EFFECT OF TETANUS TOXIN ON CONTRACTILITY OF THE ACTOMYOSIN-LIKE PROTEIN OF RAT BRAIN

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Preparation of purified actomyosin-like protein (ALP) was isolated from the coarse fraction of rat brain mitochondria. The ALP preparation contained Mg(Ca)-ATPase activity, inhibited by 0.1% deoxycholate, together with acetylcholinesterase activity and contractility (superprecipitation reaction). The contraction of ALP and ATPase activity were considerably reduced by tetanus toxin. Inactivated toxin had no effect. The possible functional role of ALP in the maintenance and secretion of mediators by nerve endings is discussed.

KEY WORDS: rat cerebral cortex; tetanus toxin; actomyosin-like protein; ATPase; acetylcholinesterase; secretion of mediators.

Many papers have recently been published on the isolation and study of the properties of an actomyosin-like protein (ALP) from nerve tissue [7, 9, 14, 20], including from the fraction of coarse mitochondria [2] and synaptosomes of the mammalian cerebral cortex [21]. The function of ALP in nerve tissue has not yet been explained.

One of the most likely suggestions is that ALP is an important element in the system for maintenance and secretion of mediators. Experiments have shown [21] that ALP in nerve endings may be localized in the membranes of the synaptic vesicles (SV). The marked increase in the passive transport of Ca^{++} ions into the synaptoplasm during conduction of the nervous impulse may initiate (through ATP hydrolysis) contraction of ALP on contact between SV and the presynaptic membranes, leading to secretion of mediators into the synaptic space. Tetanus toxin (TT) is known to disturb the secretion of mediators in central synapses [3, 5, 10-12] and in neuromuscular synapses [6, 8, 17].

It was accordingly decided to study the effect of TT on the contractility of ALP in the rat brain.

EXPERIMENTAL

ALP was isolated by Puszkin's method [9, 20, 21] with certain modifications from the fraction of coarse mitochondria (CM). The CM fraction was isolated from the gray matter of the rat cortex [1] and washed with 0.32 M sucrose containing 3 mM EDTA and then with deionized water. The washed fraction was suspended in water (30 mg protein/ml). After freezing and thawing (−10°C, 10 h), the fraction was homogenized and 1.2 M KCl in 0.01 M tris-HCl, pH 8.2, was added in the ratio of 1 : 1; after mixing for 40 min at 0-4°C the ALP was further extracted for 16 h at 0-4°C. After centrifuging (15,000 g, 1 h, 0-4°C) the P_1 fraction containing 47% of protein was obtained in the residue and the supernatant was diluted with cold water to 0.1 M KCl and the pH adjusted to 6.0 by the addition of 0.12 M Na-acetate buffer, pH 4.5. The turbid suspension was mixed for 30 min at 0-4°C and centrifuged at 15,000 g (10 min) to obtain a residue of ALP which was suspended in 0.6 M KCl, 0.01 M tris-HCl buffer, pH 7.2, or in water (depending on the object of the investigation). The yield of ALP was 0.86-0.92 mg protein/g wet weight of brain tissue or 4.8% of protein of the original CM fraction. The ALP was used immediately after isolation or 24 h after keeping at 0-4°C (or at −10°C).

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TABLE 1. Mg(Ca)-ATPase activity of Rat Brain ALP (M ± m)

KCl concentration (in moles)	ATPase activity (in μmoles P_{in}/mg protein/h)			
	Mg-ATPase	Ca-ATPase	Mg-ATPase	Ca-ATPase
	I		II	
0	4,9±0,8 (6)	4,1±1,2 (6)	3,5±1,2 (4)	4,0±1,4 (4)
0 ± TT (20 μg/ml)	3,9±0,4 (3)	3,4±0,7 (3)	—	—
0,1	3,7±0,9 (7)	2,2±0,6 (7)	1,8±0,4 (3)	1,1±0,4 (3)
0,6	3,0±0,7 (6)	2,5±1,0 (6)	1,7±0,5 (3)	1,4±0,4 (3)

Legend. Mg(Ca)-ATPase was taken to be activity of the enzyme during hydrolysis of ATP in the presence of Mg^{++} or Ca^{++} ions (5 mM respectively) only: I) without DOC treatment; II) treatment with DOC.

