

Snake Phospholipase A₂ Neurotoxins Enter Neurons, Bind Specifically to Mitochondria, and Open Their Transition Pores^{*[S]}

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Snake presynaptic neurotoxins with phospholipase A₂ activity are potent inducers of paralysis through inhibition of the neuromuscular junction. These neurotoxins were recently shown to induce exocytosis of synaptic vesicles following the production of lysophospholipids and fatty acids and a sustained influx of Ca²⁺ from the medium. Here, we show that these toxins are able to penetrate spinal cord motor neurons and cerebellar granule neurons and selectively bind to mitochondria. As a result of this interaction, mitochondria depolarize and undergo a profound shape change from elongated and spaghetti-like to round and swollen. We show that snake presynaptic phospholipase A₂ neurotoxins facilitate opening of the mitochondrial permeability transition pore, an inner membrane high-conductance channel. The relative potency of the snake neurotoxins was similar for the permeability transition pore opening and for the phospholipid hydrolysis activities, suggesting a causal relationship, which is also supported by the effect of phospholipid hydrolysis products, lysophospholipids and fatty acids, on mitochondrial pore opening. These findings contribute to define the cellular events that lead to intoxication of nerve terminals by these snake neurotoxins and suggest that mitochondrial impairment is an important determinant of their toxicity.

Two classes of neurotoxins can paralyze the neuromuscular junction through their enzymatic activity: (i) the clostridial neurotoxins, metalloproteases acting specifically on SNARE (soluble NSF attachment protein receptor) proteins to cause

tetanus and botulism, and (ii) the SPANs (1). SPANs³ play a major role in envenomation and cause a botulism-like flaccid paralysis with autonomic symptoms (2, 3). The enzymatic activity and the neurospecificity make these toxins very effective; however, like botulinum neurotoxins, SPANs do not affect the cell body and axon of the motor neuron, allowing complete recovery in most patients (4).

Impairment of neuromuscular transmission by SPANs is traditionally measured in nerve-muscle preparations isolated from the mouse hemidiaphragm or from the chicken biventer cervicis. A simpler and more sensitive assay, based on SPAN-induced irreversible bulging of nerve terminals in culture, was recently described (5). It was also shown that an early consequence of the action of SPANs is the hydrolysis of phosphatidylcholine into lysophosphatidylcholine and fatty acids and that their equimolar mixture mimics the swelling response of nerve terminals to the toxin itself (6). The SPAN-induced nerve bulges accumulate Ca²⁺, and this event is accompanied by mitochondrial rounding and depolarization (7). The cytosolic [Ca²⁺] increase could also trigger the activity of many Ca²⁺-activated hydrolases of nucleic acids, proteins, and lipids, all factors that could account for the pronounced degeneration of nerve terminals poisoned by SPANs (8–11).

Previous studies indicated that SPANs can gain access to the cell interior. Indeed, fluorescein-conjugated β -Btx was found to rapidly enter hippocampal neurons in culture and was suggested to associate at least in part with lysosomes (12). By antibody labeling, Tpx was found to localize inside chromaffin cells in culture (13). Fluorophore-conjugated ammodytoxin A (a 14-kDa PLA₂ neurotoxin isolated from the venom of *Vipera ammodytes*) was detected in the nucleus of hippocampal neurons (14) and in the cytosol of undifferentiated NSC34 cells (15), a mouse neuroblastoma \times spinal cord hybrid cell line (16). In addition, Tpx was reported to bind an endoplasmic reticulum-located protein *in vitro* (17), and ammodytoxin A was found to bind a variety of cytosolic proteins (18, 19) and R25, an integral protein of mitochondria (20). As SPANs require Ca²⁺ for their hydrolytic activity, the biological relevance of these findings was considered to be questionable. However, we recently documented that SPANs do induce the accumulation of Ca²⁺ within nerve terminals (7), and this finding reopened the possibility of a contribution of the entry of SPANs in the nerve terminal cytosol to the pathogenesis of envenomation.

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^[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1 and S2.

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³ The abbreviations used are: SPANs, snake presynaptic phospholipase A₂ neurotoxins; β -Btx, β -bungarotoxin; PTP, permeability transition pore; CGNs, cerebellar granular neurons; CRC, calcium retention capacity; CsA, cyclosporin A; mLysoPC, 1-myristoyllysophosphatidylcholine; Ntx, notexin; PLA₂, phospholipase A₂; OA, oleic acid; SCMN, spinal cord motor neurons; Tpx, taipoxin; Tetx, textilotoxin.

Here, we report that active fluorescent derivatives of Ntx, β -Btx, and Tpx enter nerve terminals and bind specifically to mitochondria, whose morphology changes from the elongated, spaghetti-like shape to a rounded one. Rounded mitochondria were detected inside the toxin-induced bulges of nerve terminals. To understand the mechanistic basis for the mitochondrial changes, we investigated the effect of these neurotoxins on isolated mitochondria and discovered that SPANs are inducers of the mitochondrial PTP, with a relative potency that matches their PLA₂ activity. These findings have important consequences in defining the molecular events that lead to the pathogenesis of peripheral nerve paralysis caused by snake presynaptic PLA₂ neurotoxins in general.

EXPERIMENTAL PROCEDURES

Neurotoxins and Lipid Mixture Preparation—Ntx, Tpx, and Tetx were purchased from Venom Supplies; fluorescein isothiocyanate-conjugated β -Btx and β -Btx were from Sigma. Their purity was controlled by SDS-PAGE. 1-Myristoyllyso-phosphatidylcholine (mLysoPC; Sigma) and an oleic acid (OA; Sigma) mixture (mLysoPC + OA) were prepared as described previously (6).

