Neurotoxicity of Ammodytoxin A in the Envenoming Bites of Vipera Ammodytes Ammodytes

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Abstract

Envenoming bites by Vipera ammodytes ammodytes (the long-nosed viper) can cause life-threatening neurotoxicity, particularly in children. We investigated the mechanisms of the neurotoxicity of ammodytoxin A, the principal toxin in the venom of these snakes, in isolated nerve-muscle preparations from mice. The toxin was bound selectively to the neuromuscular junction, and at concentrations similar to those likely to be found in the circulation of young bite victims, it blocked the response of the muscle to indirect but not direct stimulation. Electron microscopy showed that the toxin induced a small but insignificant depletion of synaptic vesicles from motor nerve terminals; nerve terminal mitochondria were swollen and damaged, but plasma membranes of terminal boutons were undamaged. Exposure to the toxin did not affect postsynaptic acetylcholine receptors or cause structural damage to preterminal motor axons or muscle fibers. Spontaneous transmitter release was similarly unaffected. Taken together, these results indicate that ammodytoxin A is the principal agent involved in the neurotoxic activity of the venom of V ammodytes ammodytes and that the underlying cause of the failure of transmission may be the de-energization of the nerve terminal resulting from mitochondrial degeneration and subsequent impairment of coupling between the action-potential-induced depolarization of the nerve terminal and the evoked transmitter release.

Key Words: Ammodytoxin A, Motor nerve terminals, Neuromuscular junction, Neurotoxicity, Vipera ammodytes ammodytes.

INTRODUCTION

Snakebite is commonly thought to be a problem only in parts of Latin America, Africa, and Asia. Current estimates by the World Health Organization for those regions are more than 5 million bites and at least 120,000 deaths per year. Although the risk of severe envenoming snakebites in Europe is relatively small, 6 recognized species of potentially dangerous viper are endemic. Three of these snakes are relatively uncommon, and interactions between them and the human population are rare. Vipera latastii is found only in Iberia, Vipera xanthina is found only in eastern Turkey, and Vipera ursinii is sparsely and discontinuously distributed through southern France, parts of Italy, Austria, and eastern Europe. The 3 most important vipers in Europe are Vipera berus, which is common across the whole of western and northern Europe; Vipera aspis, which is common to central and western Europe; and Vipera ammodytes, the long-nosed viper (also known as the Western sand viper, horned viper, nose-horned viper, or rhinoceros viper), which is largely confined to the Balkans and neighboring regions (1). The subspecies V ammodytes ammodytes (Fig. 1) is the most common viperid snake in the Balkans. It is 20 times more common than the common viper V berus berus, and it is the most dangerous of the European vipers. A recent retrospective analysis of 542 envenoming snakebites in southern Croatia (total population, 4500000) reported a mean annual incidence of bites of approximately 5 per 100,000. The most common signs and symptoms were coagulopathy, extensive swelling and edema, ecchymosis, and regional lymphadenitis, but clinical signs and symptoms of neurotoxicity such as ptosis, ophthalmoplegia, dysphagia, dysphonia, and neuromuscular weakness occurred in as many as 16% of the cases (2, 3). Neurotoxic snakebites constitute a medical emergency, and the signs and symptoms at presentation can progress from ptosis to profound neuromuscular weakness over a period ranging from 30 minutes to several hours. Treatment is often inappropriate and ineffective because the underlying pathology and pathophysiology are often poorly documented and misunderstood.

