APAMIN FROM BEE VENOM.* EFFECTS OF THE NEUROTOXIN ON SUBCELLULAR PARTICLES OF NEURAL CULTURES

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1. Introduction

Apamin, the minor active component of bee venom, is a low molecular weight peptide containing 18 amino acid residues of which 4 are half-cystines. The peptide was first isolated by Habermann and Reiz [2] and later by Shipolini and his colleagues [3]. The primary structure was elucidated in the former laboratory [4] and finally established in the latter laboratory [3,5].

Apamin is strongly basic like melittin [6], the toxic main peptide from bee venom, but in contrast to melittin [7], apamin shows no cytolytic activity against human erythrocytes. Apamin has excitatory neurotoxic effects on the central nervous system and when lethal or sublethal doses are intravenously injected into mice it causes extreme uncoordinated hypermotility, clonic convulsions, followed by respiratory distress and death. The LD_{50} is in the range of 4–5 mg/kg of body weight. In previous microscopic studies [1], it was shown that when cultures of the cerebral cortex of the embryonic mouse were treated with apamin, the almost immediate changes observed were that the neurons became round and the processes of the cells were lost or fully retracted (after 30 min of incubation). Further incubation (1,5 hr) showed reappearance of the processes, and they became longer than normal after 3 hr of incubation. This step was followed by a period of proliferation (after 4–6 hr), more cells coming out of the explant, and deformation of the neurons became apparent after 8 to 10 hr of incubation. 24 hr later there was a definite deformation of the neurons. The cells were enlarged and the processes were retracted or cut short. To promote our knowledge at the ultrastructural level and in particular to study the mode of action of this neurotoxin under experimental conditions, apamin was applied to cultures of the cerebral cortex of the mouse which subsequently were studied by electron microscopy.

* Part II. Previous communication see [1].

2. Materials and methods

Apamin was purified by ion exchanger and gel filtration according to Shipolini et al. [3]. All other chemicals and Leighton tubes [1] were purchased from Flow Laboratories, Bonn, Germany. The nutrient medium contained 60% Ham-F 10 medium [8], 20% foetal bovine serum, 4% NCTC 109 medium [9], 5% lactalbumin (0.5 g in 100 ml of Hank's solution), 10% bovine amniotic fluid, 1% glucose (0.6 g in 100 ml twice distilled water), few drops of $NaHCO_3$ (0.5 g in 10 ml twice distilled water) and Hepes buffer [1] to adjust the pH to 6.7. The constituents were aseptically transferred into a flask, mixed and filtered through a Sartorius millipore filter SM 1624 (pore size: 0.45–0.20 μm). The cortex was prepared

as previously described [1]. The 1 mm^2 explants were placed at about 1 cm distance apart. A drop of Simms balanced salt solution [10] was added to avoid drying. A piece of pretreated cellophane was placed on top of the coverslip and the coverslip was then transferred in the Leighton tube by means of a forceps. 1 ml of nutrient medium was added and the cultures were incubated at 36°C. Every 3 days the old medium was replaced by fresh one. After 14 days of growth the nutrient medium of the cultures was replaced by 1 ml of medium containing 4 μg/ml of apamin. Controls were also included. The cultures were stained with the Giemsa staining technique for light microscopic studies and photographed every 60 min.

For electron microscopic studies only the acute changes were used. Controls and incubated cultures containing 4 μg/ml of apamin were treated in a lying position as following. The cellophane was removed and the nutrient medium with or without apamin was replaced by balanced salt solution. The cultures were washed several times. The salt solution was then replaced by 2.5% glutaraldehyde in buffer, pH 7.4. After 10 min the temperature was reduced to 4°C and the cultures were left in the fixative for 1 hr. After several rinses in cold buffer the cultures were post-fixed in 2% OsO_4–sucrose mixture for 1 hr. Dehydration was carried out in alcohol. The coverslips were then removed from the Leighton tubes and embedded in Epon 812 (Polymer) on a slide. Following polymerization, the coverslips were examined under the light mocroscope and 2 mm square blocks were cut off and mounted on empty Epon pyramids. Ultrathin sections were sliced using an LKB Ultratom. They were then examined using a Zeiss 9 S-2 Electron microscope, stained with lead citrate [11] and photographed.

