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CONTRACTILE PROTEINS OF NERVE ENDINGS AS A POSSIBLE TARGET FOR THE PRESYNAPTIC ACTION OF TETANUS TOXIN

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KEY WORDS: tetanus toxin; nerve endings; contractile proteins.

Tetanus toxin (TT) is known to have a presynaptic action, leading to inhibition of mediator secretion in central and peripheral synapses [5]. It has also been shown that TT binds selectively with gangliosides of nerve ending membranes [6]. However, the mechanism of its toxic action has not yet been explained. It has been suggested that there are three centers in the TT molecule: receptor, binding with gangliosides, antigenic, and toxophore [6]. The results of electrophysiological investigations indicate that the action of TT is aimed at the final stage of exocytosis – interaction between synaptic vesicles (SV) and complementary regions of the presynaptic membrane [5]. Elucidation of the biochemical target for TT is important for the understanding not only of the pathogenesis of tetanus, but also of the process of mediator secretion itself. It has recently been postulated that mediator secretion is based on mechano-chemical processes in which a cytoskeletal network of microtubules, neurofilaments, and active microfilaments associated with the presynaptic membrane takes part [2, 9]. These structures may be responsible both for transporting SV to the active zone of the synapse and for complementary contact between SV and the presynaptic membrane, with subsequent exocytosis. Accordingly, it can be suggested that the target for TT may be the contractile proteins of nerve endings. It was shown previously [4] that TT depresses the ATPase activity of the actomyosinlike protein (AMLP) of the rat brain. The object of the present investigation was to study the effect of TT on the AMLP superprecipitation reaction and on interaction between actin-like protein (ALP) and isolated SV.

EXPERIMENTAL METHOD

The TT was purified by gel-filtration on Sephadex G-100 [8]. TT with a toxicity of $1.25 \cdot 10^5$ MLD for mice/mg protein was used in the experiments. AMLP was isolated from bovine cerebral cortex by the method in [12] with certain modifications. The tissue was homogenized in 2 volumes of isolation medium (0.9M KCl, 1.5 mM dithiothreitol, 5 mM $MgCl_2$, 50 mM Tris-HCl; pH 9.2) at 0-4°C. The homogenate was treated with 0.5% Triton X-100 and kept in the cold for 18 h, after which it was centrifuged (1 h, 10,000g). The supernatant was diluted with 1 mM $CaCl_2$ solution in the ratio of 1:5, the pH of the medium was adjusted to 6.25-6.3 with acetic acid, and centrifugation was repeated under the same conditions. The residue was washed and suspended in isolation medium, the final KCl concentration being adjusted to 0.6M. After centrifugation (30 min, 100,000g) the supernatant was diluted sixfold with dionized water, the pH adjusted to 6.25-6.3 with acetic acid, after which centrifugation was carried out for 1 h at 100,000g. The residue, consisting of AMLP, was dissolved in 0.6M KCl containing 1 mM dithiothreitol and 20 mM Tris-HCl, pH 7.4, and kept at 0-4°C for 1-2 weeks. The yield of AMLP was 10 mg protein/g wet weight of tissue. Protein was determined by Lowry's method.

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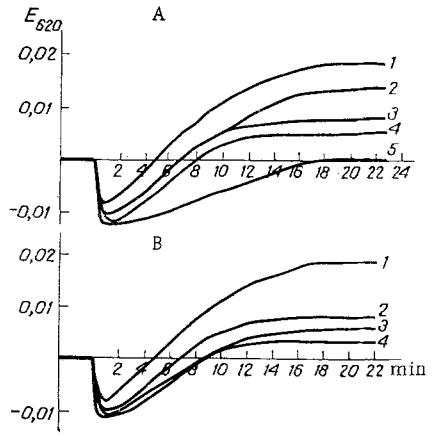

Fig. 1. Action of TT on bovine brain AMLP superprecipitation reaction. Incubation medium: KCl 0.1 M, Tris-acetate 20 mM, pH 6.8, temperature 37°C. Volume of sample 4 ml, volume of additives 50 μl. Preincubation with TT for 10 min. A: 1) Without TT, 2) 0.25 MLD/ml TT, 3) 2.5 MLD/ml TT, 4) 25 MLD/ml TT, 5) 250 MLD/ml TT; B: 1) without TT, 2) 2.5 MLD/ml TT, 3) gangliosides 0.25 μl/ml. 4) gangliosides + TT. Abscissa, time (in min); ordinate, E_{620} (in optical density units).

