Biochemical Pharmacology, Vol. 21, pp. 969–974. Pergamon Press, 1972. Printed in Great Britain

EFFECTS OF BLACK WIDOW SPIDER VENOM ON ACETYLCHOLINE RELEASE FROM RAT CEREBRAL CORTEX SLICES *IN VITRO*

N. FRONTALI, F. GRANATA and P. PARISI
Istituto Superiore di Sanità, Rome, Italy

(*Received* 22 *July* 1971; *accepted* 8 *October* 1971)

Abstract—Addition of a whole extract of *Latrodectus mactans tredecimguttatus* venom glands to rat cerebral cortex slices incubated in an eserinized low-potassium saline solution elicits over the following hour a more than 2-fold increase in the amount of acetylcholine (ACh) released into the medium, and a corresponding decrease in the ACh content of the tissue. The total amount of ACh synthesized in 1 hr is not significantly altered; however, if the tissue is incubated in a high-potassium medium, the resulting enhanced ACh synthesis is markedly reduced. Protein concentrations of venom ranging around 15 μg/ml in the incubation medium are sufficient to elicit a full response. Neurotransmitter depletion of cholinergic nerve terminals may contribute to explain the intoxication symptoms in man and in other vertebrates.

THE VENOM of the black widow spider *Latrodectus mactans tredecimguttatus* has been shown to be active on different innervated vertebrate tissues *in vitro*. Cantore[1] found that its addition to the bath caused contraction of the rabbit ileum, which was reversible upon washing and was antagonized by atropine. Repeated additions of equal amounts of venom were followed by progressively decreasing responses, while the response to acetylcholine (ACh) remained unchanged. Recently Longenecker *et al.*[2] assayed this venom *in vitro* on the frog sartorius muscle. Addition of the venom was followed, after a lag of several minutes, by a striking increase in frequency of the miniature end-plate potentials, which reached a peak and then declined to the initial values in the course of the following hour. This phenomenon, which does not require the presence of calcium ions, is interpreted as a reaction of the venom with the nerve terminal membrane inducing the release of transmitter. Morphological changes have been shown[3] to be associated with these electrophysiological findings, namely, the complete disappearance of the synaptic vesicles from the end-plates in electron microscope preparations. Similar results have been obtained with cat muscle.[4] Paggi and Rossi[5] have studied the effects of black widow spider venom on the rat superior cervical ganglion *in vitro* (extracellular recordings). Addition of the venom to the bath elicited discharges of asynchronous action potentials from the unstimulated ganglion, while, upon preganglionic stimulation, the evoked postganglionic compound action potential was depressed. Ganglia previously incubated with labelled choline showed a marked decrease of labelled ACh content following venom addition.

In the present study direct measurements were made of ACh content and release from a different section of the nervous system, namely, from rat brain cortex slices incubated *in vitro* with the venom.

MATERIALS AND METHODS

Venom glands from adult females of *Latrodectus mactans tredecimguttatus* were collected in a mortar kept at 0°, as already described,[6] and ground with 1 ml ice-cold 0·9% NaCl/50 gland couples. The homogenate was centrifuged 15 min at 9000 *g* at 0°; the supernatant was stored at −20° for several weeks without loss of activity. This preparation is routinely indicated as "venom". Its protein content was measured with the method of Lowry *et al.*[7] Its toxicity on mice was determined on the basis of the LD_{50}, calculated with logarithmic/probability paper. Groups of mice (males weighing 18–22 g) were inoculated subcutaneously with 5 μl/g of different dilutions of the venom. The percentage of deaths was recorded after 48 hr. One toxicity unit (t.u.) was defined as the amount of venom corresponding to the LD_{50}/g of mouse.