Toxin Labeling and Assay—One hundred and fifty micrograms of purified toxin (Ntx, Tpx, and Tetx) were resuspended in 150 μ l of 10 mM Hepes, 150 mM NaCl, pH 7.4; the pH of the reaction buffer was adjusted to 8.0 by adding sodium bicarbonate. Fifteen micrograms of Alexa568 dye (Molecular Probes) (from a stock solution of 10 μ g/ μ l in Me₂SO) were added to the toxin solution. The reaction was carried out in the dark at room temperature for 1 h under continuous stirring and was stopped by the addition of 15 μ l of 1.5 M hydroxylamine, pH 8.5. Excess dye was removed by extensive dialysis against 10 mM Hepes, 150 mM NaCl, pH 7.4 (Slide-A-Lyzer dialysis cassette, 10-kDa cut-off, Pierce). The conjugate was collected; its absorbance spectrum was recorded; and ratios of 0.5 Alexa568/Ntx molecule, of 1.2 Alexa568/Tpx molecule, and 3.5 Alexa568/Tetx molecule were determined. The toxicity of Alexa568-conjugated toxins was assayed in the mouse nerve-hemidiaphragm preparation as before (6). The fluorescent Ntx and Tpx derivatives, as well as the fluorescein isothiocyanate-conjugated β -Btx, were nearly as neurotoxic as their nonconjugated counterparts (supplemental Table S1). Alexa568-Tetx showed pronounced absorption onto the polylysine/polyornithine-laminin coating of the neuronal cultures and could not be used for neuron imaging.

Chemical Modifications of Notexin—Acetylation of lysine residues with acetic anhydride (Sigma) was performed as described (21) with minor modifications. Briefly, 30 μ g of Ntx were dissolved in 100 μ l of a saturated solution of sodium acetate in 50 mM sodium borate buffer, pH 8.2, and then cooled in an ice-water bath. The solution was treated with a total amount of 15 μ l of a 1:500 dilution of acetic anhydride, distributed over five additions during 1 h at 4 °C. Acetylated Ntx was then dialyzed against 150 mM NaCl, 10 mM Hepes, pH 7.4 (Slide-A-Lyzer dialysis cassette), and conjugated with Alexa568 as described above.

Histidine modifications of Ntx with diethyl pyrocarbonate (Sigma) or *p*-bromophenacyl bromide (Sigma) were performed

as described previously (22, 23). In the case of modification with diethyl pyrocarbonate, the reaction was performed in 50 mM phosphate buffer, pH 7.8, at 25 °C (toxin concentration = 0.2 mg/ml) by adding aliquots of a freshly prepared solution of diethyl pyrocarbonate in anhydrous ethanol. The reaction was followed by monitoring the absorbances at 243 and 278 nm in a Perkin-Elmer Lambda 5 spectrophotometer (22) and was stopped by the addition of imidazole (5 mM final concentration). The modified toxin was then dialyzed against 50 mM phosphate buffer, pH 7.0.

Notexin histidines were modified also with *p*-bromophenacyl bromide. Briefly, 100 μ g of Ntx were resuspended in 100 μ l of conjugation buffer (0.1 M sodium cacodylate-HCl, pH 6, 0.1 M NaCl). Incubation with *p*-bromophenacyl bromide was carried out at 30 °C at a molar reagent:protein ratio of 5:1 for 7 h and was followed by extensive dialysis against 10 mM Hepes, pH 7.4, 150 mM NaCl. Neurotoxicity, PLA₂ activity, and effects on isolated brain mitochondria of modified toxins were tested (supplemental Table S2).

Cell Culture Preparation—Rat CGNs were prepared from 6-day-old Wistar rats as described previously (24) and used 6–8 days after plating. Primary rat SCMN s were isolated from Sprague-Dawley (embryonic day 14) rat embryos and cultured following previously described protocols (25, 26). SCMN s were used after 5–8 days of neuronal differentiation *in vitro*.

Fluorescence Cell Imaging—SCMN s or CGN s were grown on 24-mm diameter coverslips and exposed to Alexa568-Tpx or Alexa568-Ntx or fluoresceinated β -Btx (25–50 nM) for different time periods at 37 °C in E4 medium (in the case of SCMN s) or Krebs-Ringer Hepes buffer (in the case of CGN s). E4 composition was 120 mM NaCl, 3 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose, and 10 mM Hepes, pH 7.4. Krebs-Ringer Hepes buffer composition was 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2 mM CaCl₂, 1.2 mM KH₂PO₄, 6 mM glucose, and 25 mM Hepes, pH 7.4. After incubation, cells were extensively washed with the same buffers, and the coverslips were placed on the stage of an inverted epifluorescence microscope (Leica ADMIRE3) equipped with a Leica DC500 CCD camera, 63 \times oil immersion objective (NA 1.4). Images were acquired using Leica FW4000 software and analyzed with Leica Deblur and ImageJ v1.35 software. For colocalization studies, neurons were loaded with the mitochondrial dye nonyl acridine orange (5 nM, Molecular Probes) for 30 min at 37 °C and then washed and incubated with the fluorescent toxins. Images were acquired at different times from toxin addition, and the fluorescent signals were superimposed.