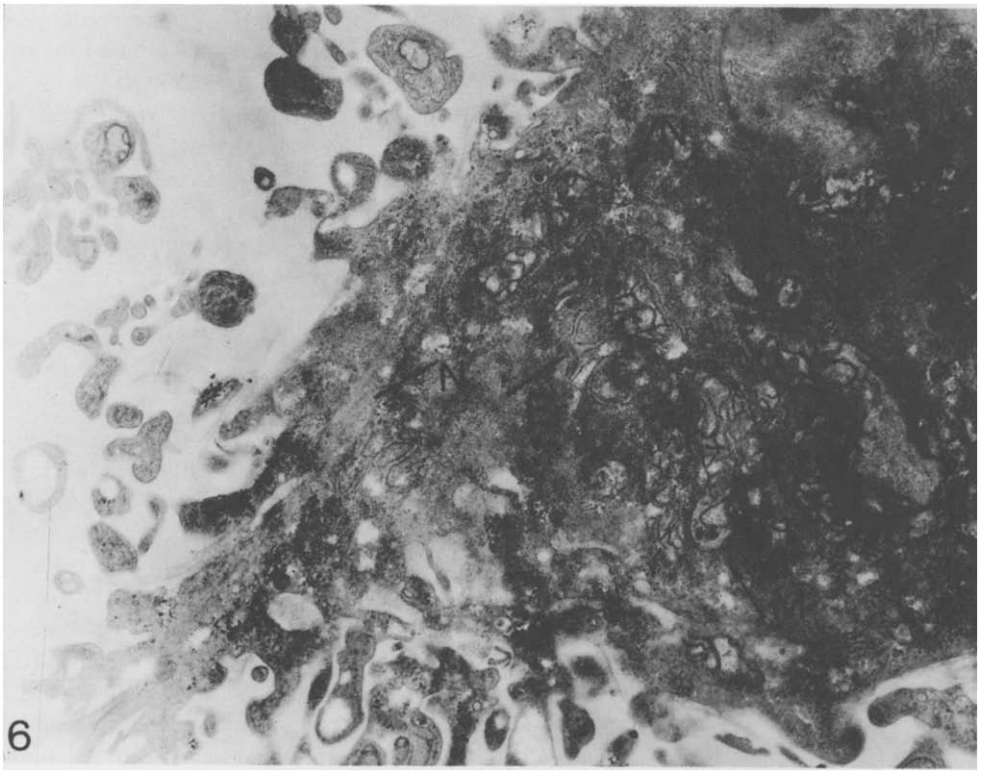

Fig.1. Electron micrograph of neural cultures after incubation with 4 μg/ml of apamin for 10 hr. Note the disordered Nissl bodies (N), the widened and curved cisternae of the endoplasmatic reticulum (Er). The mitochondria (M) are swollen, vacuolated with disrupted internal and external membranes, × 4900.

3. Results and discussion

3.1. *Light microscopy*

When cultures of the mouse cerebral cortex are incubated with 4 μg/ml of apamin the effects, demonstrated in the previous studies [1] using 50 μg/ml of apamin are also evident.

3.2. *Electron microscopy*

No degenerative changes could be observed before 8 to 10 hr of incubation with 4 μg/ml of apamin. The first changes are predominantly found in the subcellular organelles of the cells observed. 15 treated cultures and 15 controls were used and the average number of cells examined per culture were 70. The following phenomena could be seen in all cells studied.

The Nissl substance (N) is broken up, the mitochondria (M) are swollen and vacuolated and show disrupted internal and external membranes. The cisternae of the endoplasmatic reticulum (Er) are widened and are not arranged in parallel arrays (fig.1). Some cells show an increased amount of osmiophillic bodies (O), perhaps remnants of ruptured lysosomes, and vacuolation (V) of the cytoplasm is often observed (fig.2). After 24 hr of incubation the cell membrane has disappeared. A deeply infolded nucleus (Nu) and cell organelles in a non-defined surrounding are observed. The mitochondria (M) with vacuoles and disrupted cristae, elements of the Golgi complex (G) and some lysosomes (L) are clearly visible. Only remnants of the endoplasmatic reticulum (Er) can be seen (figs. 3 and 4).