Experiments in vitro. Male rats weighing approximately 200 g were decapitated after stunning. The brains were immediately removed and placed in ice-cold saline. Cerebral cortex slices[8] were quickly weighed and placed in vessels containing 2·5 ml of saline. The saline solution routinely employed had the following composition (mM): NaCl, 119·5; KCl, 4·2; $CaCl_2$, 1·3; $MgSO_4$, 1·2; Na_2HPO_4–HCl buffer pH 7·4, 16·3; glucose, 10; eserine salicylate, 0·04. When this solution was employed, the vessels were gassed with O_2. The "high-potassium" medium had the following composition (mM): NaCl, 98; KCl, 27; $CaCl_2$, 1·3; $MgCl_2$, 1·2; KH_2PO_4, 0·4; $NaHCO_3$, 24·5; glucose, 10; eserine salicylate 0·04. In this case the vessels were gassed with a mixture of 95% O_2 and 5% CO_2. Incubation was performed in an oscillating water bath at 37°. The solutions to be tested were added after 30 min preincubation, to allow for complete inhibition of the cholinesterase activity of the brain. Before and immediately after the incubation period the vessels were kept in an ice bath.

At the end of the incubation period, measured aliquots of the medium were acidified to pH 2·5 with HCl, heated for 1·5 min in a boiling water bath, cooled, neutralized with NaOH, centrifuged and employed for ACh assay. The tissue was dropped into 2·5 ml of fresh saline solution acidified to pH 2·5 with HCl and kept in a boiling water bath; heating was continued for 1·5 min, then the mixture was cooled, homogenized and treated as above. For determination of ACh content in the medium plus tissue the content of the vessels was treated as described for the tissue in fresh saline solution.

The ACh assay was performed biologically on frog rectus abdominis muscle.[9] Controls were performed for the presence of sensitizing substances.[10]

RESULTS

A series of control experiments was performed preliminarily. In the absence of the tissue, but in the presence of maximal venom concentrations, the medium, following treatment with heat in acid *milieu* (which serves also to inactivate the venom), was not found to elicit any response in the frog muscle, nor to modify its response to ACh. ACh, added to the medium at the beginning of incubation, was quantitatively recovered at the end, showing that the concentration of eserine employed is sufficient to completely inhibit the cholinesterase activity of the tissue, and that the treatment of the sample allows a good recovery of ACh. In the absence of eserine, the medium was found to be completely inactive on the frog muscle at the end of incubation (either with or without the venom). Treatment of the eserinized medium for 5 min in a boiling

water bath at pH 11 after incubation (with or without the venom) inactivated it completely.

In a first series of experiments a high concentration of venom was added to the incubation medium, namely, 10 μl/ml, which corresponded to 0·5 couples of glands, to 75 μg of protein and to 120 mouse t.u./ml. ACh content of the medium and of the tissue was measured separately at two incubation time intervals, 30 and 60 min. The results are shown in Fig. 1. Addition of the venom was followed by an approximately 3-fold increase of the amount of ACh released into the medium, while the ACh content of the tissue was correspondingly lowered. On the basis of the preliminary experiments reported above, we have considered as ACh the additional amount of ACh-like substance released into the medium as a consequence of venom addition.

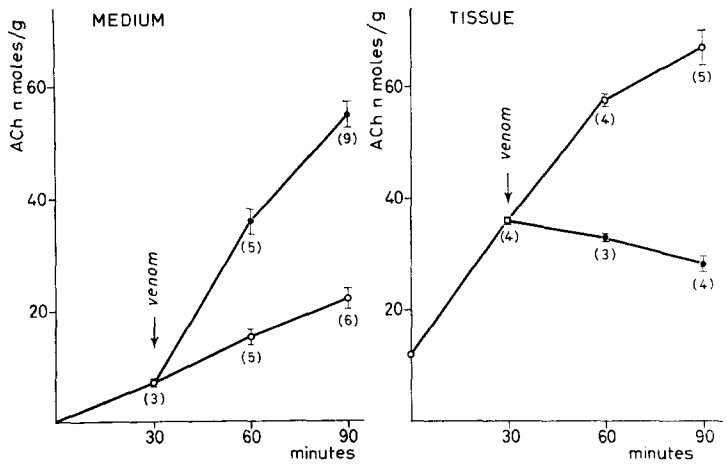

FIG. 1. Effect of venom addition to cortex slices incubated in low-potassium medium on the ACh content (±S.E.M.) of the medium and of the tissue after 30 min preincubation. Controls = white circles; with venom addition = black circles.

