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Brief Communication:

© ULTRASTRUCTURAL EFFECTS OF THE VENOM OF THE SMALL-SCALED SNAKE (*PARADEMANSIA MICROLEPIDOTUS*) ON THE NERVE TERMINALS OF THE RAT DIAPHRAGM

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Summary. Venom of the small-scaled snake (*Parademansia microlepidotus*) causes a large increase in the number of coated omega figures inserted in the axolemma of nerve terminals. It also causes both a loss of synaptic vesicles and an increase in the number of neurofilaments in the nerve terminals. The actions of the venom are very similar to taipoxin, a pre-synaptic neurotoxin from the venom of the taipan (*Oxyuranus scutellatus*). The morphological changes in the nerve terminals caused by the small-scaled snake venom are not unusual and do not explain the extreme lethality of the venom.

INTRODUCTION

For nearly a century there has been confusion over the existence of the small-scaled snake, *Parademansia microlepidotus*, as a species distinct from the taipan, *Oxyuranus scutellatus*. Recently, Covacevich and Wombey (1976) established that *P. microlepidotus* is a valid species and not a geographical variant of *O. scutellatus*. At least ten small-scaled snakes have been captured and milked so that their venom could be studied in the laboratory. Studies of this venom have strengthened the opinion that *P. microlepidotus* is a distinct species (Sutherland *et al.*, 1978; Broad, Sutherland, Tanner and Covacevich, 1979). These studies have shown that the venom of the small-scaled snake is the most toxic (as indicated by mouse lethality) terrestrial snake venom found to date (Broad, Sutherland and Coulter, 1979).

Taipoxin, a neurotoxin isolated from the venom of the taipan, *O. scutellatus*, causes a great increase in the number of "coated omega figures" in the axolemma of nerve terminals when it is applied to isolated phrenic nerve hemi-diaphragm preparations (Cull-Candy *et al.*, 1976). As the small-scaled snake is closely related to, though distinct from, the taipan and since it has an extremely potent venom, we investigated whether this venom caused any ultrastructural alterations to motor nerve terminals.

MATERIALS AND METHODS

Phrenic nerve hemi-diaphragm preparations from hooded rats (CSL strain) were set up in Krebs solution maintained at 37° and bubbled with carbogen. The hemi-diaphragms were continually stimulated via the phrenic nerve with single electrical pulses of 1 volt for 0·1 to 1 m sec at a rate of six pulses per minute. *P. microlepidotus* venom was added to the bath to attain a final concentration of 0·5 μg/ml. In 90 to 120 min, when the hemi-diaphragms no longer contracted after the phrenic nerve was stimulated, they were fixed for electron microscopy with 2·5% (v/v) glutaraldehyde in 0·1 M

cacodylate buffer (pH 7·2), containing 2·5 mM $CaCl_2$, at room temperature for at least 4 h. Control hemi-diaphragms, which were still contracting after 120 min stimulation, were also fixed. The end plate zone was dissected from the fixed hemi-diaphragms and cut into small blocks which were washed overnight in 0·1 M cacodylate buffer (pH 7·2). Washed blocks were fixed in 1% (w/v) osmium tetroxide in 0·1 M cacodylate buffer (pH 7·2) at 0° for 1 h after which they were block stained in 2% (w/v) uranyl acetate for 2 h at room temperature, dehydrated in an acetone series and embedded in Durcupan ACM (Fluka). Ultrathin sections, cut on an LKB Ultratome III, were stained with lead citrate for 5 min at room temperature before they were examined with a Philips EM301 electron microscope. Blocks from 5 venom treated hemi-diaphragms and 2 control hemi-diaphragms were examined.

RESULTS

The ultrastructure of motor nerve terminals in the rat diaphragm has been described (Winckler and Foroglou-Kerameos, 1971; Korneliussen, 1972). Typically motor nerve terminals contain numerous synaptic vesicles and some mitochondria, and a nerve terminal from a control hemi-diaphragm illustrates these points (Fig. 1). "Coated omega figures" (or coated micropits), "coated vesicles" (or complex vesicles), and "empty shells" are present in normal motor nerve terminals (Korneliussen, 1972). We did not observe these structures in our control material as frequently as Korneliussen (1972). Heuser and Reese (1973) suggested that "coated omega figures" are the first stage in the endocytosis of membrane from the axolemma. After endocytosis the "coated omega figures" become "coated vesicles" which later lose their "coats" and are reprocessed to become synaptic vesicles. "Empty shells" are pieces of "coat" material that are not surrounding vesicles.