ATPase activity was determined [1] by the accumulation of inorganic phosphorus [16] in the incubation medium (20 min, 37°C). The composition of the incubation samples for Mg(Ca)-ATPase was (in millimoles): ATP-Na_2 3, tris-HCl (pH 7.4) 50, $MgCl_2$ ($CaCl_2$) 5, protein 200-300 μg/ml. After preincubation (5 min, 37°C) the reaction was started by the addition of ATP and stopped by the addition of 10% CPA (1 : 1). Acetylcholinesterase (ACE) activity was determined by Ellman's method [13]. Protein was determined by Lowry's method. The contractility of the ALP was judged by the superprecipitation test: the increase in optical density (E) was recorded at 520 nm and 20°C. The incubation sample (2.5 ml) contained tris-HCl (pH 7.4) 20 mM, ALP 425 μg protein, and 0.1 or 0.6 M KCl. After preincubation (5 min, 20°C) the reaction was started by the addition of 0.025 ml Mg^{++}(Ca^{++}) and ATP-Na_2 in a final concentration of 5 and 3 mM, respectively. The reaction reached saturation after 3-5 min and thereafter was unchanged for 30 min. During the test the reagents were periodically stirred. Disk electrophoresis in 7.5% polyacrylamide gel (PG) was carried out in 5 mM tris-glycine buffer, pH 8.3, at 20°C in an apparatus of the firm "Reanal" (Hungary). The PG also contained 1% Na dodecylsulfate, 8 M urea, and 1 mM mercaptoethanol (solution No. 1). When solubilized in solution No. 1 containing 5 mM tris-HCl pH 7.95, the ALP was applied in a dose of 120 μg to the separating gel. The initial current was 1 mA/gel and its duration 40 min; later, when the protein had entered the PG, the strength of the current was increased to 3 mA/gel and the duration of electrophoresis to 120 min. The PG was fixed with 20% sulfosalicylic acid (16 h, 0-4°C), washed with water, and stained for 60 min with 0.2% Coomassie in a solution of $CH_3COOH-CH_3OH-H_2O$ (1 : 1 : 2), and then rinsed in a solution of $CH_3COOH-CH_3OH-H_2O$ (1 : 10 : 30). Quantitative analysis of the fractions was carried out on a Chromoscan (England) densitometer.

RESULTS AND DISCUSSION

Anodic disk electrophoresis on PG revealed the electrophoretic homogeneity of the solubilized ALP. No staining of the PG with Coomassie was found at the start, further evidence of the high degree of homogeneity of the ALP preparation. Incidentally, if the ALP preparation was suspended in 0.6 M KCl, pH 7.2, instead of water, it was impossible later to solubilize the protein in solution No. 1. According to data in the literature, ALP isolated from bovine, cat, and rat brain, and also from bovine brain synaptosomes, also possessed electrophoretic (and sedimentation) homogeneity [9, 20, 21].

It will be clear from Table 1 that the ALP had Mg(Ca)-ATPase activity; hydrolysis of ATP in the presence of Mg^{++} was at a higher intensity than in the presence of Ca^{++} ions only (in the same concentration). The Mg(Ca)-ATPase activity of ALP in different states of aggregation, i.e., in the presence of 0.1 and 0.6 M KCl, differed, as it also did for ALP of nerve tissue isolated by other workers [9], but in the present experiments this difference between the activities was not significant. However, the Mg-ATPase activity, like the contractility (Table 2), was a little higher in 0.1 M KCl than in the suspension with 0.6 M KCl; this suggests that in solutions with low ionic strength polymerization and aggregation were intensified. Treatment of the ALP suspended in water or in salt medium with 0.1% Na deoxycholate (DOC) for 30 min at 20°C, with a protein concentration of 1-3 mg/kg, led to a decrease in the activity of the ATPases studied. It also follows from Table 1 that TT inhibited the Mg(Ca)-ATPase activity of ALP. It follows from Fig. 1 that the adsorption in the ultraviolet region (especially in the region 250-280 nm) of a suspension of ALP in 0.1 M KCl was much less than in 0.6 M KCl; this was probable evidence of differences in the state of aggregation of the ALP in media with different ionic strengths. The addition of 0.1% DOC to the medium led to a sharp change in the aggregation properties of the ALP: a decrease in the adsorption of the suspension in the 250-280 nm region in 0.1 M KCl and an increase in 0.6 M KCl. The data shown in Fig. 1 also point to the possibility of

TABLE 2. Action of TT on Contractility of ALP (M ± m)