PLA₂ Activity—The enzymatic activity of the four SPAN s was measured with a commercial kit based on the use of the 1,2-dithio analogue of diheptanoylphosphatidylcholine as substrate (Cayman Chemicals). The hydrolysis of the thioester bond at the *sn*-2 position by PLA₂ generates free thiols that interact with 5,5'-dithiobis(nitrobenzoic acid), leading to an increase in the absorbance at 405 nm. ΔA_{405} was measured with a Beckman SpectraCount.

Rat Brain Mitochondrial Preparation—Two adult Wistar rat forebrains were used for each mitochondrial preparation. Rats were killed by cervical dislocation, and forebrains were immediately transferred to ice-cold isolation medium (250 mM

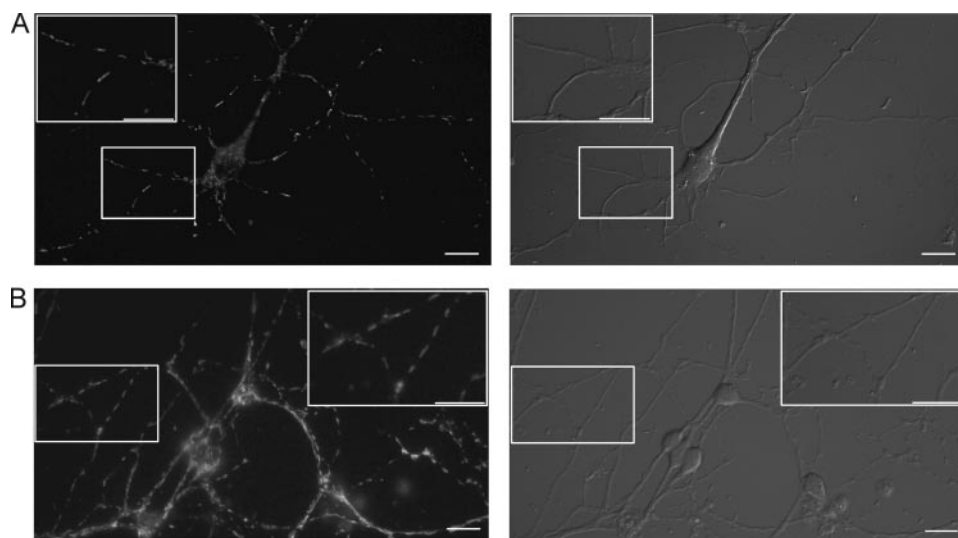


FIGURE 1. Intracellular localization of SPANs in different primary neuronal cultures. A, left panel: the intracellular distribution of Alexa568-Ntx in spinal cord motor neurons after a 5-min incubation at 37 °C (50 nm) is shown. Right panel: the corresponding bright field is shown. The insets show selected areas at higher magnification. Scale bar = 10 μ m. B, a similar intracellular distribution was found also in cerebellar granular neurons and with another SPAN, i.e. Alexa568-Tpx. Scale bar = 2 μ m.

four different SPANs and two different primary neuronal cultures. Alexa568 fluorescent derivatives of three SPANs with different quaternary structure, Ntx (monomeric, 14 kDa), Tpx (trimeric, 42 kDa), and Tetx (pentameric, 70 kDa), were prepared and their toxicities were tested. In the case of β -Btx (heterodimeric, 21 kDa) we used a commercial fluoresceinated toxin. The fluorescent derivatives were nearly as active as the native toxins; however, Alexa568-Tetx was strongly absorbed by the culture plate coating and could not be used for fluorescence imaging (see "Experimental Procedures"). Because the end plates of motor neurons *in vivo* are not readily accessible to investigation, we have studied the entry of fluorescent toxins in primary cultures of SCMN, which are closer to peripheral motor neurons (26), and in a very homogeneous population of CGNs.

sucrose, 10 mM Tris-HCl, pH 7.4, 0.1 mM EGTA). Dissected forebrains were chopped with scissors and homogenized with 5–7 strokes of a loose-fitting Wheaton pestle. The homogenate was centrifuged for 3 min at $2,000 \times g$ in isolation medium + 0.5% bovine serum albumin to precipitate the nuclei, and the supernatant was centrifuged twice for 8 min at $12,000 \times g$. The resulting pellet was resuspended in isolation medium without bovine serum albumin and centrifuged for 8 min at $12,000 \times g$. The resulting pellet was finally resuspended in isolation buffer to a protein concentration of 50–60 mg/ml. Protein concentration was quantified with the biuret assay.

Assessment of Permeability Transition in Isolated Mitochondria—Onset of the permeability transition was monitored as the fast Ca^{2+} release following accumulation of multiple 10 μM Ca^{2+} pulses at 1-min intervals (27). Extra-mitochondrial Ca^{2+} concentration was monitored with the Ca^{2+} indicator Calcium Green-5N (excitation/emission, 505/535 nm, Invitrogen) with a PerkinElmer 650–40 fluorescence spectrometer. Mitochondria were resuspended to a final protein concentration of 1 mg/ml in 2 ml of the following medium: 120 mM KCl, 10 μM EGTA, 5 mM glutamate, 2.5 mM malate, 1 mM Tris phosphate, 10 mM Tris-HCl, pH 7.4, 1 μM Calcium Green-5N. A quartz cuvette with continuous stirring through a magnetic bar was employed to ensure rapid mixing. The number of 10 μM Ca^{2+} pulses retained by the mitochondrial suspension before PTP opening was counted and set to 100% mitochondrial CRC. Similar experiments were carried out in the presence of the indicated toxins at concentrations ranging between 0.5 and 50 nM. Where indicated, 0.8 μM CsA (Sigma) was added to inhibit the opening of the PTP. CRC experiments were performed within 3 h of mitochondria isolation.