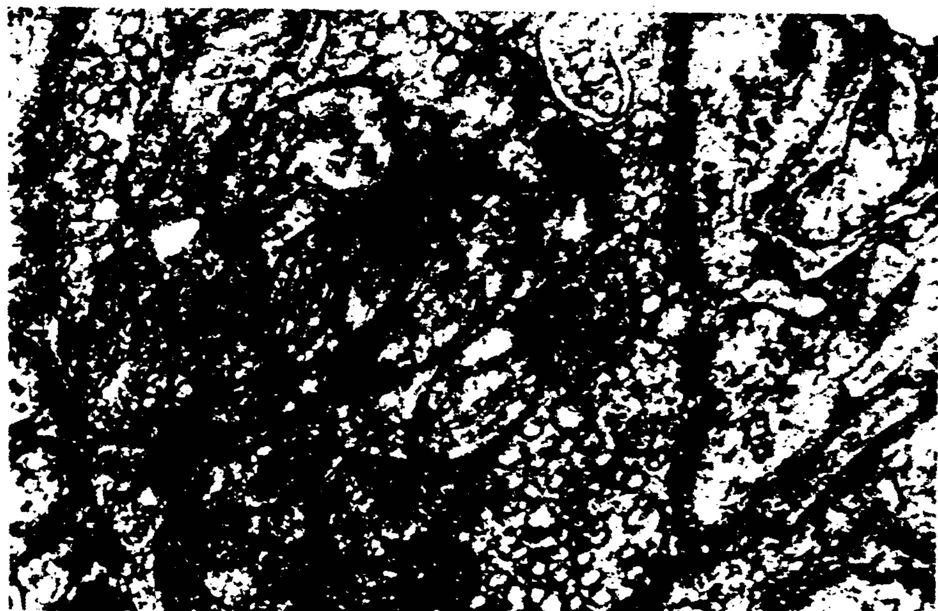

Fig. 1. Electron micrograph of a motor nerve terminal from a rat diaphragm stimulated *in vitro* for 120 min. Note the numerous synaptic vesicles (S). 65,000 ×.

The nerve terminals that had been exposed to the *P. microlepidotus* venom showed a large increase in the number of "coated omega figures" which were inserted in the axolemma (Fig. 2). These "coated omega figures" were not only adjacent to the synaptic cleft, but also were adjacent to the Schwann cell

enveloping the axon terminal. They were present throughout the axolemma in the axon terminal but were not present in the myelinated pre-terminal axon. As well as the increase in the numbers of "coated omega figures", there was a decrease in the number of synaptic vesicles within the terminals and an increase in the number of neurofilaments within the terminals.

Fig. 2. Electron micrograph of a motor nerve terminal from a rat diaphragm treated *in vitro* with whole *P. microlepidotus* venom. Note the numerous coated omega figures (arrows) in all parts of the axolemma, the few synaptic vesicles (S), and the numerous neurofilaments (N). 65,000 ×.

DISCUSSION

The ultrastructural actions of *P. microlepidotus* venom are very similar to those demonstrated for the purified neurotoxins beta-bungarotoxin, a neurotoxin isolated from the venom of the krait *Bungarus multicinctus* (Tsai, Chang and Lee, 1976), taipoxin, and notexin, a neurotoxin isolated from the tiger snake *Notechis scutatus* (Cull-Candy *et al.*, 1976). It has been possible to demonstrate ultrastructural presynaptic actions of a whole snake venom on an isolated nerve-muscle preparation *in vitro*. It was not necessary to fractionate the venom to demonstrate its ultrastructural pre-synaptic activity

Covacevich and Wombey (1976) established that *P. microlepidotus* is a species separate from *O. scutellatus*, and Sutherland *et al.* (1978), Broad, Sutherland and Coulter (1979) and Broad, Sutherland, Tanner and Covacevich (1979) have shown that *P. microlepidotus* venom is the most potent venom derived from a terrestrial snake. However, its pre-synaptic action, as demonstrated here, is not unique and is similar to several other elapid venoms. Therefore, its extreme potency cannot be explained by any unique mechanism of action on nerve terminals, which is demonstrable by electron microscopy.