The total amount of ACh synthesized by the tissue in 1 hr (cross-striated columns in Fig. 2) is unaffected in the presence of the venom ($P > 0{\cdot}05$): what is drastically modified is the distribution of the transmitter between the tissue and the medium (white and striped columns in Fig. 2).

Addition of 2 μl of venom/ml (corresponding to 0·1 gland couples, to 15 μg of protein and to 24 mouse t.u./ml) was followed by a 2- to 3-fold increase in ACh release; addition of an amount of venom 10 times lower (1·5 μg protein/ml) had no effect on ACh release.

The total ACh content in the medium plus tissue was measured before and at different time intervals after the addition of the venom (0·5 gland couples/ml) to cerebral cortex slices incubated in a "high-potassium" medium. Vessels containing slices from one hemisphere served as controls for vessels containing the slices from the controlateral hemisphere which were treated with the venom (Fig. 3). The formation of new ACh in these conditions of potassium-stimulated synthesis is definitely decreased by the addition of the venom.

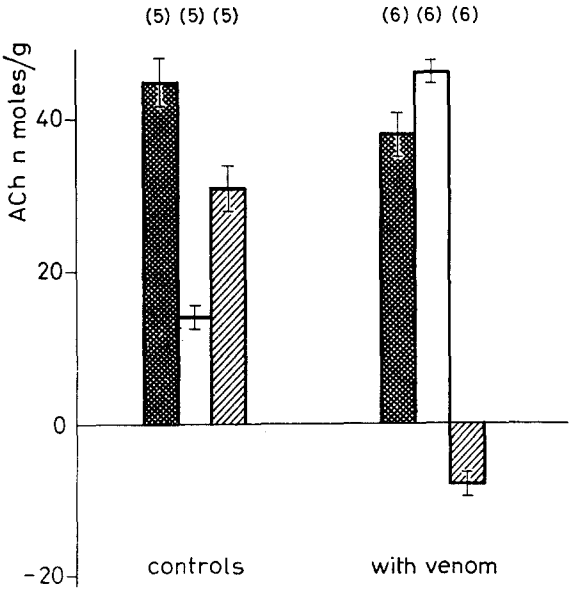

FIG. 2. Change in ACh content (±S.E.M.) of medium plus tissue (cross-striated columns), medium (white columns) and tissue (striped columns) after 1 hr, as a consequence of venom addition (low-potassium medium).

DISCUSSION

Addition of black widow spider venom elicits a striking increase in the output of ACh from rat cerebral cortex slices into the medium, when this contains a physiological concentration of KCl. At this KCl concentration, ACh release ranged in control vessels around 15 nmoles/g tissue in 1 hr. After addition of the venom in amounts as low as 15 μg protein/ml, the release of ACh was increased by a factor of

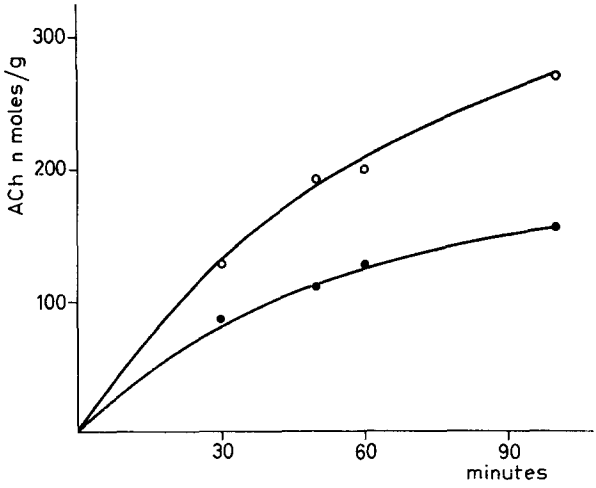

FIG. 3. Venom addition (after 30 min preincubation) to brain cortex slices incubated in a high-potassium medium. Effect on the total ACh content of medium plus tissue. Controls = white circles; with venom addition = black circles.