Two classes of toxin are generally considered to be involved in venom-related neurotoxicity following bites by viperid snakes: the presynaptically active phospholipases A2, which disrupt transmission at the motor nerve terminal (4), and the much rarer postsynaptically active phospholipases A2 (5, 6). The ammodytoxins, A, B, and C are the principal toxins of the crude venom of V ammodytes ammodytes. Ammodytoxin A, initially known as fraction k2, is the most toxic of the ammodytoxins, with an intravenous median lethal dose in mice of 0.021 μg/g (7). It comprises up to 30% of the protein content of the crude venom (B. Halasy, personal communication, 2008), and it has neurotoxic activity (8). It seems reasonable to postulate, therefore, that ammodytoxin A is primarily responsible for the neurotoxic signs and symptoms seen in many victims of severe envenoming bites by V ammodytes ammodytes.
In this study, we used electrophysiology, light and electron microscopy, and immunocytochemistry to investigate the mechanisms of acute neurotoxicity in mammalian neuromuscular systems exposed in vitro to ammodytoxin A. We discuss the possible role of ammodytoxin A in the expression of neurotoxic envenoming in patients bitten by *V ammodytes ammodytes* and the possible role of ammodytoxins in neurotoxic bites by a small subgroup of *V aspis* located in southeastern France (6).

### MATERIALS AND METHODS

#### Animals and the Nerve-Muscle Tissue

Adult female BALB/c mice obtained from Charles River Ltd (Margate, UK) were maintained and humanely killed according to the *Guiding Principles in the Use of Animals in Toxicology* (Society of Toxicology, 1999, Guidelines are available at www.toxicology.org) and the Animals (Scientific Procedures) Act 1986. Either soleus or diaphragm muscles were removed into a balanced salt solution (BSS composition [mmol/L]: NaCl, 118.4; NaHCO3, 25; glucose, 11; KCl, 4.7; MgSO4, 1.2; KH2PO4, 1.2; and CaCl2, 2.5) and bubbled with 5% CO2 in oxygen. Diaphragm muscles were prepared for functional studies (see Functional Studies below). Soleus muscles were mounted in a chamber containing BSS bubbled as above. Ammodytoxin A (726 nmol/L [10 μg/mL]) was introduced to the bath for 3 to 3.5 hours. Ammodytoxin A was not added to the control preparations. At the end of the incubation period, muscles were removed and processed for electron microscopy, immunocytochemistry, or cytochemistry (see Electron Microscopy, Immunocytochemistry and Cytochemistry below). Experiments were performed either at room temperature (19–22°C) or at 37°C. The neuropathologic consequences of the exposure did not vary according to temperature; therefore, data from these muscles are not further differentiated.

#### Functional Studies

Hemidiaphragms and accompanying phrenic nerves were dissected from freshly killed mice as described earlier and mounted in 10-mL organ baths containing BSS maintained at pH 7.2 to 7.4. Twitches of the muscle were evoked indirectly by stimulating the attached phrenic nerve at 0.2 Hz with pulses of 0.2-ms duration and a supramaximal voltage from a Grass S84 stimulator via a stimulus isolation unit, model SIU 5A (Grass Instrument Co, West Warwick, RI). For direct muscle stimulation, the muscle was stimulated at 0.1 Hz with pulses of 2-ms duration and a supramaximal voltage. When the twitch responses were stable, ammodytoxin A was introduced into the tissue bath. Control preparations were maintained in BSS alone.