RESULTS

Snake Presynaptic PLA_2 Neurotoxins Enter Nerve Terminals—To obtain results of rather general value, we have used here

which are closer to peripheral motor neurons (26), and in a very homogeneous population of CGNs.

Fig. 1A shows that Alexa568-Ntx rapidly entered neuronal projections of SCMN. Remarkably, fluorescent neurotoxin was not homogeneously distributed in the cytosol but rather localized to elongated, spaghetti-like structures that are clearly reminiscent of mitochondria. A similar staining pattern was found also in cerebellar granular neurons and with Alexa568-Tpx (Fig. 1B) and fluoresceinated β -Btx (data not shown), indicating that the mitochondrial-like staining is a rather general feature of SPANs.

With time, SPANs induce bulging of neuronal projections (5). Fig. 2 shows the staining of SCMN with Alexa568-Tpx at 30 min; similar patterns were obtained with fluorescent Ntx and β -Btx (data not shown). The shape of the structures stained by the toxin changed during intoxication, and after 30 min, labeled organelles appeared as rounded bodies, which were always localized inside toxin-induced bulges.

SPANs Bind Specifically to Mitochondria within Neurons—The identification of the intracellular organelles stained by these neurotoxins as mitochondria is supported by the findings of Fig. 3, which shows a close superimposition between the staining patterns of Alexa568-Tpx and the mitochondrial dye nonyl acridine orange in SCMN. Similar findings were obtained in CGNs and with fluorescent β -Btx and Ntx (data not shown). This latter observation is only apparently different from that of Herkert *et al.* (12) in hippocampal neurons, which was interpreted as partial localization of fluoresceinated β -Btx to lysosomes. In fact, a close inspection of the figures shows that the spotty distribution found in the neuronal projections is compatible with a staining of mitochondria after 30 min of incubation with the neurotoxin (see below). These observations are consistent with the electron microscopy pictures of motor neurons and CGNs exposed to these neurotoxins (3, 7–11, 28), whose mitochondria

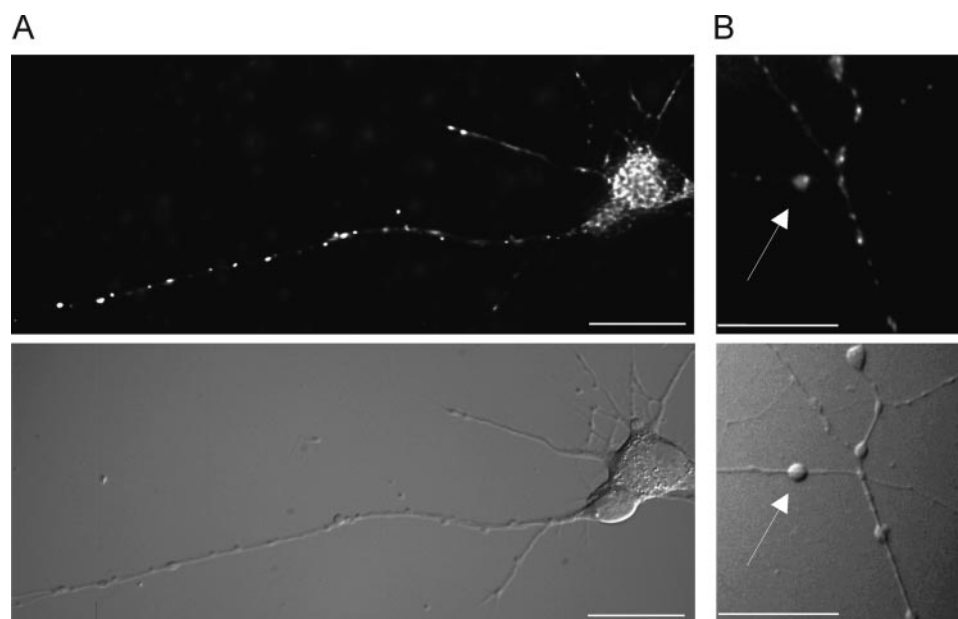


FIGURE 2. Accumulation of SPAN staining within toxin-induced membrane enlargements with time. Spinal cord motor neurons were incubated with 50 nM Alexa568-Tpx for 30 min at 37 °C and washed, and images were acquired. Alexa568-Tpx fluorescent signal accumulates within the toxin-induced membrane bulges, which can be better appreciated in *B*. The same results were obtained in cerebellar granular neurons and after Alexa568-Ntx and fluoresceinated β -Btx exposure (not shown). Scale bar = 10 μ m in *A* and 5 μ m in *B*.