Heuser and Reese (1973) have suggested that "coated omega figures" and "coated synaptic vesicles" are membranes that are undergoing endocytosis and are an important stage in the recycling of synaptic vesicle membranes. *P. microlepidotus* venom, like the other elapid pre-synaptic toxins, causes an enormous increase in "coated omega figures" inserted in the axolemma. It may be that the venom slows down the recycling of synaptic vesicle membranes. The venom may slow down the pinching-off, or endocytosis of the "coated omega figures", to become "coated vesicles" within the nerve terminals.

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The venom may be involved in receptor mediated endocytosis (Goldstein, Anderson and Brown, 1979), binding to a receptor on the nerve terminal membrane, being internalized, and then exerting its toxic action within the nerve terminal.

When there was a reduction in synaptic vesicle numbers from the nerve terminals there appeared to be an increase in the number of neurofilaments within the terminals. Perhaps the filaments were always present and only became apparent when vesicle numbers were significantly reduced. The filaments may be associated with a homeostatic mechanism which attempts to maintain synaptic vesicle numbers within the nerve terminals.

Fohlman (1979) fractionated *P. microlepidotus* venom by gel filtration and compared the results with those obtained with gel filtration of *O. scutellatus* (taipan) venom. The chromatograms were very similar and a taipoxin analogue paradoxin was purified from the venom. It had an amino-acid analysis very similar to taipoxin. Fohlman (1979) speculated that the higher toxicity of *P. microlepidotus* venom when compared with taipan venom may be due to its higher content of "notexin-like" molecules. However, Coulter (personal communication) found very little cross-reaction between anti-notexin antibodies and taipan venom when tested by radio immunoassay. This suggests there are few, if any, "notexin-like" molecules in taipan venom. Because notexin and taipoxin have similar ultrastructural effects on nerve terminals (Cull-Candy *et al.*, 1976), a venom which contains a toxin analogous to taipoxin and perhaps contains toxins analogous to notexin would not be expected to have any different ultrastructural effects.

If the "coated omega figures" do represent receptor mediated endocytosis of pre-synaptic neurotoxin, the "coated omega figures" would be the last stage at which antivenom could neutralize these venom components. Once the neurotoxin was within the terminal it would be unavailable to neutralization by antivenom. Recovery of the injured nerve terminal after this stage would presumably involve the nerve terminal healing itself, perhaps, by axonal sprouting, or perhaps by axonal transport of undamaged components to the injured nerve terminal. However, this does not mean that antivenom should be withheld from severely envenomated patients, as the antivenom would still be of use mopping up circulating venom and neutralizing post-synaptic neurotoxins.

REFERENCES

BROAD, A. J., SUTHERLAND, S. K., and COULTER, A. R. (1979): *Toxicon*, **17**, 661.

BROAD, A. J., SUTHERLAND, S. K., TANNER, C., and COVACEVICH, J. (1979): *Mem. Queensl. Mus.*, **19**, 319.

COVACEVICH, J., and WOMBEY, J. (1976): *Proc. R. Soc. Queensl.*, **87**, 29.

CULL-CANDY, S. G., FOHLMAN, J., GUSTAVSSON, D., LULLMAN-RAUCH, R., and THESLEFF, S. (1976): *Neuroscience*, **1**, 175.

FOHLMAN, J. (1979): *Toxicon*, **17**, 170.

GOLDSTEIN, J. L., ANDERSON, R. G. W., and BROWN, M. S. (1979): *Nature*, **279**, 679.

HEUSER, J. E., and REESE, T. S. (1973): *J. Cell Biol.*, **57**, 315.

KORNELIUSSEN, H. (1972): *Z. Zellforsch.*, **130**, 28.

SUTHERLAND, S. K., BROAD, A. J., TANNER, C., and COVACEVICH, J. (1978): *Med. J. Aust.*, **1**, 288.

TSAI, M. C., CHANG, C. C., and LEE, C. Y. (1976): 'The relation between the blockage of transmitter release and the ultrastructural changes of the motor nerve terminals caused by β-bungarotoxin.' In "Memorial volume to President Chiang Kai Shek". S. L. Chien (ed.), Academia Sinica, Taiwan, p. 289.

WINCKLER, G., and FOROGLOU-KERAMEOS, C. (1971): *Acta Anat.*, **78**, 444.