### Toxins and Other Reagents

Ammodytoxin A was purified from *V ammodytes ammodytes* venom (9). Alexa546-AtxA was prepared by conjugating ammodytoxin A (200 μg) and Alexa546 succinimidyl ester (25 μg) in the presence of dimethyl sulfoxide (7.6 μL) and triethylamine (1.0 μL) at room temperature in the dark for 1 hour with constant stirring. The reaction was stopped by adding 15 μL of taurine (pH 8) followed by incubating for 30 minutes at room temperature. After labeling, the components of the conjugation mixture were separated by reverse-phase high-pressure liquid chromatography (Hewlett Packard series 1100 system, Hewlett Packard, Bracknell, UK) using an Agilent BU 300 column (30 × 4.6 mm, Perkin Elmer Brownlee, Waltham, MA). Conjugates were eluted at moderately separated peaks after the nonmodified toxin and were analyzed by electrospray ionization mass spectrometry (high-resolution magnetic-sector AutopsacQ mass spectrometer; Micromass, GU Instruments, Manchester, UK). Monodervative ammodytoxin A (Alexa546-AtxA) comprised approximately 60% of the total protein input and was characterized by its receptor binding properties, phospholipase activity (10), estimation of murine median lethal dose, and expression of neurotoxicity on a mouse phrenic nerve-hemidiaphragm preparation. Tetramethylrhodamine isothiocyanate-conjugated α-bungarotoxin, fluorescein isothiocyanate (FITC)-conjugated α-bungarotoxin, and Alexa Fluor566 carboxylic acid (succinimidyl ester) were obtained from Molecular Probes Inc, Invitrogen, Paisley, UK. Triethylamine (sequanal grade) was purchased from Pierce (Rockford, IL). Chicken polyclonal anti-neurofilament protein antibodies (Chemicon International, Temecula, CA) and rabbit polyclonal anti-synaptophysin antibodies (Neomarkers, Thermo Fisher Scientific, Runcorn, UK) were used as primary antibodies. Swine anti-rabbit FITC-conjugated polyclonal antibody and donkey anti-chicken FITC-conjugated polyclonal antibodies (DAKO, Ely, UK) were the secondary antibodies. All secondary antibodies were incubated with normal rat serum (DAKO) and centrifuged to yield a clear supernatant before use. Primary and secondary antibodies were routinely used in conjunction with 3% (wt/vol) bovine serum albumin and 0.1 M lysine and at a dilution of 1:100 or 1:1000. Vectashield HardSet mounting medium was supplied by Vector Laboratories (Peterborough, UK). All other reagents were obtained from regular commercial sources and were routinely Analar grade.
Intracellular Recording

The electric activity of the muscle was recorded using an Electro 705 unitary gain electrometer (World Precision Instruments, Sarasota, FL). Signals were amplified by a high-input impedance CED1902 amplifier (CED Ltd., Cambridge, UK), displayed and simultaneously stored on the hard disk of a Pentium (Intel Corporation, Santa Clara, CA) 200-MHz desktop computer for further analysis, with a suite of purpose-designed programs (see Electrophysiological Data Analysis below). Resting membrane potentials and miniature endplate potentials (mepps) were recorded by inserting a glass microelectrode (resistance, 10–15 MΩ; filling solution, 3 M KCl) close to endplate regions. Miniature endplate potentials with rise times 1 ms or less were recorded in control tissues or toxin-treated tissues. Recording sites were rejected if the membrane potential was less than $-60$ mV on the initial impalement or the membrane potential varied by more than 10% during the first minute of recording. Miniature endplate potentials were recorded for approximately 2 minutes before moving to a new recording site. Data were collected from between 6 and 12 cells from each preparation ($n = 3$ controls and 3 exposed to ammodytoxin A). At the end of the experiment, the nerve-muscle preparations were processed for electron microscopy.

### Electrophysiological Data Analysis

Abnormal signals due to electrical interference or spiking were rejected. Accepted signals were subject to further analysis using WinEDR V2.8.8 (Windows Electrophysiology Disk Recorder) or when appropriate using WinWCP V3.8.9 (Windows Whole Cell Programme). Miniature endplate potential amplitudes were normalized to a resting membrane potential of $-80$ mV, but because of their small sizes, correction for nonlinear summation was not required (11).

**FIGURE 2.** Ammodytoxin A caused a progressive fall in the amplitude of an indirectly elicited twitch response in the mouse hemidiaphragm after a latent phase lasting approximately 50 minutes. The response of control preparations was well maintained. Each point represents mean ± SE ($n = 3$). AtxA, ammodytoxin A.

