Snake presynaptic neurotoxins with phospholipase A2 activity induce punctate swellings of neurites and exocytosis of synaptic vesicles

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Summary

The mechanisms of action of four snake presynaptic phospholipase A2 neurotoxins were investigated in cultured neurons isolated from various parts of the rat brain. Strikingly, physiological concentrations of notexin, β-bungarotoxin, taipoxin or textilotoxin induced a dose-dependent formation of discrete bulges at various sites of neuronal projections. Neuronal bulging was paralleled by the redistribution of the two synaptic vesicle markers synaptophysin I (SypI) and vesicle-attached membrane protein 2 (VAMP2) to the bulges, and by the exposure of the luminal domain of synaptotagmin on the cell surface. These neurotoxins induced glutamate release from cultured neurons similarly to the known evoked release of acetylcholine from neuromuscular junctions. In addition, partial fragmentation of F-actin and neurofilaments was observed in neurons, but not in astrocytes. These findings indicate that these snake presynaptic neurotoxins act with by same mechanism and that the observed phenotype results from the fusion of synaptic vesicles with the plasma membrane not balanced by an adequate membrane retrieval. These changes closely resemble those occurring at neuromuscular junctions of intoxicated animals and fully qualify these primary neuronal cultures as pertinent models for studying the molecular mode of action of these neurotoxins.

Key words: Snake presynaptic neurotoxins, Phospholipase A2, Synaptic vesicle recycling, Neurotransmitter release, Synaptic vesicles

Introduction

Several presynaptic neurotoxins endowed with phospholipase A2 (PLA2) activity have been isolated from the venom of four major families of venomous snakes (Crotalidae, Elapidae, Hydrophiidae and Viperidae). These neurotoxins play a role in envenomation of the prey (Harris, 1997) by causing a persistent blockade of the neuromuscular transmission (Kini, 1997; Schiavo et al., 2000). The presynaptic neurotoxins of Elapid venoms show a range of structural complexity and of specific PLA2 activity (Rosenberg, 1997). Notexin, from the Australian tiger snake *Notechis scutatus* is a single chain phospholipase A2 (Karlsson et al., 1972; Westerlund et al., 1992), whereas β-bungarotoxin (β-Btx) from *Bungarus multicinctus* consists of two covalently linked subunits, the larger of which is a PLA2 (Kelly and Brown, 1974; Kwong et al., 1995). By contrast, only one of the three similar, non-covalently linked subunits of taipoxin, from *Oxyuranus scutellatus* venom, has PLA2 activity (Fohlman et al., 1976). Textilotoxin is purified from the venom of the Australian elapid *Pseudonaja textilis* and consists of five subunits, all endowed with PLA2 activity (Su et al., 1983).

The development of neuromuscular transmission failure in isolated nerve muscle preparations exposed to these toxic phospholipases is triphasic. An initial phase of weak inhibition of acetylcholine release is followed by a second prolonged phase of facilitated release, and then by a third phase of progressive decline of neurotransmission (Su and Chang, 1984; Harris, 1997). Electron-microscopic pictures taken at the third stage show swollen, enlarged axon terminals with depletion of synaptic vesicles (SVs) and with the appearance of several clathrin-coated Ω-shaped plasma-membrane invaginations in areas not facing the muscle. At later stages, swollen mitochondria and vacuoles develop (Chen and Lee, 1970; Cull-Candy et al., 1976; Harris et al., 2000). The kinetics and morphological changes induced by PLA2 neurotoxins at neuromuscular junctions (NMJs) suggest that they might promote fusion of SVs with the presynaptic membrane and, at the same time, inhibit their retrieval (Montecucco and Rossetto, 2000).