between 2 and 3. The meaning of this increase in ACh output into the medium is explained by the parallel determinations of ACh content in the tissue (Fig. 1). The increase in ACh output is not supported by an increase in ACh synthesis; on the contrary, the tissue is correspondingly depleted. As a result, at physiological KCl concentrations, the addition of the venom does not significantly alter the total amount of ACh, newly synthetized in 60 min incubation (Fig. 2). The venom therefore seems to interfere rather with the distribution of ACh between tissue and medium, i.e. with its release. This is in good agreement with the interpretation Longenecker *et al.*[2] give of their electrophysiological work on frog neuromuscular junctions and with the findings on the disappearance of the synaptic vesicles from the end-plates of frog[3] and cat muscle[4] treated with the venom.

In the conditions of stimulated ACh release and synthesis obtained by incubating cerebral cortex slices in a "high potassium" medium, the addition of the venom was followed by a marked reduction in ACh synthesis. It has been shown[2] that a burst of increased frequency of the miniature end-plate potentials may be recorded from frog muscle end-plates as a consequence of venom application also in the complete absence of calcium ions; the authors therefore consider it unlikely that this effect is caused entirely by a depolarization of the nerve ending membrane. It is well known that a high potassium concentration of the medium stimulates instead transmitter release through a physiological mechanism requiring calcium ions.[11] (Formation of new ACh is stimulated secondarily, in all probability as a consequence of its lowered concentration at the site of synthesis.[12]) This physiological mechanism is therefore completely different from that whereby black widow spider venom increases transmitter release. This difference may help to explain why the presence of the venom does not enhance the effects of potassium on ACh release from cerebral cortex slices, but actually counteracts it. If we suppose that also in the brain cortex the venom causes a disintegration of the synaptic vesicles, and these are not rebuilt (at least for many hours), it is not surprising to find that also ACh synthesis might be secondarily reduced, as a consequence of disruption of its storage sites. The present work deals only with short term effects, and is therefore centered on ACh release; but if the depleted storage sites are not restored, the release should be followed by a block of ACh release. The stimulation obtained with the potassium-rich medium probably allows this situation to be reached more quickly.

The mechanism whereby black widow spider venom acts on the cholinergic nerve terminals in the rat cerebral cortex is therefore probably basically the same as on vertebrate neuromuscular junctions[2] and on the nerve terminals in the superior cervical ganglion of the rat,[5] which are also cholinergic. The venom concentrations in the physiological media employed by these authors are similar to ours, i.e. about 0·1 gland couples/ml; concentrations ten times lower were inactive also in their hands. The concentrations found to be active *in vitro* are only one order of magnitude higher than those which are lethal to mice *in vivo* (for this calculation an even distribution of the venom throughout the body has been supposed).

Of the many types of nerve endings contained in the brain cortex the present work considers only those containing ACh; it could be that the venom acts in the cortex on the release of other neurotransmitters too, as shown for peripheral nerve fibres (rat iris adrenergic fibres[16]).

The symptoms of latrodectism in vertebrates can all be basically reconducted to an

impairment of the central and the peripheral nervous system.[13] The hypothesis of a systemic disturbance of cholinergic and adrenergic nerve terminals, where the release of transmitters is first increased and then blocked, may help to understand the mechanisms underlying these symptoms. Also the contracting effect of the venom on rabbit ileum *in vitro*[1] could be due to transmitter release.

Other animal venoms seem to elicit similar effects on vertebrate preparations *in vitro*: the venom of the scorpion *Tityus serrulatus*,[14] and a toxin (β-bungarotoxin) which has been separated from the venom of the snake *Bungarus multicinctus*.[15]

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