**FIGURE 3.** (A) Miniature endplate potentials amplitude distributions in a control hemidiaphragm preparation (upper) and in a hemidiaphragm preparation after a transmission failure after exposure to AtxA (lower). Note the appearance of a population of very large mepps in muscle fibers exposed to AtxA (arrow). (B) Images of complex and very large mepps recorded after exposure to AtxA. Similar observations were made in 2 other control and ammodytoxin-treated preparations. mepps, miniature endplate potential; AtxA, ammodytoxin A.
One-Micrometer-Thick Sections and Electron Microscopy

Soleus muscles or longitudinal slivers of diaphragm muscles were pinned out at approximately 1.2 times the resting length and fixed in Karnovsky fluid (12) for 1.5 hours. They were then rinsed in phosphate-buffered saline (PBS, pH 7.4) and teased into bundles of 10 to 15 fibers. Tissue blocks 1 mm thick were cut out of the endplate containing region of the muscle, postfixed in OsO4, dehydrated in increasing concentrations of acetone, and embedded in TAAB resin (TAAB Laboratory Equipment, Berkshire, UK). One-micrometer-thick sections were cut and stained with toluidine blue, and ultrathin sections were cut at 50 to 70 nm (gold/silver in color) collected onto grids stained with uranyl acetate, hydrated and counterstained in lead citrate, and examined in a Philips E500 microscope (FEI, Oregon, USA). Neuromuscular junctions were identified by a colleague (Tracey Scott-Davey) who was blinded to the experimental status of the individual muscles. All junctions were photographed, provided that they were not obscured by grid bars and were sitting in a well-defined, deeply folded, postjunctional trough.

Because neuromuscular junctions in control soleus and diaphragm muscle fiber preparations were indistinguishable in all respects and junctions on muscles exposed to ammodytoxin A exhibited identical pathology, data from the 2 muscle sources are not further differentiated.

Immunocytochemistry

Transverse and longitudinal sections (6–10 and 15–25 μm thick, respectively) were cut from the belly of frozen soleus muscle and mounted onto chrome-alum-coated slides. The sections were permeabilized in absolute ethanol (10 minutes) followed by absolute methanol and 0.1% (vol/vol) Triton X-100 (Sigma Aldrich, Gillingham, Dorset, UK) (15 minutes) all at −20°C. The sections were then rinsed, air dried, and exposed to anti-synaptophysin and anti-neurofilament primary antibodies in a closed moist chamber at 4°C overnight. The sections were then rinsed and counterlabeled with the secondary antibodies (see above) in PBS containing 3% (wt/vol) bovine serum albumin, 0.1 M lysine, and 1:200

TABLE. Nerve Terminals and Synaptic Vesicles

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<th>Controls (n = 16)</th>
<th>Toxin treated (n = 11)</th>
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<tr>
<td>Nerve terminal area, μm²</td>
<td>31.0 ± 8.5</td>
<td>26.0 ± 3.7</td>
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<tr>
<td>Synaptic vesicles, volume fraction</td>
<td>0.44 ± 0.10</td>
<td>0.38 ± 0.04</td>
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Mean areas of nerve terminal boutons and volume fractions of the boutons occupied by synaptic vesicles in the control terminals and terminals of muscles exposed to ammodytoxin A were determined. There was no change in nerve terminal area or the volume fraction occupied by synaptic vesicles after exposure to ammodytoxin A (p < 0.05).
FITC-conjugated α-bungarotoxin, rinsed and mounted in Vectashield HardSet.

Cytochemistry

Intact soleus muscles were exposed to 726 nmol/L Alexa\textsuperscript{546}-AtxA in BSS for 3 hours in the dark. The muscles were then fixed in 4% paraformaldehyde in PBS for 20 minutes and frozen in liquid N\textsubscript{2}. Longitudinal sections were cut, and the sections were collected on subbed slides and counterstained with FITC-conjugated α-bungarotoxin (1:200 in 3% [wt/vol] bovine serum albumin and 0.1 M lysine in PBS) for 1 hour in the dark at room temperature. The sections were washed 3 times in PBS for 30 minutes before mounting in Vectashield HardSet.