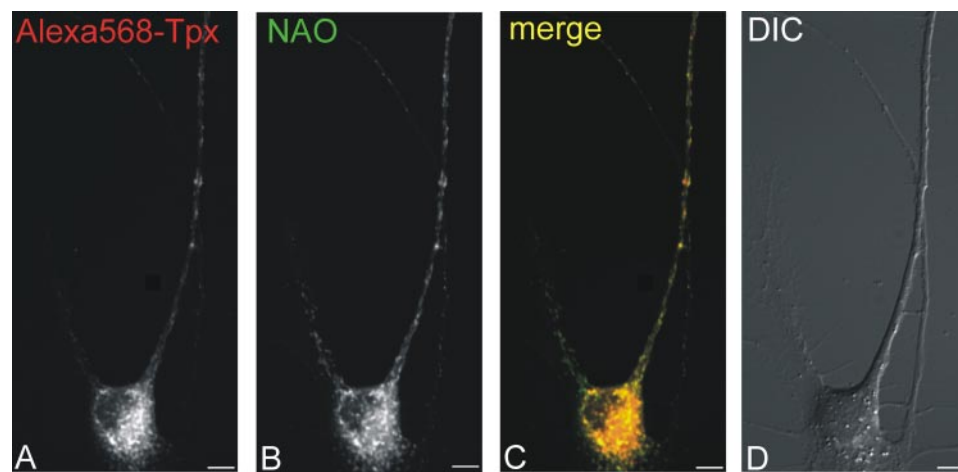


FIGURE 3. Colocalization between SPANs and mitochondria. Spinal cord motor neurons were incubated with 50 nM Alexa568-Tpx and 5 nM nonyl acridine orange (NAO) for 30 min at 37 °C and washed, and images were acquired. *A* and *B* show the fluorescence images at the single excitation wavelengths. *C* shows the superimposition between the two emitted wavelengths; *D* represents the corresponding differential interference contrast (DIC). Scale bar = 5 μ m.

show rounding and alteration of cristae indicative of their loss of function.

The action of SPANs is known to be very specific for the presynaptic nerve terminals *in vivo*. Also in our cultures SPANs staining appears to be very specific for mitochondria within neurons, as shown by lack of toxin staining in non-neuronal cells (Fig. 4). These findings prompted us to investigate the effects of SPANs in mitochondria isolated from rat brain.

SPANs Open the Mitochondrial Permeability Transition Pore—A common cause of mitochondrial swelling and depolarization *in situ* is the opening of the mitochondrial PTP, an inner membrane high-conductance channel that can be desensitized by CsA (29). The propensity of the PTP to open in a population of mitochondria can be monitored with a sensitive

technique based on the CRC, *i.e.* the amount of Ca^{2+} that can be taken up by mitochondria in the presence of inorganic phosphate before onset of PTP opening (27). Untreated, control mitochondria accumulated 10 pulses of 10 μM Ca^{2+} before onset of the permeability transition, which is readily detected by a precipitous release of the previously accumulated Ca^{2+} (Fig. 5*A*). Addition of as little as 1 nM Ntx dramatically decreased the threshold for PTP opening, which was observed after accumulation of three pulses of Ca^{2+} (Fig. 5*B*). It should be noted that prior to PTP opening, the rate of Ca^{2+} uptake in Ntx-treated mitochondria was indistinguishable from that of controls, indicating that, in the absence of added Ca^{2+} , Ntx does not affect energy coupling. As expected for a PTP-dependent event, treatment with CsA increased the CRC both in the absence (Fig. 5*C*) and presence (Fig. 5*D*) of Ntx.

We then investigated the effects of the four SPANs on the CRC and their relative potency. Ntx was the most effective, β -Btx and Tpx displayed an intermediate PTP sensitizing activity, whereas Tetx was nearly ineffective (Fig. 6*A*). This order of potency correlates well with the PLA_2 activity of the four SPANs measured by an *in vitro* assay (Ntx, 371 $\mu\text{mol}/\text{min}/\text{mg}$; β -Btx, 218 $\mu\text{mol}/\text{min}/\text{mg}$; Tpx, 100 $\mu\text{mol}/\text{min}/\text{mg}$; Tetx, 10 $\mu\text{mol}/\text{min}/\text{mg}$; see “Experimental Procedures”).

To test the hypothesis that the enzymatic activity is indeed responsible for facilitation of PTP opening by SPANs, we determined the direct effect of the products of the PLA_2 activity, mLysoPC, and OA. These were added to rat brain mitochondria either individually or in the 1:1 molar mixture that is produced by SPANs (Fig. 6*B*). Consistent with our hypothesis, the equimolar mixture of mLysoPC + OA (1 μM) facilitated PTP opening, whereas mLysoPC alone was less effective. OA had a strong effect, in line with previous observations, demonstrating that fatty acids (*i.e.* arachidonic and palmitic) are effective inducers of the PTP in isolated mitochondria and intact cells (30). Together with our previous findings that fatty acid alone has a minor inhibitory effect on the transmission of the nerve impulse to the muscle (6), the present result indicates that very little fatty acid is able to partition from the plasma membrane