Despite many studies (Kini, 1997), the molecular mechanism of action of snake presynaptic PLA2 neurotoxins (SPANs) remains elusive. Owing to the complexity of the anatomically fine structure of NMJ and to the inherent limited possibility of experimental manipulation of this tissue preparation, further progress requires the validation of an in vitro neuronal model amenable to biochemical and imaging

investigations. Therefore, we performed a detailed study of the neurotoxic effects of four major elapid SPANs (notexin, β-BTx, taipoxin and textilotoxin) on three different cultured primary neurons (cerebellar granule cells, cortical neurons and hippocampal neurons). These primary neuronal cultures are relatively homogeneous and, after a few days in culture (6-7 days in vitro), they form a network of functional synaptic contacts, acquiring the characteristics of mature neurons (Dotti et al., 1988; Cox et al., 1990; Baughman et al., 1991). Such cultured primary neurons were exposed for short time periods to toxin concentrations in the same range as their 50% lethal dose (LD_{50}) for mice. Their morphology was examined by light and field-emission scanning electron microscopy (FESEM), and the distribution of cytoskeletal, vesicular and membrane markers was analysed by immunofluorescence. SPAN-induced SV exocytosis was assayed by following the exposure of a lumenal epitope of the vesicular protein synaptotagmin I (Matteoli et al., 1992) and by measuring the release of glutamate. These data indicate that all four SPANs tested here acted similarly by inducing bulging of neuronal projections coupled to stimulated SV exocytosis with release of neurotransmitter. These neuronal models open the possibility of clarifying the molecular mode of action of SPANs and might advance our understanding of the presynaptic control mechanisms for neurotransmitter release.

Materials and Methods
Materials
Notexin, taipoxin and textilotoxin were purchased from Venom Supplies (Tanunda, South Australia); β-BTx was from Sigma (Saint Louis, MS, USA). Purity was checked by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE). Basal Eagle's medium (BME), minimal essential medium (MEM), Neurobasal-A and B27 cell culture media, and gentamycin were obtained from Gibco (Burlington, ON, USA). Cytosine arabinoside, poly-L-lysine, trypsin inhibitor and resazurin were purchased from Sigma, trypsin was from ICN Biomedicals (Aurora, OH, USA); DNase I from Boehringer Mannheim (Mannheim, Germany), foetal bovine serum (FBS) from Euroclone (Milan, Italy), bovine serum albumin (BSA) from Roche (Indianapolis, IN, USA), and paraformaldeyde from Merck (Darmstadt, Germany). L-Glutamic acid, L-glutamic dehydrogenase and β-nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$) were from Sigma. Aristolochic acid, 12-epi-sc<ladial and manoalide from Biomol (Plymouth, PA, USA). The sPLA2 assay kit was from Cayman (Ann Arbor, MI, USA).

Monoclonal antibody against neurofilaments (gift from M. Vittadello, University of Padova, Padova, Italy) was used at a 1:1000 dilution. Polyclonal antibody against tubulin and phalloidin-TRITC (Sigma) were used at dilutions of 1:300 and 1:100, respectively. Polyclonal antibody against SNAP25 (produced in our laboratory against the 12 C-terminal amino acids of SNAP25) was used at a dilution of 1:300; monoclonal antibody against syntaxin (Synaptic Systems, Göttingen, Germany) was used at a dilution of 1:1000; polyclonal antibody against vesicle-attached membrane protein 2 (VAMP2) (Rossetto et al., 1996) was used at a dilution of 1:300; monoclonal antibody against synaptophysin (DAKO, Glostrup, Denmark) was used at a dilution of 1:10; monoclonal antibody against synaptotagmin 1 lumenal domain (Synaptic Systems) was used at a dilution of 1:100; polyclonal antibody against glial fibrillar acidic protein (GFAP) (DAKO) was used at a dilution of 1:1000. Fluorescein- or Texas-Red-conjugated secondary antibodies (Calbiochem, San Diego, CA, USA) were used at a working dilution of 1:100.