Confocal Microscopy

A Leica TCS SP2 UV dual-channel microscope controlled by the LCS 2.51 Build 1537 software (Leica, Heidelberg, Germany) was used for all confocal microscopies. Fluorescein isothiocyanate labeling was excited at 488 nm and detected at 515 nm, tetramethylrhodamine isothiocyanate labeling was excited at 547 nm and collected at 576 nm, and Alexa\textsuperscript{546} labeling was excited at 543 nm and collected at 560 nm.
Statistics

Data are expressed as mean ± SE. Where indicated, statistical significance was determined by the Student unpaired t-test or, where n < 4, the Mann-Whitney U test. In all cases, statistical significance is indicated by p < 0.05.

RESULTS

Functional Studies and Electrophysiology

Indirectly stimulated control muscles generated a twitch response that was well maintained over a period of 300 minutes. The twitch response of muscles exposed to ammodytoxin A (726 nmol/L [10 μg/mL]) was maintained for approximately 50 minutes. Thereafter, the response began to fall progressively and failed totally after 213 ± 25 minutes (Fig. 2). At this point, direct stimulation of the muscle resulted in a full twitch response (data not shown). Miniature endplate potentials could still be recorded at motor endplates of the diaphragm muscle fibers after exposure to ammodytoxin A and the complete failure of the response to indirect stimulation. Miniature endplate potential frequency in muscle fibers exposed to ammodytoxin A (53.0 ± 10.8 per minute) was not statistically different (p > 0.05) from control preparations that had not been exposed to ammodytoxin A (60.0 ± 6.2 per minute). Mean mepp amplitude was a little larger in the muscle preparations exposed to ammodytoxin A (mean mepp amplitude in control fibers, 1.39 ± 0.01 mV; mean amplitude in muscles exposed to ammodytoxin A, 1.53 ± 0.01 mV). This difference is statistically significant and seems to be due largely to the spontaneous release of multiple quanta in the toxin-treated muscles, mepps occasionally reaching an amplitude in excess of 6 mV that was never seen in control preparations (Fig. 3). The mean resting membrane potential of the muscle fibers exposed to ammodytoxin A (−68.0 ± 0.9 mV) was significantly (p < 0.05) less negative than that of control muscle fibers (−72.0 ± 1.2 mV).

Electron Microscopy

The typical endplate in both control and ammodytoxin A-treated muscles is composed of 1 or 2 (occasionally 3 or 4) terminal boutons, each bouton sitting in a deeply folded synaptic trough. In control tissues, the boutons were typically filled with synaptic vesicles and mitochondria and were capped by Schwann cell and fibroblast-like cell processes (Fig. 4A). Endplates exposed to ammodytoxin A were similar in most respects to control except that mitochondria were often swollen, sometimes shrunken and electron dense, and their associated cristae were frequently disrupted. These morphological changes were seen only rarely in the mitochondria of control tissues. Mitochondria in the skeletal muscle cells and Schwann cells of tissues exposed to ammodytoxin were also sometimes affected by exposure to ammodytoxin A, but the proportion of the mitochondria affected did not seem to be as great as in the terminal boutons. There was a statistically insignificant 13.6% reduction in the volume fraction occupied by synaptic vesicles, whereas mean terminal areas were not different between treated and control samples (Table). Small aggregations of synaptic vesicles were sometimes seen within the cytosol and at release sites on the inner face of the plasma membrane of the terminals. O-shaped profiles (indicative of slowly recycling synaptic vesicles) were not seen in either control or poisoned nerve terminals. Typical images of terminal boutons after exposure to ammodytoxin A are shown in Figures 4B to D. Intramuscular nerves were regularly

FIGURE 7. (A) Longitudinal section of a mouse soleus muscle exposed to Alexa546-AtxA (red) counterlabeled with fluorescein isothiocyanate-conjugated α-bungarotoxin to localize junctional acetylcholine receptors (green). There is specific synaptic localization of the Alexa546-AtxA. A laser beam was used to scan the image alternately in the red and green mode from top right to bottom left. (B) The scans confirm the specific binding of Alexa546-AtxA to the region of the synapse.
encountered in the tissue sections. Neither the nerve bundles nor individual axons within the bundles showed any abnormalities because of the exposure to ammodytoxin A (Fig. 5).