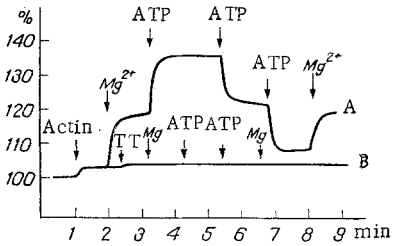

Fig. 2. Action of TT on changes in scattering of light by synaptic vesicles induced by Mg and ATP, in the presence of brain actin. Incubation medium: 50 mM KCl, 20 mM Tris-HCl, pH 7.4. ALP 80-100 μg protein per sample, SV 100 μg protein per sample, volume of sample 2.5 ml, temperature 22°C. $MgCl_2$ and ATP were added each in a volume of 10 μl of the 250 mM solution. A) Without TT, B) 1600 MLD/ml TT. Abscissa, time (in min); ordinate, intensity of scattering of light (in %).

The AMLP superprecipitation reaction was studied by determining the change in optical density (E) at 620 nm and 37°C. The reaction was started by the addition of 50 μl Mg-ATP, in a final concentration of 1 mM. The apparatus for continuous recording of E_{620} consisted of a photoelectric colorimeter (FEK-56) with inbuilt magnetic mixer and constant-temperature cuvette, dc amplifier (pH-340), and KSP-4 automatic writer [1].

The SV fraction was isolated from rat cerebral cortex or bovine hypothalamus by De Robertis' method with certain modifications [7]. Interaction between SV and ALP in the presence of Mg–ATP was studied by measuring the change in intensity of scattering of light at an angle of 90° at 520 nm and 22°C on a Hitachi-204 (Japan) spectrofluorometer with automatic recorder [7]. The total ganglioside fraction was isolated from bovine brain [13]. Noradrenalin was determined fluorometrically [14].

EXPERIMENTAL RESULTS

In the experiments of series I the effect of TT on superprecipitation of AMLP from bovine brain (Fig. 1a), initiated by the addition of Mg–ATP in medium with low ionic strength, was studied. This reaction is important

TABLE 1. Action of TT on Liberation of Noradrenalin into Incubation Medium on Interaction of SV and ALP (M ± m)

Substances added	Liberation of noradrenalin, %
SV	100±6 (n=9)
SV+ALP	130±10 (n=10)
SV+ALP+TT (1000 MLD/ml)	87±7 (n=5)
SV+ALP+TT+antitetanus serum (150 i.u./ml)	120±13 (n=4)

Legend. Composition of incubation medium (30 min, 37°C): 50 mM KCl, 0.2 mM $MgCl_2$, 0.2 mM ATP-Na_2; 1 mM $CaCl_2$; Tris-HCl; pH 7.4; SV 90-120 μg protein/ml; ALP 70-90 μg protein/ml. Volume of sample 1 ml. Quantity of noradrenalin liberated from SV during incubation without ALP (45% of its content in SV) taken as 100.

evidence [3] of the contractile ability of the AMLP isolated from brain. It consists of two phases: rapid (1-2 min) – the phase of clearing, corresponding to dissociation of AMLP into ALP and myosin-like protein; slow (10-15 min) – the phase of true superprecipitation, i.e., an increase in optical density above the initial level, corresponding to the formation of the AMLP complex and its polymerization and aggregation. As Fig. 1a shows, TT interacts with AMLP, thereby significantly changing the course of the superprecipitation reaction; clearing is intensified somewhat and true precipitation considerably weakened. It is important to note that the inhibitory action of TT was manifested in comparatively low concentrations (2-200 mg protein/ml, equivalent to 0.25-25 MLD/ml), but in a concentration of 250 MLD/ml the value of E_{620} in the second phase of the reaction did not increase above its initial level. In the absence of Mg–ATP, TT in the concentrations studied did not affect aggregation of the AMLP preparation. In control experiments, human serum albumin in concentrations of up to 2 μg protein/ml did not affect aggregation or superprecipitation of bovine brain AMLP.

