in a dose of 38 mg/100 g body weight and unithiol in a dose of 25 mg/100 g body weight showed that Na,K-ATPase can be reactivated in the preclinical period of poisoning, but only by unithiol. Each preparation was injected immediately after intraperitoneal injection of the toxin.

The results of these experiments thus indicate inhibition by BT of active transport of monovalent cations through erythrocyte membranes and that under certain conditions this effect can be abolished by donors of sulfhydryl groups.

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ELIMINATION OF AUTOLOGOUS CLOTTING FACTOR Xa FROM THE BLOOD STREAM OF INTACT AND THYMECTOMIZED RATS

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The intensity of elimination of active enzymes from the blood stream is determined by various mechanisms: the state of the reticuloendothelial system, the velocity of interaction with substrates and inhibitors, and also the immune potential of the body. This last factor is confirmed by the writers' previous investigations, which demonstrated disinhibition of enzyme activity in the hemostasis system of thymectomized rats [2, 3, 6].

The object of the present investigation was to determine the rate of elimination of an active enzyme (factor Xa) from the blood stream of intact and thymectomized rats.

EXPERIMENTAL METHOD

Stuart–Prower factor (blood coagulation factor Xa) was isolated from pooled plasma obtained from 110 rats. The plasma was coagulated by the addition of thromboplastin (from Kaunas) and 1% $CaCl_2$ solution. The fibrin thus formed was removed and the enzyme isolated from the serum by ion-exchange chromatography on DEAE-Sephadex A-50 (from Pharmacia, Sweden), using stepwise elution gradients of phosphate buffer (0.05 M, pH 7.0; 0.2 M, pH 7.0; 0.45 M, pH 8.0). Fractions obtained with the last gradient of buffer were pooled and rechromatographed. The isolated preparation was desalted on Acrilex P-60 (from Reanal, Hungary) and lyophilized. The enzyme possessed BAME-esterase activity, converted prothrombin into thrombin in a prothrombin-factor Xa–Ca^{++} system, and was homogeneous on electrophoresis in polyacrylamide gel. Prothrombin was isolated from pooled bovine plasma by the method in [7]. Factor Xa was labeled with ^{131}I by the method described previously [5]. The specific radioactivity was 0.05 μCi/ml. The preparation completely preserved its enzymic properties for

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