**Cytochemistry and Immunocytochemistry**

Preincubation of tissue sections with ammodytoxin A did not prevent the binding of fluorescent conjugates of α-bungarotoxin to junctional acetylcholine (ACh) receptors. In control muscles, 95% to 98% of endplate sites labeled with fluorescent conjugates of α-bungarotoxin were also labeled with fluorescent conjugates of anti-synaptophysin. There was no apparent loss of immunolabeling for synaptophysin in the preparations exposed to ammodytoxin A. The synaptic organization of neuromuscular junctions as revealed by the combined labeling of ACh receptors, synaptophysin, and neurofilament was indistinguishable from that of controls. Specifically, there was no morphological evidence of damage to the terminal branches of the axon. Typical images are shown in Figure 6. Neither frozen muscle sections stained with hematoxylin and eosin nor ultrathin muscle sections showed any evidence of structural damage to skeletal muscle (not shown).

**Localization of Binding of Ammodytoxin A**

Longitudinal sections of muscle incubated with Alexa 546-AtxA were counterlabeled with FITC-conjugated α-bungarotoxin. Punctate labeling by Alexa 546-AtxA was identified at numerous synapses (Fig. 7). Labeling was always perisynaptic, but the resolution of these experiments was too low to allow us to differentiate between presynaptic and postsynaptic sites for the binding of the toxin.

**DISCUSSION**

Exposure of murine nerve-muscle preparations to ammodytoxin A caused a failure of neuromuscular transmission within 3 to 3.5 hours. There are several possible explanations for the failure of neuromuscular transmission. The most obvious are a loss of muscle contractility and excitability, ACh receptor blockade, axonal degeneration, depletion of synaptic vesicles from the nerve terminals, degeneration of the terminal bouton, and the inhibition of transmitter release. We consider each possibility in turn. Although the paralyzed muscle fibers were depolarized, the fall in mean resting membrane potential was very small (∼4 mV), and the paralyzed muscles did exhibit a twitch-like response to direct electrical stimulation. Thus, there was no loss of muscle contractility or excitability. There was also no morphological evidence of toxin-induced myotoxicity.

Miniature endplate potentials recorded at neuromuscular junctions after exposure to ammodytoxin A were of normal average amplitude, and the labeling of junctional ACh receptors by FITC-conjugated α-bungarotoxin was unimpaired in muscles exposed first to either ammodytoxin A or Alexa 546-AtxA. These data suggest both that the ACh binding sites on the α subunits of the ACh receptor were not a target for ammodytoxin A and that ammodytoxin did not affect the packaging of transmitter in the individual synaptic vesicles. The appearance of a population of large and complex mepps in toxin-treated muscles may, however, represent the occasional discharge of the small aggregates of synaptic vesicles seen in the terminal boutons of the paralyzed muscles (Fig. 4D).

There was no evidence of degeneration of the terminal portions of the motor axons after exposure to ammodytoxin A on direct examination of the intramuscular nerve bundles and terminal boutons. Moreover, combined immunolabeling of neurofilament protein and synaptophysin in preparations exposed to the toxin suggested that there was no disaggregation of neurofilaments or other evidence of axon degeneration.

