CONCANAVALIN A BLOCKS BLACK WIDOW SPIDER TOXIN STIMULATION OF TRANSMITTER RELEASE FROM SYNAPTOSOMES

A. GRASSO, S. RUFINI and I. SENNI
Laboratory of Cell Biology, CNR, Via Romagnosi 18A, 00196 Rome, Italy

Received 28 October 1977

1. Introduction

Black widow spider toxin (BWSTx), is a neurotoxin purified from the venom of the spider *Latrodectus mactans tredecimguttatus* [1]. BWSTx when tested on synaptosomes [1,2], seems to reproduce the physiological events shown to occur at the neuromuscular junction of the frog [3], lobster [4] and locust [5] after treatment with crude venom: namely a massive release of transmitters. A superfusion method of synaptosomes, based on the detection in the perfusate of previously accumulated radioactive transmitter has proved to be a highly successful technique for the study of transmitter release mechanism [6,7].

The recent report [8], that preincubation of cultured neuromuscular junctions with low concentration of Concanavalin A (Con A), inhibits the transmitter release caused by the spider venom, prompted us to study this effect on the efflux of previously accumulated γ-amino [^{14}C]butyrate, from synaptosomes.

We present here evidence that BWSTx stimulates the release of radioactive γ-amino butyrate (GABA) from synaptosomes and that preincubation of synaptosomes with micromolar concentrations of Con A abolishes the effect of the toxin.

2. Experimental procedures

BWSTx was purified from venom glands extract by gel permeation and ion exchange chromatography as reported [1]. Toxicity data as well as comparison of the chemical and physical properties of the toxin suggest considerable similarity with B_5 fraction purified [9].

Synaptosomes were prepared from male rat forebrain, in a sucrose Ficoll system, according to [10]. After washing the synaptosomes were resuspended in a buffered medium at a concentration of about 2 mg total synaptosomal protein ml^{-1}, and incubated with a GABA mixture giving a final concentration of 1 μCi γ-amino [^{14}C]butyrate and 1 μM GABA in presence of 10 μM aminooxy acetic acid (AOAA) for 10 min at 37°C. Synaptosomes were diluted to 1 mg total proteins ml^{-1}, and 1.5 ml aliquots were transferred into 0.65 μm Millipore filters (DAWP02500) prepared on teflon superfusion units similar in design to those described [11]. Synaptosomes superfusion was carried out essentially by the method described [6,11], except that the temperature was kept at 25°C. After a double wash by vacuum suction the plated synaptosomes were perfused by the constant suction of a peristaltic pump run at about 40 ml/h. About 0.7 ml effluent was collected, every minute, directly in the scintillation vials and the radioactivity counted in 8 vol. Aquasol. Since the presence of 10 μM AOAA was kept constant throughout the experiment the radioactivity released represents more than 95% total γ-amino [^{14}C]butyrate as assayed by amino acid analysis [6].

For Con A experiments, the final synaptosomal suspension was incubated in the presence of Con A (A grade Calbiochem), for 30 min, at 25°C in a water bath under mild agitation (100 rev/min, New Brunswick Scientific Co.). The final Con A concentration was 0.1 μM. The Con A treated synaptosomes were washed twice with incubation medium by successive centrifu-

gation at 40 000 X *g* for 15 min and the final pellet resuspended in incubation medium. The uptake and superfusion of radioactivity GABA was performed as mentioned above.

As a control synaptosomes were treated exactly the same way except that 0.2 M α-methyl-D-mannoside, a competitive inhibitor of Con A binding to sugars, was included in the Con A pretreatment step. Preliminary experiments indicated that Con A-treated synaptosomes take up GABA as efficiently as untreated ones. With the exception of the experiments in presence of Con A for which an incubation medium free of glucose was used, the composition of incubations and perfusion media was : 128 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.4 mM $MgCl_2$, 10 mM glucose and 20 mM *N*-2-hydroxyethylpiperazine-*N*-2 ethanesulfonic acid (Hepes) buffered at pH 7.4. All solutions were prepared in deionized, glass-distilled water from analytical grade reagents.

Protein was estimated by the method [12] after precipitation with 100% trichloroacetic acid to eliminate sucrose and Hepes. Each experimental result presented in this paper was obtained at least three times on duplicate samples: representative experiments are illustrated.

3. Results

As shown in fig.1, BWSTx markedly stimulated the release of γ-amino [^{14}C]butyrate. In the 3–5 min perfusion during which BWSTx was present at concentration varying from 0.05, 0.5 and 3 μg/ml, an increase in transmitter release from 1.5- to almost 5-fold that of controls was demonstrable. The effect of the toxin was rapid and the increase in transmitter release noticeable in the perfusate within 1–2 min after toxin addition and lasted for 5–6 min. In the same experimental conditions, a perfusing medium having 56 mM KCl replacing an equimolecular concentration of NaCl exerted a stimulatory effect on GABA similar in time response to that obtained with BWSTx.