PLA2 activity
The PLA2 activity of the four presynaptic neurotoxins and of the bovine pancreas enzyme were determined with the Cayman secretory PLA2 assay kit (Reynolds et al., 1992) based on the use of the 1,2-dithio analogue of diheptanoyl phosphatidylcholine. Upon hydrolysis of the thioester bond at the *sn*-2 position by PLA2, free thiols are detected with 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB) by monitoring the increase in absorbance at 405 nm. The analysis was performed according to the manufacturer's instructions. Activities of the neurotoxin preparations used here were found to be comparable to those reported previously (Rosenberg, 1997). Several reported PLA2 inhibitors were tested on these neurotoxins. All inhibitors were themselves neurotoxic and so these experiments were abandoned.

Hemidiaphragm preparations
Mouse phrenic nerve hemidiaphragms were isolated from male Swiss-Webster mice weighing about 20 g, and mounted in 10 ml oxygenated (95% O_2, 5% CO_2) Krebs-Ringer solution (137 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 24 mM $NaHCO_3$, 1 mM NaH_2PO_4 and 11 mM glucose, pH 7.4). The phrenic nerve was stimulated via two ring platinum electrodes with supramaximal stimuli of 3-6 V amplitude and 0.1 millisecond pulse duration with a frequency of 0.1 Hz. Isometric muscle contraction was monitored with a displacement force transducer connected to a recorder. In control experiments with no added toxin, the amplitude of muscle contraction under stimulation was constant for at least 8 hours. Prior to toxin addition, the concentration of magnesium ions ($[Mg^{2+}]$) was increased to 10 mM to reduce the indirect twitch response by approximately 50%. β-BTx (3 µg ml^{-1}), notexin, taipoxin or textilotoxin (1 µg ml^{-1}) were added to the nerve-muscle preparations in tissue bath at 37°C under nerve stimulation and paralysis times were monitored. At least three different preparations were used for each neurotoxin.

Primary neuronal cultures and transfections
Cerebellar granule neurons
Rat cerebellar granule neurons (CGNs) were prepared from 6-day-old Wistar rats as previously described (Levi et al., 1984). Briefly, neurons were isolated from freshly dissected cerebella by mechanical disruption in the presence of trypsin and DNase I and then seeded onto six-, 24- or 96-well culture plates covered with poly-L-lysine (10 µg ml^{-1}). Cells were seeded at a density of $1.2{\times}10^6$ (six-well plate), $3{\times}10^5$ (24-well plate) and $1{\times}10^5$ (96-well plate) in BME supplemented with 10% FBS, 25 mM KCl, 2 mM glutamine and 50 µg ml^{-1} gentamycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Cytosine arabinoside (10 µM) was added to the culture medium 18-24 hours after plating to arrest the growth of non-neuronal cells. Experiments were performed at 6 days in vitro.

Cortical neurons
Rat cortical neurons were prepared from 1-day-old Wistar rats following the same protocol used for CGNs. Cells were seeded at a density of $0.8{\times}10^6$ (six-well plate), $2{\times}10^5$ (24-well plate) and $0.7{\times}10^5$ (96-well plate) in Neurobasal-A medium supplemented with B27 (2% v/v), L-glutamine 0.5 mM and 50 µg ml^{-1} gentamycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Cytosine arabinoside (10 µM) was added to the culture medium 24-36 hours after plating. Experiments were performed at 6 days in vitro.

Hippocampal neurons
Low-density primary cultures of hippocampal neurons were prepared from Sprague-Dawley rat embryos (Charles River Italica, Calco, Italy)

as described previously (Banker and Cowan, 1977). Neurons were co-transfected at 3 days in vitro with expression vectors encoding fluorescent proteins [enhanced cyan fluorescent protein (ECFP) fused to VAMP2 (ECFP-VAMP2) and SypI fused to enhanced yellow fluorescent protein (EYFP) (SypI-EYFP)] by using 25 kDa polyethylenimine (PEI 25) (Sigma-Aldrich, Steinheim, Germany) as previously described (Pennuto et al., 2002). For fluorescence analysis, cells at 15-18 days in vitro were washed once with Krebs-Ringer buffer (KRH; 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 6 mM glucose and 25 mM HEPES, pH 7.4) and incubated in the same solution in either the presence or the absence of 5 nM taipoxin for 1 hour at 37°C in 5% CO_2.