Considering that the gangliosides of nerve ending membranes are the receptors for binding TT [6] and that glycolipids can play the role of regulators of the function of contractile proteins of nerve endings [2, 7], the combined action of TT and gangliosides on the superprecipitation reaction was studied. It will be clear from Fig. 1b that total brain gangliosides, in a concentration of 0.25 μg/ml, have a similar action to TT. During the combined action of TT and gangliosides, their effect on true superprecipitation of AMLP is additive in character. Hence, it can be postulated that TT, when bound with the receptor (gangliosides), remains capable of acting on AMLP. Consequently, the toxophore center of the TT molecule is different from its ganglioside-binding center. These data confirmed, on a different model, the previous conclusion [6] of spatial separation of the toxophore and ganglioside-binding centers.

In the experiments of series II the effect of TT on interaction between isolated SV and the preparation of bovine brain ALP was studied. It has been shown that membranes of SV contain a myosinlike protein, whereas presynaptic membranes contain ALP. Interaction between SV and ALP can evidently lead to the formation of the AMLP complex, which was confirmed by the increase in Mg–ATPase activity of SV in the presence of ALP [6, 11], by the increase in scattering of light by SV and ALP initiated by Mg^{++} and ATP [6], and also by liberation of endogenous noradrenalin [6] and of labeled glutamate [11] from SV into the incubation medium in response to addition of ALP. It is shown in Fig. 2 that TT (1600 MLD/ml) blocks the formation of the complex in a suspension of SP from rat cerebral cortex and of bovine brain ALP on the addition of Mg^{++} and ATP, as judged from the intensity of scattering of light.

Interaction of SV of bovine hypothalamus with bovine brain ALP in medium containing Mg-ATP was accompanied by increased liberation of endogenous noradrenalin from SV (Table 1). This process was inhibited by TT in a dose of 1000 MLD/ml, whereas preliminary incubation of TT with antitetanus serum (Diaferm, USSR; final activity 150 i.u./ml) abolished the action of TT.

Of toxins with a known mechanism of action, phalloidin (the toxin of the death-head Amanita phalloides) and staphylococcal toxin affect contractile proteins directly. The toxic action of phalloidin is explained by stimulation of polymerization of liver G-actin into the fibrillary form, as a result of which the interaction of

F-actin with myosin is disturbed [15]. Staphylococcal toxin disturbs the contractile power of actomyosin of smooth muscles [10]. The action of TT is probably similar to the action of these poisons. The results described above indicate that the contractile proteins of nerve endings, responsible for exocytosis of mediators, are the probable target for TT. The immediate target for TT is probably the ALP built into the presynaptic membrane. The result of binding of TT and ALP is blocking of contact of SV with the presynaptic membrane and of mediator secretion. It is not yet clear whether the TT molecule penetrates through the membrane of nerve endings or whether it acts on the cytoskeletal network and on SV in the cytosol of nerve endings.

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PATHOGENESIS OF EXPERIMENTAL CEREBRAL HEMORRHAGE DURING HYPERTENSION IN THE LIGHT OF MICROCIRCULATION STUDIES

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Problems concerning with the pathogenesis of cerebral hemorrhage in hypertension have recently been examined in the light of microcirculation studies [1, 3].

Microcirculation problems include not only the principles of transcapillary exchange and the structure and ultrastructure of the walls of the microvessels, but also the state of the blood itself [10]. It is accordingly interesting to study the morphology of the microcirculation and indices of blood clotting and fibrinolysis in the early stages of experimental hemorrhages which, by their neurohumoral mechanisms, have features of similarity with hemorrhages due to apoplexy in man. Audiogenic apoplexy, excluding the possibility of limiting inhibition [5], and improved by the author [2] by the addition of hypoxic hypoxia, was used as the model of cerebral hemorrhage associated with hypertension.

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