In order to exclude that BWSTx action could be simply explained by synaptosomes disruption, lactate dehydrogenase activity was measured in the supernatant after treatment of synaptosomes with the toxin and compared with the activity released after treatment with 0.1% Triton X-100. After 20 min incubation lactate dehydrogenase activity was 3% that of Triton X-100 treated samples and 1.5 times that of untreated controls.

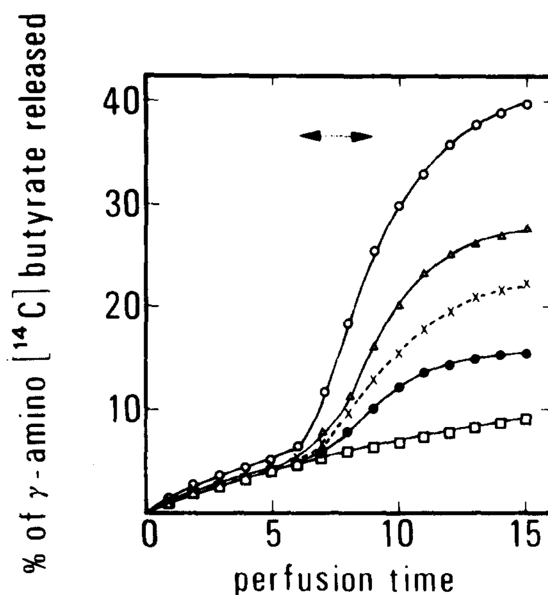

Fig.1. Effect of BWSTx on the release of γ-amino [^{14}C]butyrate from rat brain synaptosomes. After plating on Millipore filters, synaptosomes aliquots were filtered washed and superfused with incubation medium, as described in the text. At 7 min various concentrations of toxin were simultaneously introduced by a complete change of perfusing medium in parallel chambers. A medium containing 56 mM K^+ was used as an indicator that synaptosomes were viable and responded to depolarization. The double arrow indicates the period during which the toxin and high K^+ were present in the media perfusing synaptosomes. The superfusion rate was about 0.7 ml/min, and the radioactivity released is expressed as percentage of total radioactivity (fractions plus filter at the end of superfusion). (□——□) Control; (●——●) 0.05 μg BWSTx/ml; (△——△) 0.5 μg BWSTx/ml; (○——○) 3 μg BWSTx/ml; (X– –X), 56 mM KCl. Cumulative curves are given.

Figure 2 shows the effect of pre-treating the synaptosomes with Con A on the toxin-dependent release of γ-amino [^{14}C]butyrate. In one set of experiments synaptosomes treated with Con A and loaded with radioactive GABA were perfused and the effect of 1 μg/ml BWSTx and depolarizing concentrations of K^+ tested. Synaptosomes treated with the same amount of Con A but in presence of 0.2 M α-methyl-

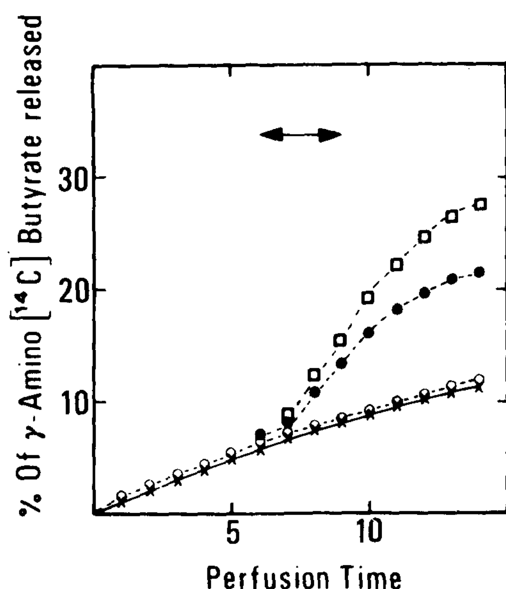

Fig.2. Effect of Con A pretreatment on the release of γ-amino [^{14}C]butyrate stimulated by BWSTx, in rat brain synaptosomes. The synaptosomal fraction was incubated with 0.1 μM Con A or 0.1 μM Con A in presence of 0.2 M α-methyl-D-mannoside. The synaptosomes were then centrifuged washed and incubated for γ-amino [^{14}C]butyrate uptake. Aliquots of the suspension were plated on Millipore filters, filtered washed and superfused with perfusion medium as described in fig.1. (X —X) Control synaptosomes pretreated with Con A; (○- -○) 1 μg BWSTx/ml on synaptosomes pretreated with Con A; (• - -•) 56 mM KCl on synaptosomes pretreated with Con A; (□- -□) 1 μg BWSTx/ml on synaptosomes pretreated with Con A but in presence of 0.2 M α-methyl-D-mannoside. The molecular weight of Con A was assumed to be that of the tetramer (110 000).

