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Modulation of functional activities of the neurotoxin from black widow spider venom

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We have studied the action of an α-latrotoxin (α-LTX) complex of two polypeptides (LTX 130 kDa and low molecular weight protein (LMWP) 8 kDa) and the action of a venom fraction containing LTX with excess LMWP on calcium influx into synaptosomes and PC12 cells as well as on [^{14}C[GABA release from synaptosomes. Both preparations considerably activate calcium influx and stimulate [^{14}C]GABA release from synaptosomes. Preincubation of both preparations with antibodies against a 14 amino acid residue C-terminal peptide of LMWP differentially modulates these effects. Antibodies inhibit induced calcium influx and enhance induced GABA release.

α-Latrotoxin; Rat brain synaptosome; PC12 cell; Calcium influx; GABA release

1. INTRODUCTION

α-Latrotoxin (α-LTX) isolated from black widow spider venom selectively binds to presynaptic nerve endings of vertebrates and stimulates massive neurotransmitter release from virtually all types of synapses [1]. The action of α-LTX increases membrane calcium permeability and subsequent transmitter release [1,2]. Recently it was shown that highly purified toxin preparations contain two components – polypeptides of 1401 (LTX) and 70 (low molecular weight protein, LMWP) amino acid residues [3,4]. This study examines the functional activity of LMWP and the process of its specific interaction with LTX in complex.

2. EXPERIMENTAL

2.1. *Purification of α-LTX, LMWF and LMWP*

Crude *Latrodectus mactans tredecimguttatus* venom was obtained by water extraction of fresh spider venom glands. Isolation of α-LTX was performed as described earlier [5]. LMWF corresponds to fraction S5 from gel filtration of crude venom on Sephacryl S-300 [5]. For isolation of LMWP, 10% trichloroacetic acid was added to LMWF and precipitate removed by centrifugation. Supernatant was fractionated by reverse phase HPLC on an Ultrapore RPMC column (5 μ, 4.6 × 250 mm) (Beckman, USA) in 0.1% TFA with an acetonitrile concentration gradient. Obtained fractions were lyophilized and LMWP dissolved in suitable buffer.

2.2. *Generation of antibodies*

Polyclonal antibodies against synthetic peptide corresponding to the 57–70 C-terminal fragment of LMWP were raised in rabbits after coupling to Keyhole-limpet haemocyanine by the glutaraldehyde method [6]. Anti-peptide antibodies (Abs) were affinity purified with immobilized synthetic peptide.

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2.3. *Calcium influx*

Freshly prepared synaptosomes [7] were resuspended at 4 mg of protein/ml in standard saline medium. $^{45}Ca^{2+}$ (0.5 mCi/ml) was added after 15 min preincubation at room temperature to ensure a return to steady-state conditions. Abs modified (1 h preincubation) or unmodified α-LTX, LMWF or LMWP was added to synaptosomal suspension simultaneously with $^{45}Ca^{2+}$. To terminate $^{45}Ca^{2+}$ uptake at definite time intervals, 100 μl aliquots were layered onto Sephadex C-50 columns (1 ml bed volume) pre-equilibrated with 50 mM Tris- HCl, pH 7.4, 2 mM $CaCl_2$ and eluted with 0.8 ml of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl. Synaptosomes were solubilized in SDS (final concentration 1%) and synaptosomal $^{45}Ca^{2+}$ was quantitated by scintillation counting using an aqueous cocktail in a Delta 300 counter (Tracor Analytic, USA).

PC cells cultured in Eagle's minimal essential medium (Sigma, USA), containing 10% horse serum ('Vector', Novosibirsk) and 5% fetal calf serum ('Bacpreparat', Minsk) as monolayer were detached, washed and resuspended in standard saline medium at 1 mg of cell protein/ml. $^{45}Ca^{2+}$ influx was stopped by filtration of 0.25 ml aliquots through Whatman GF/C filters, which were then washed twice with 4 ml of ice-cold buffer containing 126 mM NaCl, 5 mM KCl, 20 mM HEPES, pH 7.4.