We assume that the use of tissue culture, allowing the study of the reactions of living neurons and glial cells is specially rewarding, although a differentiation between neurons and astrocytes is difficult at light microscipical level, as both cell types present similar pictures. However electron microscopical studies allow

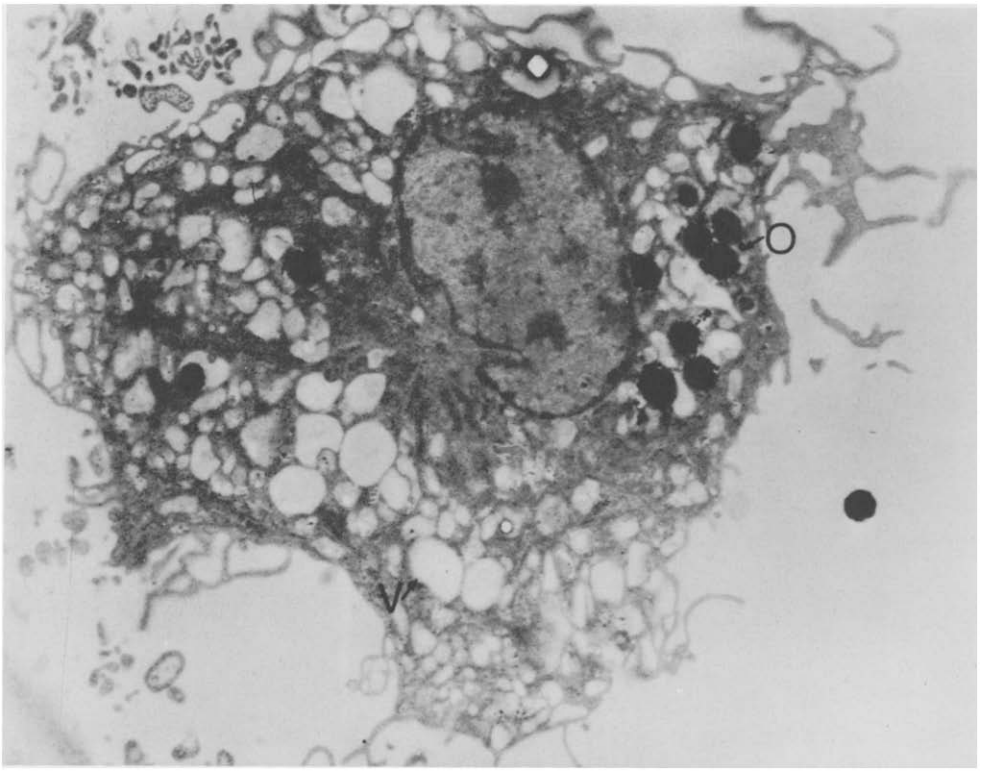

Fig.2. Another neuron from a treated culture showing a highly vacuolated cytoplasm (V), and an increase in the number of osmiophilic bodies (O), × 2900.

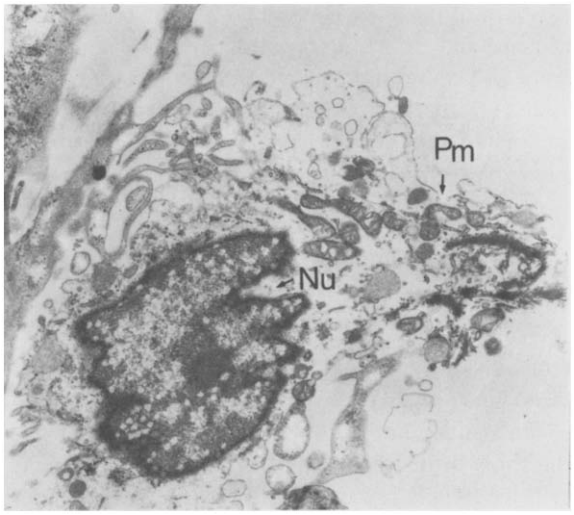

Fig. 3. After 24 hr of application with 4 μg/ml of apamin. Note the deeply infolded nucleus (Nu) and the destruction of the cell membrane (Pm), × 2500.