Viability assay

The assessment of neuronal viability was based on a colorimetric reaction using the dye resazurin, which is an indicator of the metabolic activity of living cells. Cells were seeded onto 96-well plates and exposed to the four SPANs at 5 nM concentration for different time periods (10 minutes, 30 minutes, 60 minutes and 180 minutes) at 37°C in BME without serum. After extensive washing to remove residual toxin, they were incubated for further 4 hours at 37°C with resazurin (10% in complete medium). The proportion of viable cells was determined by monitoring spectrophotometrically their capacity to reduce the dye; the amount of living cells after exposure to the toxins was calculated from the ratio of light absorbance at 540/620 nm and expressed as a percentage of the untreated control.

FESEM analysis

Cells seeded onto 24-well plates were exposed to the neurotoxins at concentration between 0.1-5 nM for 60 minutes at 37°C in serum-free medium, then washed and fixed overnight with paraformaldeyde 4% in Sorensen's buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, pH 7.4). Samples were then extensively washed with Sorensen's buffer and processed for FESEM. Samples were scanned with a JEOL 6700F field-emission scanning electron microscope (Jeol, Akishima, Japan). Secondary emission images were taken at 5 kV with a probe current of 10 μA. For the quantification of the bulges induced by the neurotoxins, linear portions of neurites characterized by uniform diameter from treated and control CGN cultures were imaged at constant magnification (×2000) and their surface determined following a standard stereology protocol using a lattice constant of 20 mm (Lucocq, 1994; Mayhew, 1992). Neurite enlargements with a diameter at least 150% the neurite thickness were counted and expressed as bulge density (bulges μm^{-2}).

Glutamate release

Glutamate release was monitored using an enzyme-linked fluorescent assay based on the conversion of released glutamate to α-ketoglutarate catalysed by glutamate dehydrogenase coupled to the formation of $NADPH^+$ (Nicholls and Sihra, 1986). Briefly, CGNs (at 6 days in vitro) plated onto poly-L-lysine-coated 24-mm coverslips (1.4×10^6 cells per well) were washed in warmed KRH buffer and exposed for different times at 37°C to KRH containing $NADP^+$ 1 mM, L-glutamic dehydrogenase (36 U ml^{-1}) and 5 nM SPANs. $NADPH^+$ formation was determined in a Perkin-Elmer LS-50 spectrofluorimeter (340 nm excitation wavelength, 460 nm emission wavelength). For each sample a baseline was established for ~5 minutes. A standard curve was constructed by measuring fluorescence changes associated to addition of known amounts of glutamate. The relationship between glutamate concentration and fluorescence was linear over the range of concentrations of glutamate detected. Cells treated with toxins were lysed at the end of the experiment with 0.5% Triton X-100 to determine the total glutamate content.

Immunofluorescence assays

Experiments were performed at 6 days in vitro. Cells seeded onto 24-well plates were incubated with each of the four SPANs for 60 minutes at 37°C in serum-free medium, washed, fixed for 20 minutes at room temperature with 4% paraformaldehyde in PBS, quenched (0.38% glycine, 0.24% NH_4Cl in PBS, twice for 10 minutes each) and permeabilized with 5% acetic acid in ethanol for 20 seconds at –20°C. After saturation with 0.5% BSA in PBS for 20 minutes, samples were incubated with the primary antibodies (diluted in 3% BSA in PBS) for 60 minutes at room temperature, washed with 0.5% BSA in PBS and then incubated with the correspondent Texas-Red- or fluorescein-conjugated secondary antibodies diluted in 3% BSA in PBS. To test the effect of the four SPANs on SV recycling, both control cells and cells treated with the toxins (60 minutes at 37°C) were incubated with a primary antibody directed against the lumenal domain of synaptotagmin 1 (SytI-ecto Abs); incubation was carried out for 5 minutes at 37°C and then samples were extensively washed, fixed, quenched and incubated without permeabilization with Texas-Red-conjugated anti-mouse secondary antibody. Coverslips were mounted in 90% glycerol in PBS containing 3% N-propylgallate and examined by confocal (Bio-Rad MRC1024ES) or epifluorescence (Leica DMIRE2) microscopy.