2.4. *GABA release from synaptosomes*

Suspensions of synaptosomes were preloaded with [^{14}C]GABA in buffer containing 10^{-5} M amino oxyacetate according to the described method [8]. After 5 min incubation at 30°C, initial levels of [^{14}C]GABA were measured (zero point). Abs-modified or unmodified α-LTX, LMWF or LMWP were added at the zero point. At definite times 0.5 ml aliquots were removed and filtrated through Whatman GF/C filters. Before scintillation counting, pellets were solubilized in 1% SDS. Protein recovery of synaptosomal suspension was 93–96%.

3. RESULTS AND DISCUSSION

Independent of isolation method [5,9] highly purified α-LTX consists of two polypeptides with m.m. 130 kDa (LTX) [3] and 8 kDa (LMWP) [4]. N-terminal amino acid sequence analysis showed that LTX and LMWP are present in α-LTX complex in practically equal quantity. Attempts to separate these two polypeptides in native conditions by different chromatographic methods were unsuccessful. Homogeneous LMWP was prepared in this study using a low molecular weight fraction of venom (LMWF; m.m. 5–10 kDa) obtained by gel filtration of crude venom [5]. Purified LMWP did not exhibit any biological activity peculiar for α-LTX. To elucidate the role of LMWP in α-LTX complex, rabbit polyclonal antibodies (Abs) were raised against synthetic peptide corresponding to a C-terminal fragment of LMWP with the structure:

Asn-Lys-Val-Tyr-Glu-Glu-Lys-Asp-Thr-Pro-Pro-Val-Gln-Glu

These Abs were highly specific to LMWP and could not recognize other proteins in the spider venom. Electrophoretic analysis of venom and its fractions followed by Western blots using these Abs revealed LMWP in purified α-LTX and in LMWF of the venom (Fig. 1). PAAG electrophoresis and reverse-phase HPLC chromatography showed that LMWF contains LMWP (about 50% of total protein) along with a mixture of other low molecular weight proteins. Western blots with A4 monoclonal antibodies against LTX [2] showed the presence of no more than 1% LTX in LMWF (Fig. 1). This could be accounted for by non-specific LTX binding to matrix during gel filtration of the crude venom on Sephacryl S-300.

Previously, in studies of the modification of toxin effects by monoclonal antibodies against α-LTX, it was

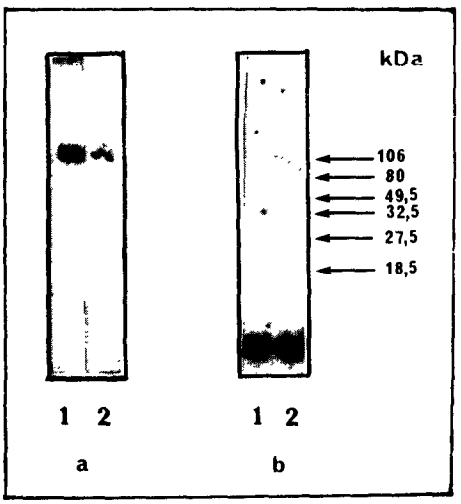

Fig. 1. Immunoblot detection with monoclonal antibodies A-4 (a) and polyclonal antibodies Abs (b) of α-LTX (lane 1) and LMWF (lane 2).

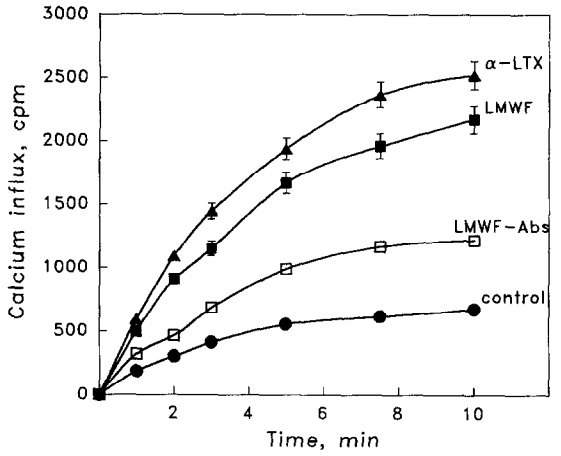

Fig. 2. Effect of α-LTX (1.3 μg/ml), LMWF (40 μg/ml) and LMWF–Abs complex (1:2 molar ratio) on calcium influx into synaptosomes. Each point represents the mean value of duplicate assay. The figure shows a representative of eight experiments.