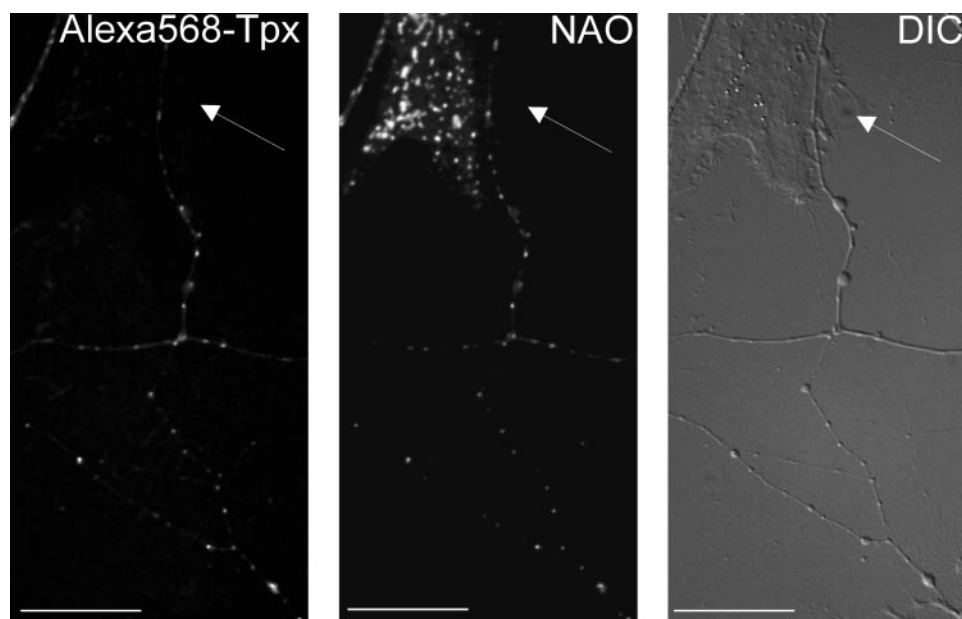


FIGURE 4. **SPANs specifically localize within neurons.** Alexa568-Tpx (25 nM) intracellular localization in primary cultures of spinal cord motor neurons after a 30 min incubation at 37 °C is confined to neuronal cells, as demonstrated by the lack of fluorescent signal in fibroblasts, whose mitochondria are stained well with nonyl acridine orange (NAO). Scale bar = 5 μ m. DIC, differential interference contrast.

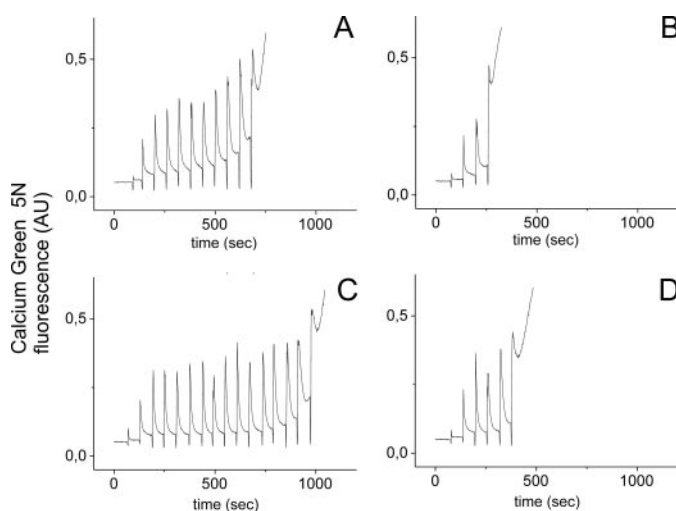


FIGURE 5. **Influence of Ntx and CsA on mitochondrial CRC.** Purified mitochondria were resuspended in the presence of Calcium Green-5N as described under "Experimental Procedures," and CRC was tested. At 60-s intervals, 10 μ M Ca^{2+} pulses were added until occurrence of the permeability transition, which is marked by a fast release of the previously accumulated Ca^{2+} . A and B, reduced mitochondrial CRC in Ntx-treated mitochondria (B, 1 nM) compared with control (A). C and D, the ability of CsA to delay mitochondrial Ca^{2+} release of control (C) and Ntx-treated mitochondria (D, 1 nM). AU, arbitrary units.

into the mitochondria of the nerve terminals after its release by the PLA_2 activity of these neurotoxins. Further evidence that the PLA_2 enzymatic activity of the SPANs is instrumental in inducing the mitochondrial change in permeability was obtained in experiments performed with chemically inactivated Ntx. The toxin was acetylated, and this derivative retained $2.9 \pm 2.5\%$ of the PLA_2 activity of the unmodified toxin ($n = 3$). Acetylated Ntx did not significantly inhibit neurotransmission of the mouse hemidiaphragm preparation, did not stain or induce any bulging of neurons in culture, and failed to

induce opening of the mitochondrial PTP. Notexin was also chemically modified with the histidine-specific reagents diethyl pyrocarbonate and *p*-bromophenacyl bromide following established procedures (22, 23), which led to partial loss of PLA_2 activity (supplemental Table S2). Importantly, the percentage of loss of enzymatic activity correlated well with the percentages of loss of neurotoxicity and of capability of opening the mitochondrial PTP. These data strongly support the proposal that the PLA_2 activity of SPANs is involved in their effect on the mitochondrial PTP.

Fig. 7 reports the relative protective effect of the PTP inhibitor CsA in the absence or presence of the four SPANs. This parameter has similar values whether or not a SPAN was present, whichever

SPAN is considered. This indicates that SPANs do not directly permeabilize the mitochondrial membrane, with ensuing unspecific Ca^{2+} leak. On the other hand, the effect of SPANs appears to be rather specific for the PTP channel, as CsA inhibited the effect of the toxins to a similar extent, with relative values close to those of the controls.

DISCUSSION

The main findings of the present study are (i) presynaptic snake neurotoxins of different size (from 14 to 42 kDa) endowed with PLA_2 activity enter neurons within a short time of addition; (ii) they bind specifically to mitochondria and induce a shape change within regions of nerve terminals that undergo swelling to form round bulges of the plasma membrane; and (iii) these neurotoxins induce opening of the mitochondrial PTP, which leads to release of Ca^{2+} , with an order of potency that matches their PLA_2 enzymatic activities.