D-mannoside were used as controls. While Con A treated synaptosomes were susceptible to the depolarizing action of 56 mM KCl they were not susceptible to the toxin action and the consequent stimulation of GABA release was abolished. Control synaptosomes on the contrary were responsive to the toxin. Neither Con A nor α-methyl-D-mannoside added to the perfusion fluid had a striking effect on the release of GABA from perfused synaptosomes. Affinity chromatography experiments of the purified toxin run at 4°C, indicated that it has no specific affinity for Con A covalently bound to Sepharose 4B.

4. Discussion

Two main findings are reported here: one is that a toxin purified from black widow spider venom markedly increases the release from synaptosomes preparations of previously accumulated radioactive GABA, the second is that this effect is blocked by pretreatment of the synaptosomes with Con A. The effect on GABA release was obtained with low concentrations of toxin; a 0.38 nM solution of toxin, based on the est. mol. wt 130 000 [1,9] was still effective. The action of the toxin is also remarkably rapid since immediately after its addition to the perfusing medium an increased amount of transmitter was noticeable in the perfusate. It is also clear that in the experimental system used, depolarization achieved by superfusing synaptosomes with high K^+ medium, caused release of GABA with a pattern having a similar time-lag. The toxin does not appear to cause a non-specific membrane damage as judged by comparing the amount of lactate dehydrogenase released from synaptosomes after toxin with that induced by treatment with the detergent Triton X-100.

There is evidence that a given lectin reacts with a unique population of oligosaccharides and does not show common receptors with other lectins [13]. Con A binds to mannopyranosyl and glycopyranosyl residues, therefore treatment of synaptosomes with Con A probably brings to the formation of glycoconjugates with membrane proteins containing these sugars. The formation of these complexes prevents the releasing action of the toxin, but does not affect the release induced by depolarization. It is tempting to speculate the existence in the synaptic plasma membrane of a glycoprotein actin as a receptor for the toxin. The possibility that BWSTx has affinity for mannopyranosyl groups can however be ruled out since incubation of the toxin with exceeding concentrations of methyl-D-mannoside did not affect the venom action. It is more probable that as a result of the lectin–membrane interaction, changes in the arrangement of surface and glycoproteins occur [14]. This could prevent the interaction of the toxin with some membrane component; alternatively it could prevent either the modification or the redistribution of the same component, a step probably necessary for toxin stimulated release. BWSTx may provide a means of characterising a structure of the presynaptic mem-

brane which appears to be involved in transmitter release functions.

Acknowledgements

The authors wish to express their appreciation to Dr S. Alemà for his helpful comments and suggestions. They are greatly indebted to B. Capparella, S. Marinella, Italy, for collecting (Summer 1976) the female black widow spiders used in this study.

References

[1] Grasso, A. (1976) Biochim. Biophys. Acta 439, 406–412.
[2] Baba, A., Sen, I. and Cooper, J. R. (1977) Life Sciences 20, 833–842.
[3] Clark, A. W., Hurlbut, W. P. and Mauro, A. (1972) J. Cell. Biol. 52, 1–14.
[4] Kawai, N., Mauro, A. and Grundfest, H. (1972) J. Gen. Physiol. 60, 650–664.
[5] Cull-Candy, S. G., Neal, H. and Usherwood, P. N. R. (1973) Nature 241, 353–354.
[6] Levi, G. and Raiteri, M. (1974) Nature 250, 735–737.
[7] Levi, G. and Raiteri, M. (1976) Int. Rev. Neurobiol. 19, 51–74.
[8] Rubin, L. L., Gorio, A. and Mauro, A. (1976) Abstr. IVth Ann. Meet. Am. Soc. Neuroscience, Toronto (Canada) no. 1031 pag. 717.
[9] Frontali, N., Ceccarelli, B., Gorio, A., Mauro, A., Siekevitz, P., Mu-Chin Tzeng and Hurlbut, W. P. (1976) J. Cell. Biol. 68, 462–479.
[10] Gurd, J. W., Jones, L. R., Mahler, H. R. and Moore, W. J. (1974) J. Neurochem. 22, 281–290.
[11] Raiteri, M., Angelini, F. and Levi, G. (1974) Eur. J. Pharmacol. 25, 411–414.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 266–275.
[13] Lis, H. and Sharon, N. (1973) Annu. Rev. Biochem. 42, 541–574.
[14] Matus, A. I., De Petris, S. and Ratt, M. C. (1973) Nature 244, 278–280.