hypothesized that α-LTX complex has separate functional sites which facilitate high affinity binding to a membrane receptor, toxin-induced Ca^{2+} uptake and toxin-stimulated neurotransmitter release [2]. Analysis of functional properties of LMWF showed that this fraction, like α-LTX [2], activates calcium influx into synaptosomes and PC12 cells. The effects of LMWF are illustrated in Figs. 2 and 3. As shown, LMWF proved very active, but at concentration 5×10^{-7} M (4 μg/ml), which is much higher than concentrations of α-LTX which produce the same effect. Homogeneous LMWP was inactive in this procedure (results not shown). Experiments in which LMWF was preincubated with Abs before application to nerve endings exhibited substantially decreased calcium influx relative to unmodified LMWF. It is noteworthy that in the experiments with α-LTX, a slight inhibition of induced calcium influx could be observed only in the case of very high excess of Abs. At the same time, mAbs A4 against α-LTX [2], which eliminates the ability of α-LTX to increase cal-

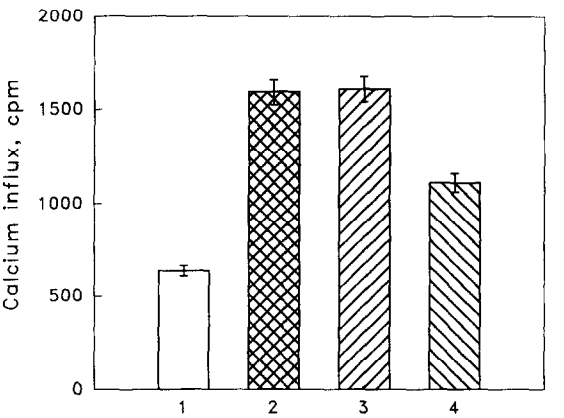

Fig. 3. Calcium influx into PC12 cells for 15 min: 1, control; 2, α-LTX (0.13 μg/ml); 3, LMWF (4 μg/ml); 4, LMWF–ABs complex (1:2 molar ratio).

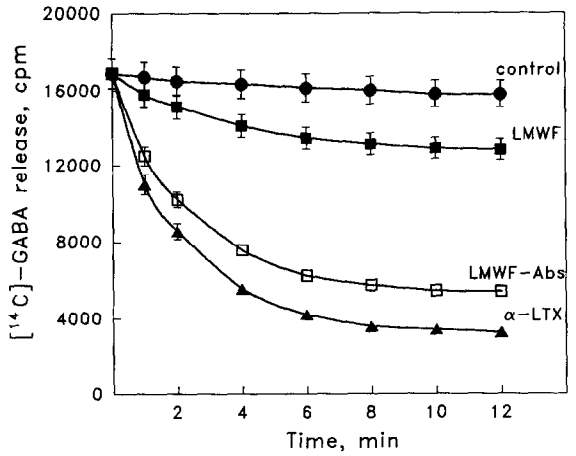

Fig. 4. Stimulation of [^{14}C]GABA release from synaptosomes by α-LTX (0.4 μg/ml), LMWF (4 μg/ml), LMWF–Abs complex (1:2 molar ratio).

cium influx into synaptosomes, also blocks this ability of LMWF (data not shown).

LMWF, but not purified LMWP, induced [^{14}C]GABA release from rat synaptosomes, although this effect was less pronounced than in experiments with α-LTX (Fig. 4). The dose–effect of LMWF on synaptosome neurosecretion was investigated. LMWF at concentration less then 10 μg/ml induces slightly detectable secretion. When the concentration of LMWF was increased its secretagogue action became more pronounced. Surprisingly, the effect of LMWF–Abs complex on [^{14}C]GABA release was significantly enhanced, relative to unmofidied LMWF, to levels approximately equivalent to those induced by α-LTX (Fig. 4).

In spite of the absence of direct results in this study with LTX free from LMWP or with LMWP free from LTX, one can assume that LMWP contributes to secretagogue and ionophore actions of purified α-LTX. The enhanced effectiveness against calcium influx and the stimulation of secretagogue activity by antibodies against the C-terminal fragment of LMWP in experiments with LMWF, which is enriched in LMWP relative to LTX, suggests that an understanding of the relationship between LTX and LMWP will be possible only with experiments using expressed LTX and LMWP.

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