The entry of SPANs inside cells was reported before. β -Btx and amodytoxin A were detected within hippocampal neurons (12, 14), amodytoxin A was recently found also in NSC34 cells (15), and Tpx staining by antibody labeling was reported within chromaffin cells (13). It was also previously established that endocytosis inside acidic intracellular compartments, as is the case of botulinum neurotoxins, is not involved in the intoxication by SPANs (31). The recent observation that amodytoxin A localizes inside vesicles in the cytosol of undifferentiated NSC34 cells may be due to tumor transformation itself and/or to the fact that the cells used were not differentiated and had no neuronal appearance (15). Although toxin endocytosis cannot be excluded, the early detection of SPANs inside the cell cytosol is not consistent with the time course of endocytosis. We are left with the possibility that SPANs enter directly by crossing the plasma membrane. It is not known whether this is an intrinsic property of these molecules or whether an initial

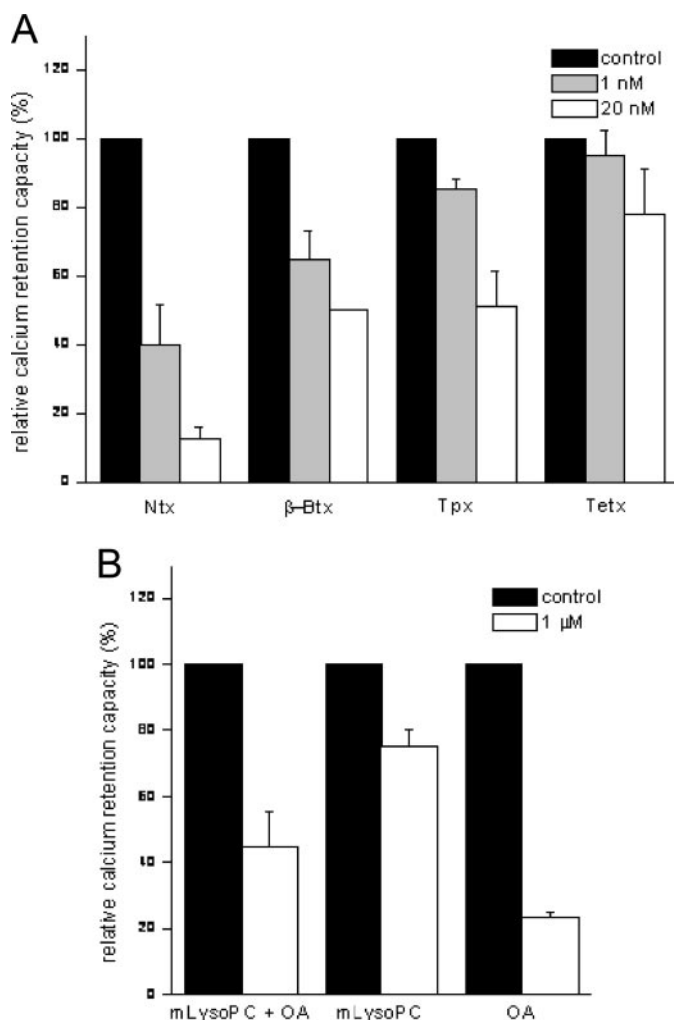


FIGURE 6. Effect of SPANs and PLA₂ activity products at different concentrations on Ca²⁺ uptake of purified rat brain mitochondria. A, mitochondria were resuspended as described under "Experimental Procedures," and CRC was tested in the presence of the four snake neurotoxins at high (20 nM, white bars) and low (1 nM, gray bars) concentrations. CRC decrease of toxin-treated mitochondria is concentration-dependent. B, the hydrolytic products of PLA₂ activity (mLysoPC and OA) were tested both alone or in an equimolar mixture (1 μ M). Data represent mean CRC values of intoxicated mitochondria normalized to control samples (black bars). For each condition, trials were performed in triplicate.

hydrolysis of the phospholipids of the outer layer of the plasma membrane is a prerequisite for their entry. The finding that Sr²⁺, which does not sustain the PLA₂ activity of SPANs, inhibited ammodytoxin A entry supports the latter possibility (15). Also, the present finding that PLA₂ inactivation of Ntx leads to loss of neurotoxicity and loss of effect on mitochondria is consistent with this possibility.

Here, we report the first evidence that SPANs inside neurons bind to mitochondria and induce their change of shape from the physiological elongated to the rounded, swollen form, which is observed in many pathological states. Given that the entry of SPANs inside excitatory cells was already reported (12–15), why was their specific binding to mitochondria not detected before? A key issue is that the typical spaghetti-like staining is maintained for a very short time following toxin addition. Because mitochondrial rounding and impairment readily follow SPAN nerve terminal poison-

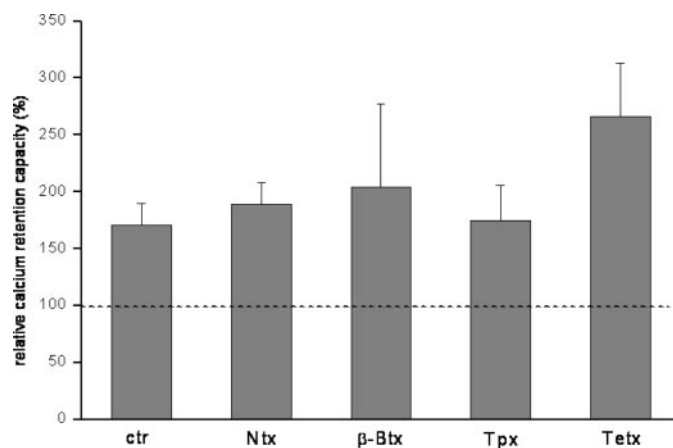


FIGURE 7. Delayed SPAN-induced PTP opening by CsA. Calcium retention capacity of control and SPAN-treated (20 nM) mitochondria was measured in the absence or presence of CsA (0.8 μ M). CRC values of CsA-treated mitochondria (in the absence or presence of toxins, respectively) are normalized to those of samples not treated with CsA (dotted line). An increased threshold for PTP opening is observed for both control and SPAN-treated mitochondria. For each condition, trials were performed in triplicate.