Previous studies have shown that neurotoxic phospholipases A2 also cause no loss of axonal conduction at acute stages of poisoning (13, 14), but the absence of any evidence of degeneration of terminal axons and boutons distinguishes ammodytoxin A from the presynaptically active neurotoxic phospholipases A2 of elapid venoms such as notexin, taipoxin, and β-bungarotoxin; degeneration of intramuscular axons, terminal boutons, and neurofilaments after exposure to these toxins is common (15–17). There was a 13.6% reduction in synaptic vesicle density in terminal boutons exposed to ammodytoxin A. This reduction was small compared with the reduction of more than 80% seen in preparations exposed to the elapid toxins (18) and was not sufficient to cause either a loss of immunolabeling of synaptophysin or a fall in mepp frequency at the neuromuscular junctions of paralyzed muscles. Therefore, we do not consider the loss of synaptic vesicles to be the major cause of transmission failure in the case of poisoning by ammodytoxin A. This contrasts with work on the presynaptically active neurotoxic phospholipases A2 where the definitive features of toxicity have been described as an increase in both spontaneous and evoked transmitter release (13); more recent work indicates that this occurs within 20 to 40 minutes and is associated with an increase in the mobilization of synaptic vesicles (Harris, unpublished data) and the later near-total depletion of synaptic vesicles by 180 minutes and the ultimate destruction of the nerve terminal (16, 17).

All of the work to date on the pathophysiology and neuropathology of venom phospholipase A2 poisoning has involved studies on the phospholipases A2 from elapid venoms rather than studies on those from viperid venoms. The elapid and viperid phospholipases A2 share many structural features but are quite different molecules; most obviously, those from elapid venoms (Group IA) have the so-called elapid loop, which is absent in phospholipases A2 from viperid venoms (Group IIA), and those from viperid venoms possess a C-terminal extension that is absent in phospholipase A2 neurotoxins from elapid venoms. There is no particular reason why the 2 neurotoxins would necessarily exploit identical mechanisms of action, and the effort often made to find a common mechanism of action may be misapplied. On the basis of our own data relating to ammodytoxin A, we have rejected the idea that the depletion of synaptic vesicles leads to neuromuscular paralysis and conclude that the primary events in ammodytoxin A-induced neuromuscular paralysis are the selective binding of the toxin to the motor nerve
terminal and the loss of coupling between action-potential-induced depolarization of the nerve terminal and evoked transmitter release. It has often been suggested that a basis for the loss of evoked transmitter release is the slowed recycling of synaptic vesicles (19, 20), but in the present study the absence of Ω-shaped profiles from the terminal boutons suggests that this was not a factor. The degenerative changes in nerve terminal mitochondria were prominent and are ubiquitous features of nerve terminals exposed to a wide range of neurotoxic phospholipases A2 (16–20). It can be postulated that the degenerative changes that we saw in mitochondria were artifactual and caused by the incubation of isolated tissues for prolonged periods of time or during fixation. There was, however, no widespread loss of the morphological integrity of the mitochondria in control preparations incubated for similar periods of time in the absence of ammodytoxin A. Therefore, we consider that suggestion unlikely. Mitochondria and synaptic vesicles are exquisitely sensitive to fatty acids, lysophosphatides, and phospholipases A2, and it is possible that the loss of evoked release of transmitter is related to a fall in ATP levels and a deenergization of the terminal after damage to the mitochondria (21–24).

Ueno and Rosenberg (25, 26) have suggested that the neurotoxic phospholipases A2 prevent synaptic vesicle mobilization by inhibiting the phosphorylation of synapsin and other synaptosomal proteins, but quite how this might happen is unclear. There is no doubt that the neurotoxic phospholipases A2 attack the nerve terminal plasma membrane, causing a loss of ionic homeostasis and the entry of Ca2+ into the nerve terminal (18–27). Is it possible that the neurotoxic phospholipases A2 are also internalized? There has been much discussion of this topic (reviewed in References 4, 20, 28), but direct evidence in favor of internalization at the nerve-muscle junction is currently lacking. We have tried more than several years to confirm the uptake of notoxin, taipoxin, β-bungarotoxin, and ammodytoxin A into the intact motor nerve terminals of rodents using, at various times, Au-conjugated toxin and Au-conjugated primary and secondary antibodies without success (unpublished data). There is, however, growing evidence that in a number of cell systems, ammodytoxin A might be internalized. For example, Križaj and colleagues demonstrated the internalization of ammodytoxin A in primary hippocampal neurons and cultured neuron-like cells of the murine NSC34 cell line (10, 29). Internalization of the neurotoxic phospholipases A2 at the motor nerve terminal would require both a specific receptor and a carrier of some kind, which might be recycling synaptic vesicles (28, 30) identified in the motor nerve terminal. If the neurotoxic phospholipases A2 were to be internalized and were able to enter the cytosol, possibly after the hydrolysis of synaptic vesicles carrying the toxins (18), the resulting nerve terminal pathology would be explicable because the synaptic vesicles and the mitochondria are particularly sensitive to phospholipases A2, lysophosphatides, and free fatty acids (31, 32). We are currently working on the possible uptake of neurotoxic phospholipase A2 toxins into the terminal boutons of motor neurons.