Results

Activity of the four snake presynaptic neurotoxins on isolated nerve muscle preparations

The mouse phrenic nerve-hemidiaphragm preparation was used for a comparative test of the biological potency of the four SPANs used in the ensuing cell biology experiments. As reported in Table 1, β-BTx (3 μg ml^{-1}), taipoxin or textilotoxin (1 μg ml^{-1}) reproducibly facilitated neurotransmitter release. This transient effect was followed by a gradual and irreversible blockade of neurotransmission with muscular paralysis. Maximal facilitation occurred after comparable time periods for the three neurotoxins with similar values of the twitch tension. This facilitation phase was not apparent in the case of notexin, as reported previously (Harris et al., 2000), which nevertheless caused complete neuromuscular blockade. These data indicate that the neurotoxins used in this study have neurotoxicities comparable to or higher than those reported in previous studies (for a review, see Hodgson and Wickramaratna, 2002). They also have PLA2-specific activity similar to those reported previously (Rosenberg, 1997) (see Materials and Methods).

Effect of SPANs on the survival of cerebellar and cortical neurons

The effect of the four SPANs on the survival of CGNs and

Table 1. Effect of phospholipase A2 neurotoxin intoxication on the twitch of mouse phrenic-nerve hemidiaphragm preparations

	Concentration	Maximum facilitation (% ctr)*	Duration of facilitation (minutes)	Paralysis time (minutes)
β-BTx	3 μg ml^{-1}	140.7±7	17±2.6	148±6.1
Taipoxin	1 μg ml^{-1}	138.8±9	19.5±1.3	56.4±6.7
Notexin	1 μg ml^{-1}	ND	ND	42±2.8
Textilotoxin	1 μg ml^{-1}	127±6.2	24.3±5.1	76.7±5.7

Data are means of three different experiments; ND, not determined.
*Percentage with respect to the control twitch (i.e. before adding tonin).

cortical neurons as a function of time of exposure was then determined. Cells were allowed to bind the neurotoxins (5 nM) at 37°C for 10 minutes, 30 minutes, 60 minutes or 180 minutes. After washing, cells were incubated for an additional 4 hours at 37°C in culture medium in the presence of the vital dye resazurin. As shown in Fig. 1, a time-dependent neurotoxicity was evident in both CGNs and cortical neurons. A 10-minute exposure is sufficient to enable each neurotoxin to bind to the neuronal surface and subsequently to cause an effect leading to the death of a large proportion of neurons during the

following 4 hours of incubation. CGNs and cortical neurons were similarly sensitive to β-BTx, taipoxin and textilotoxin, whereas notexin was the least cytotoxic in CGNs and the most cytotoxic in cortical neurons.

Morphological features of primary neuronal cell cultures exposed to snake neurotoxins