ing (7–9), the affected organelles are no longer easily identified as mitochondria. It is also possible that these neurotoxins bind efficiently to mitochondria only within neuronal cells, as suggested by the study of Ng and Howard (32), who found that β -Btx only affected brain mitochondria. This would explain why mitochondrial staining was not detected in chromaffin cells (13) and in undifferentiated NSC34 cells (15), but does not explain the lack of this type of staining in hippocampal neurons by ammodytoxin A (14). However, it should be considered that the fluorescent ammodytoxin A used in NSC34 cells displayed a 5-fold lower binding affinity for the mitochondrial R25 protein (15), suggesting that the toxin was significantly modified by the procedure used for fluorescent dye coupling, which may in turn have affected its subcellular interactions. As mentioned above, a re-inspection of hippocampal neurons intoxicated with β -Btx (12) is compatible with the mitochondrial staining found here. The mitochondrial binding of SPANs re-evaluates the biochemical finding that ammodytoxin A binds to a mitochondrial protein termed R25 (20), which could be involved as well in the mitochondrial interactions detected here in intact cells.

Bulging is the result of an excess of membrane accumulation and the positive curvature of the plasma membrane caused by the presence of lysophospholipids on the outer layer (1, 6, 33, 34). SPANs induce bulging in different neurons in culture, an event that was found to be associated with induced exocytosis and inhibition of the retrieval of synaptic vesicles (5–6, 35). The same phenotype is induced by the addition to neurons of the PLA₂ hydrolysis products (6). Interestingly, the mitochondrial shape changes in toxin-treated neurons take place within bulges of the neuronal projections and may well be caused by PTP opening mediated by the very same products of phospholipid hydrolysis. Accordingly, we found that the addition of an equimolar mixture of mLysoPC + OA induced the opening of the mitochondrial PTP (Fig. 6B).

The *in vivo* mitochondrial targeting of SPANs called for a reappraisal of their direct action on mitochondria, in light of the

current knowledge regarding the role of mitochondria in controlling the Ca^{2+} concentration of the cytosol (36, 37) as well as the fact that SPANs do induce a high increase of $[\text{Ca}^{2+}]$ within nerve terminals (7). It has long been known that Ntx and β -Btx affect isolated mitochondria and are capable of decreasing Ca^{2+} uptake by brain mitochondria and sarcoplasmic reticulum (32, 38). Our finding that SPANs sensitize the PTP to opening by Ca^{2+} readily provides an explanation for these previous observations, but also clarifies a novel and important aspect of the mitochondrial effects of SPANs. Indeed, our results demonstrate that (i) SPANs do not directly affect mitochondrial coupling or their Ca^{2+} uptake systems because the kinetics of mitochondrial Ca^{2+} uptake is not affected until the PTP opens, and (ii) Ca^{2+} release (and impairment of further Ca^{2+} uptake) is caused by PTP opening. This mitochondrial impairment caused by SPANs may then substantially worsen the deregulation of Ca^{2+} homeostasis. This sequence of events is entirely consistent with the observation that toxin-treated mitochondria are no longer able to buffer cytosolic Ca^{2+} and that they contribute to the triggering of an apoptotic program of cell death that was actually shown to occur in the hippocampal neurons exposed to the action of β -Btx (12).

Are the present findings relevant to the pathogenesis of envenomation by snakes whose venoms include SPANs as a major toxin component? As discussed in detail elsewhere (4, 11), available data suggest that blockade of peripheral nerve terminals with ensuing flaccid paralysis is mainly due to the SPAN-catalyzed hydrolysis of phospholipids of the presynaptic membrane, followed by massive entry of Ca^{2+} . Together, these two events would induce exocytosis of the synaptic vesicles of the affected nerve terminals not followed by endocytosis. It has been argued that the entry of SPANs inside the nerve cytosol must have a role (39). Clearly, much depends on the kinetics of entry. If the SPAN enters rapidly by itself, then it may begin to act as soon as the cytosolic Ca^{2+} concentration has risen to a level sufficient to support the PLA_2 activity. On the other hand, the inhibitory effect of Sr^{2+} mentioned above (15) and the inability to enter of the inactive acetylated Ntx indicate that SPANs may require phospholipid hydrolysis to alter the membrane in such a way as to promote their own translocation into the cytosol. Further investigations are needed to discriminate between these two possibilities.

In summary, we can safely conclude that the specific action of SPANs on the mitochondrial PTP channel well accounts for the rounding and swelling of mitochondria detected by all electron microscopy investigations of SPAN-intoxicated neurons (8–10, 28, 40–42). Our results provide a molecular explanation for these morphological observations and indicate that SPANs play a direct role not only in the blockade of nerve terminals, but also in the ensuing mitochondrial degeneration (8–11). The present findings call for detailed investigations of the kinetics of action of SPANs with an analysis of the various steps of intoxication, with particular attention to binding, entry into the cytosol, and effects on mitochondria.

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