Parameters	KCl (in moles)	Control	Concentration (in μg proetin /ml)							
			TT 20	TT 30	TT 40	TT 60	TT 80	inactivated TT 50	albumin 50
Initial E_{520}	0,1	0,06	0,07	0,07	0,07	0,075	0,075	0,07	0,065
	0,6	0,055	0,06	0,06	0,06	0,06	0,075	0,06	0,050
Increase E_{520} on addition of Mg^{++} and ATP	0,1	132±7,6 (18)	84,1±6,75* (12)	70,6±7,3* (3)	66,5±9,6* (12)	63,1±7,3* (12)	52,7±10,1* (8)	122,0±9,0 (11)	119,5±9,6 (9)
(in % of corresponding initial value of E_{520})	0,6	115,7±11,1 (17)	61,0±8,7* (11)	44,3±8,1* (3)	39,4±9,5* (11)	42,5±11,0* (12)	43,6±14,0* (6)	106,0±6,9 (9)	128,1±7,1 (8)

Legend. Preincubation with TT for 20 min at 20°C. The increase in E_{520} was calculated (in %) by comparison with the corresponding control. Number of experiments shown in parentheses. Values for which $P \leq 0.05$ (compared with the control) are marked by an asterisk.

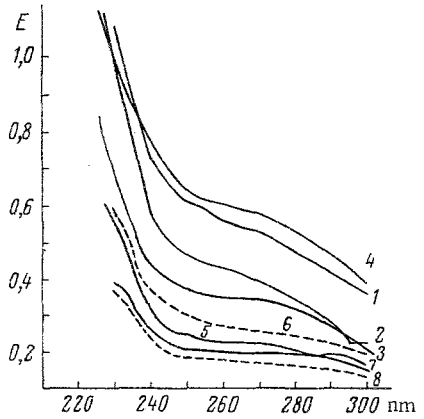

Fig. 1. Effect of DOC and tetanus toxin on adsorption of ALP in the ultraviolet region: ALP 300 μg protein, DOC 0.1%, TT 50 μg. Preincubation for 5 min at 20°C. 1) 0.1 M KCl; 2) 0.6 M KCl; 3) 0.1 M KCl + DOC; 4) 0.6 M KCl + DOC; 5) 0.6 M KCl + TT; 6) 0.1 M KCl + TT; 7) 0.6 M KCl + TT + DOC; 8) 0.1 M KCl + TT + DOC. To each test a corresponding control (without ALP) was set up. Mean results of 4-5 experiments are shown.

specific binding of TT with ALP. The toxin clearly caused a decrease in adsorption in the 240-260 nm region in the ALP suspension. The decrease was particularly marked in 0.1 M KCl (by 50%); a decrease was also produced by the toxin in the presence of 0.1% DOC, and this was particularly marked in 0.6 M KCl (by 60-70%).

The experiment showed that the ALP preparation had ACE activity (92.8 μmoles substrate/mg protein/min), evidently in the myosin-like component. Muscle myosin is known to possess ACE activity [4].

Table 2 showed that purified TT, starting with doses of 20 μg/ml (1000 MLD/ml, the dose specified for mice), reduced the contractility of ALP, the reduction being greater in a suspension containing 0.6 M KCl. In a concentration of 60-80 μg/ml the toxin reduced the contractility of the ALP by 50% or more. The action of the toxin was specific, for toxin inactivated by heating (20 min at 120°C, concentration 70-90 μg/ml) in a concentration of 50 μg/ml and human serum albumin (50 μg/ml) did not affect the contraction process. In high ATP concentrations (6-9 mM) the effect of TT was weakened, most of all for ALP in 0.6 M KCl.

TT (or its toxophore group) probably blocks the ATP-hydrolyzing center of the myosin component of the ALP, but does not prevent the binding of ATP with the regulating (allosteric) center of the myosin component with the participation of the active center. The presence of two centers of contact with ATP has been demonstrated for muscle myosin [15]. Confirmation of this possibility is given by the moderate inhibition

(20%) of Mg-ATPase activity of ALP by the toxin (Table 1). ALP isolated by the writers from the CM fraction of rat brain is basically identical in its properties with ALP isolated from the synaptosome fraction [21]. According to Puszkin [21], ALP is absent from the fractions of microsomes, mitochondria, and myelin fragments and is localized in the membranes of SV. The discovery that the contractility of ALP is inhibited by TT, which disturbs mediator secretion [3, 5, 6, 8, 11, 12, 18], can be regarded as evidence of the participation of ALP in the process of mediator secretion. The possibility that ALP may play a role in the maintenance and secretion of mediators and hormones has also been postulated by other workers [19, 21, 22].

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