The morphology of CGNs, cortical and hippocampal neurons was observed after 1 hour of exposure to 5 nM of SPANs with both Nomarski optics and FESEM. In all cultures, with all toxins, neuronal extensions assumed a characteristic bead-shaped structure showing many bulges of variable size (Figs 2, 3). By contrast, careful morphometric estimations of many neurons indicated that the volume of cell bodies was unaffected by the toxins. We attempted to evaluate the involvement of the PLA2 activity of SPANs in neuronal bulging in two different ways. The first was the use of PLA2 inhibitors (aristolochic acid, 12-epi-scraladial and manoalide), but we were unable to find an inhibitor of the enzymatic activity of these neurotoxins that is not toxic to CGNs and cortical neurons. In the second approach, SPANs were added to neurons in medium without Ca^{2+} and containing 3 mM Sr^{2+}, conditions that inhibit PLA2 activity (Simpson et al., 1993). In this case, the formation of bulges was greatly delayed, indicating that the PLA2 activity is required for the neuronal bulging. Under the same conditions, no morphological changes were detected in controls (not shown). The number of bulges per μm^2 induced by taipoxin, β-BTx and the non-neurotoxic pancreatic PLA2 was determined by FESEM analysis of CGNs (Fig. 4). As shown in Fig. 4A, only neurotoxic PLA2s caused neurite enlargements – neuronal cultures treated with a very active pancreatic PLA2 at a 40-fold higher concentration were indistinguishable from controls. The formation of bulges was found to be dose dependent and was already apparent in the presence of 0.5 nM taipoxin (Fig. 4B).

Effect of SPANs on the neuronal cytoskeleton

The establishment and maintenance of neuronal projections depend on the integrity of the cytoskeleton, and so the distribution of F-actin, neurofilaments and α-tubulin in intoxicated cells was determined and compared with that of control neurons. F-actin was labelled with rhodamine-coupled phalloidin, whereas neurofilaments and tubulin were stained with specific antibodies. The microtubule content of CGNs was not altered by a 1 hour exposure to β-BTx (5 nM) (Fig. 5). By contrast, the characteristic filamentous staining of both F-actin and neurofilaments was largely lost, appearing highly fragmented with respect to control cells. Interestingly, these hot spots localized along neurites, suggesting that the bulges observed upon SPANs treatment might accumulate actin and neurofilament subunits. Similar results were obtained in cortical neurons and by using the other three snake neurotoxins studied here (not shown). These findings obtained in neuronal primary cultures parallel the recent report describing the loss of the ring-like distribution of F-actin upon incubation of chromaffin cells with similar concentrations of taipoxin (Neco et al., 2003) and the alteration of the axonal cytoskeleton detected in neurons 1 hour after the inoculation of taipoxin into the rat hind limb (Harris et al., 2000).
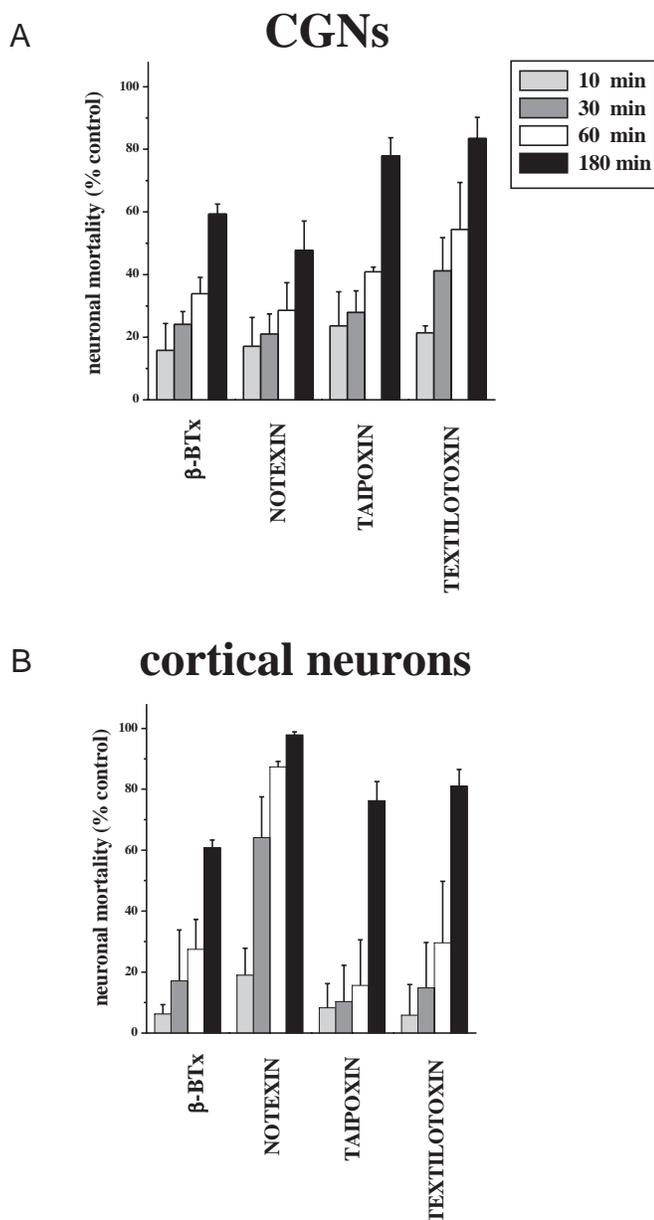