a separation of the two cell types. Neurons have a larger nucleus and contain well developed endoplasmatic reticulum (Nissl bodies), while astrocytes possess significant numbers of thick bundles of glial filaments and contain glycogen granules [12]. The above decribed phenomena were observed in both cell types. In the intact animal apamin known to enter the central nervous system may act locally on the neurons, the cell membrane in particular, causing increased permeability and depolarization and thus possibly may account for hypermotility and convulsion. It will be of interest to study the effect of apamin on isolated lysosomes and other neuronal cultures, which have been treated with membrane stabilizing agents, e.g. anticonvulsive drugs.

Apamin resembles in its amino acid composition the snake venom neurotoxins, which are highly basic single chain polypeptides. They consist of 60–74 amino acid residues and a high half-cystine content

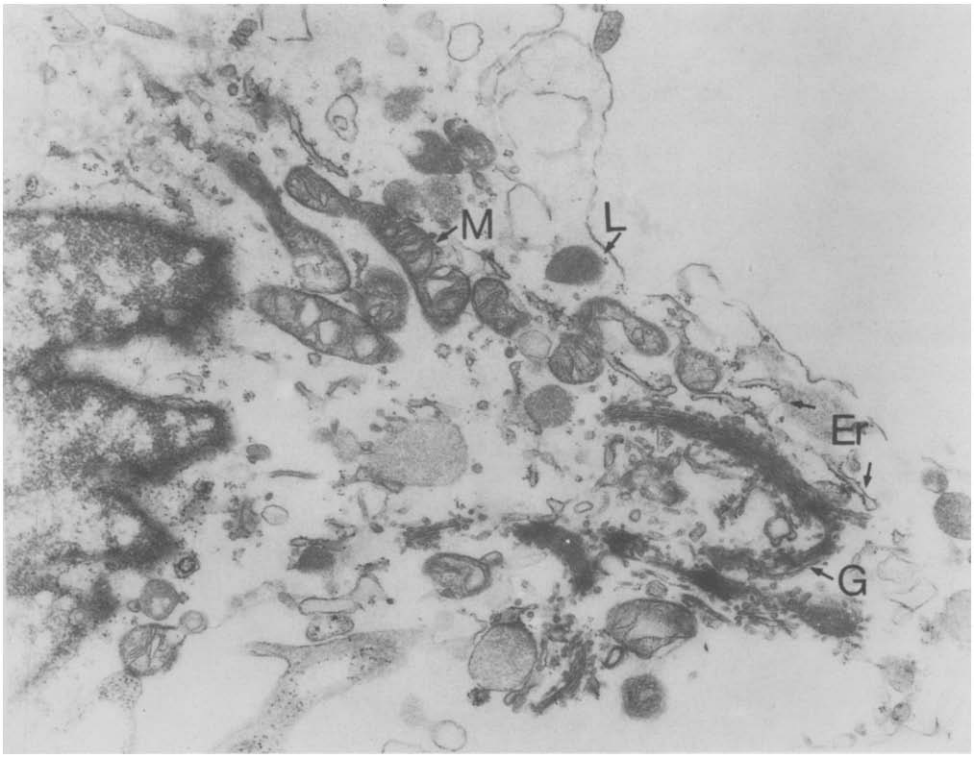

Fig. 4. Another neuron shows mitochondria with vacuoles, deformed and disrupted cristae (M), remnants of the endoplasmatic reticulum (Er), intact Golgi complex (G) and finely granular osmiophilic bodies, probably lysosomes (L), in a non defined surrounding, × 4900.

[13,14]. The presence of 4 half-cystine residues (22%) in apamin indicates a high degree of intramolecular crosslinking. The toxin is highly basic: 4 of the 18 amino acid residues (22%) are basic ones and almost all of the acidic residues are in the amide form. Apamin differs from snake venom toxins and bee venom melittin [6,15] in its biological activity, in the content of hydrophobic amino acids (28%) and in its amino acid arrangement. In the strongly basic melittin the amino acid residues are arranged as in a peptide-invert soap [15]. But apamin shows no detergent-like properties against red blood cells.

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