We now consider the clinical implications of our results. We have documented the neurotoxic activity of ammodytoxin A and have shown that it selectively blocks the evoked release of transmitter from the motor nerve terminal at a concentration of 10 μg/mL. Could such a concentration appear in the circulation of a severely envenomed victim of a bite by V ammodytes ammodytes? Young people are particularly susceptible to snakebite primarily because snakes do not match the volumes of venom ejected to the body mass of the intended prey item or predator. The case reported by Lukšič et al (3) was of a child aged 4 years who weighed 23 kg at the time of admission (B. Lukšič, personal communication) and would, therefore, have a blood volume of approximately 1.5 L (33, 34). The venom yield of an adult specimen of V ammodytes ammodytes is 15 to 45 mg, and ammodytoxin A comprises 15% to 30% of the total protein content of the venom (B. Halassy, personal communication), and if we assume that all of the venom delivered during a bite enters the circulation, the concentration of ammodytoxin A in the circulation would be between 1.5 and 9.1 μg/mL. Despite the caveats involved in this kind of calculation, we consider this to be close to the concentrations of toxin used in our study and that it seems reasonable to suggest that ammodytoxin A (probably in combination with ammodytoxins B and C) is responsible for the neurotoxic signs and symptoms seen in severely envenomed victims of bites by V ammodytes ammodytes. Support for this suggestion may be found from other studies of bites by European vipers. For example, Ferquel and colleagues (6) have shown that neurotoxic signs, including ptosis and ophthalmoplegia, are common after bites by V aspis in southeastern France but not elsewhere in Europe. In a specific case in which an envenomed patient experienced severe neurotoxicity, the offending animal was captured, and genes encoding ammodytoxins A, B, and C were identified in the venom glands. These investigators also reported that “a correlation was found between the expression of neurological symptoms in humans and the intensity of the cross-reaction of venoms with anti-Atx antibodies, which is correlated with the level of neurotoxin expression” (6). The genes encoding the ammodytoxins are not found in pure V aspis/V ammodytes hybrids in which the genes for the ammodytoxins have been preserved (6, 35).

We acknowledge that our study was performed on non-human tissues. Ideally, studies on the neuropathology of the motor nerve terminal in human victims of envenoming snakebite would be made on motor point biopsies, but this is an invasive procedure that is potentially dangerous in subjects with a coagulopathy and, therefore, clearly unethical. There are good precedents for making such toxicological studies on animal tissues (16–36), and the present study is the first to do this in relation to the neuropathology and pathophysiology of neurotoxic snakebite caused by a European viperid snake. We also acknowledge that our work relates only to the acute stages of neurotoxicity. Patients presenting neurotoxic signs and symptoms after envenoming bites by V ammodytes ammodytes are frequently weak for several days, and full recovery may take up to 28 days (B. Lukšič, personal communication). Many neurotoxic phospholipases A2 cause a slowly developing degeneration of the motor nerve terminal,
and the regeneration of the nerve terminal seems to be related to the return of neuromuscular function (36). We are currently planning to study the longer term effects of exposure in vivo to ammodytoxin A.

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