Fig. 1. Time-dependent induction of cell death of CGNs (A) or cortical neurons (B) by β-BTx, notexin, taipoxin and textilotoxin. Neurons were incubated for the indicated times with 5 nM of each toxin, washed extensively and further incubated for 4 hours in the presence of resazurin to quantify the mitochondrial activity of viable cells. Values are averages of three independent experiments with triplicate measurements; error bars indicate s.d.

Neurospecificity of the morphological changes caused by SPANs

Primary cultures of CGNs from 7-day-old rats are highly homogeneous and contain a small proportion of glial cells (5% of astrocytes). By contrast, cortical neurons prepared from 1-day-postnatal rats contained 20% of non-neuronal cells. To test whether the activity of the four snake toxins is specific for neurons, cerebellar and cortical primary cultures were co-stained with phalloidin (which binds neuronal and non-neuronal actin) and the glial-cell-specific marker GFAP. Upon exposure to taipoxin, the actin staining changed from filamentous structures to dots only in neurons, whereas the actin appearance of GFAP-positive astrocytes was unaltered (Fig. 6). Similar results were obtained with the other neurotoxins (not shown), indicating that the actin filament destabilising effect of SPANs is indeed neurospecific.

Effect of SPANs on the distribution of small synaptic vesicles markers

It was recently suggested that the snake PLA2 presynaptic neurotoxins promote the fusion of SVs with the presynaptic
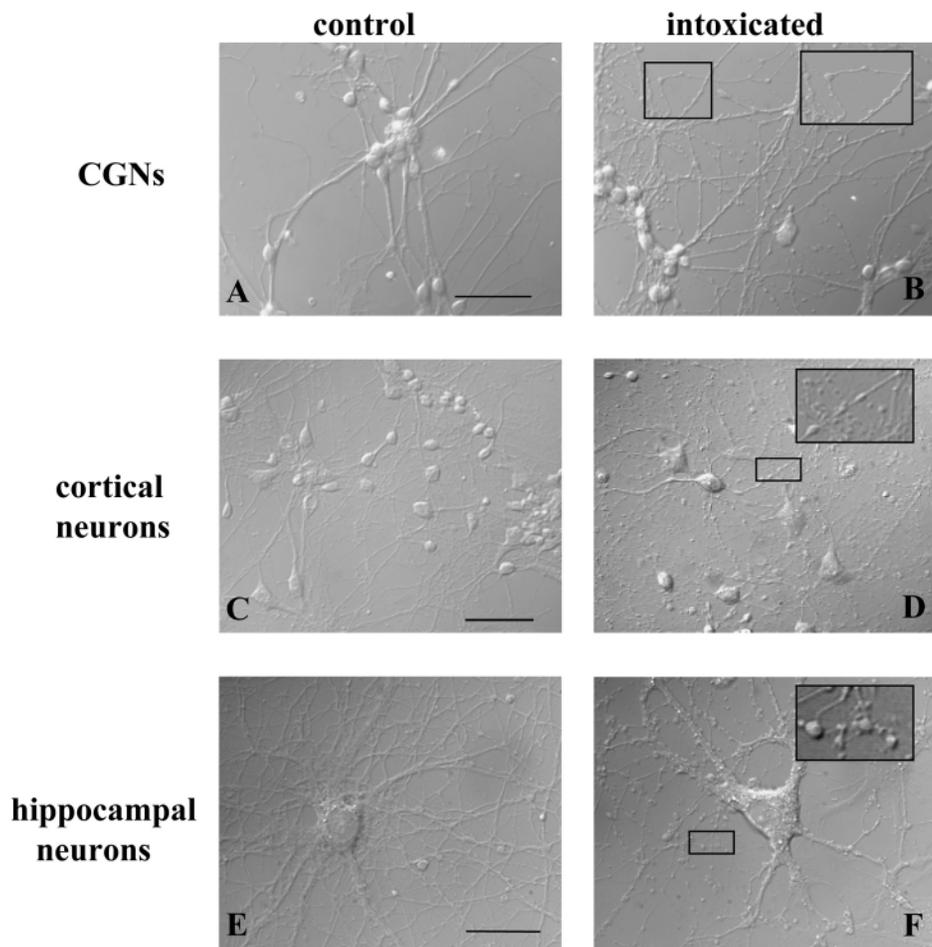

Fig. 2. Morphological changes induced by β-bungarotoxin in intoxicated primary neuronal cultures. CGNs (A,B) and cortical (C,D) neurons at 6 days in vitro or hippocampal neurons (E,F) at 15 days in vitro were examined by Nomarski optics after incubation for 1 hour in either control medium (A,C,E) or medium with 5 nM β-BTx. Notice the presence of many bulges in the neuronal extensions of the toxin-treated samples. Scale bars, 50 μm (A-D), 10 μm (E,F). Insets are higher magnifications of the areas indicated by the square.
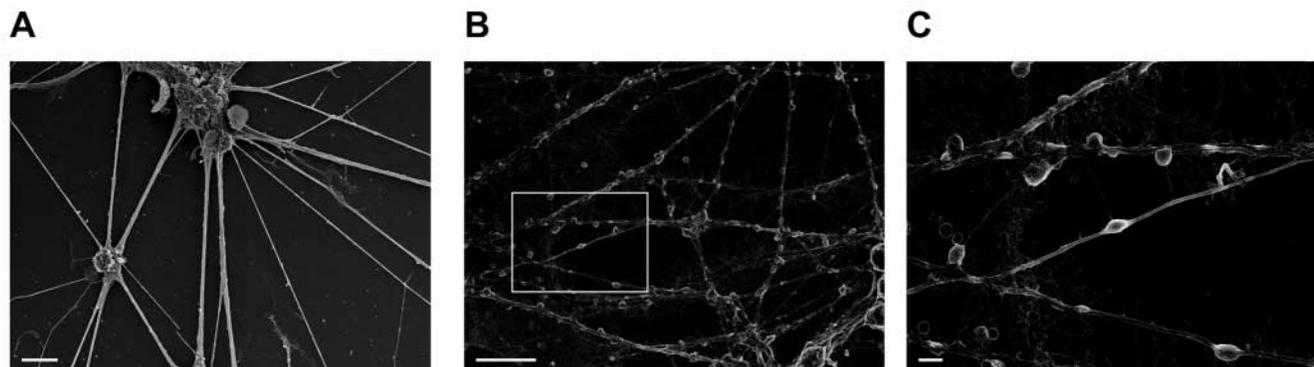

Fig. 3. FESEM analysis of control and intoxicated CGNs. Control neurons (A) and neurons treated for 1 hour with 5 nM taipoxin (B,C) were processed and analysed by FESEM. (C) Higher magnification of the area indicated by the square in (B). Scale bars, 10 μm (A,B), 1 μm (C).
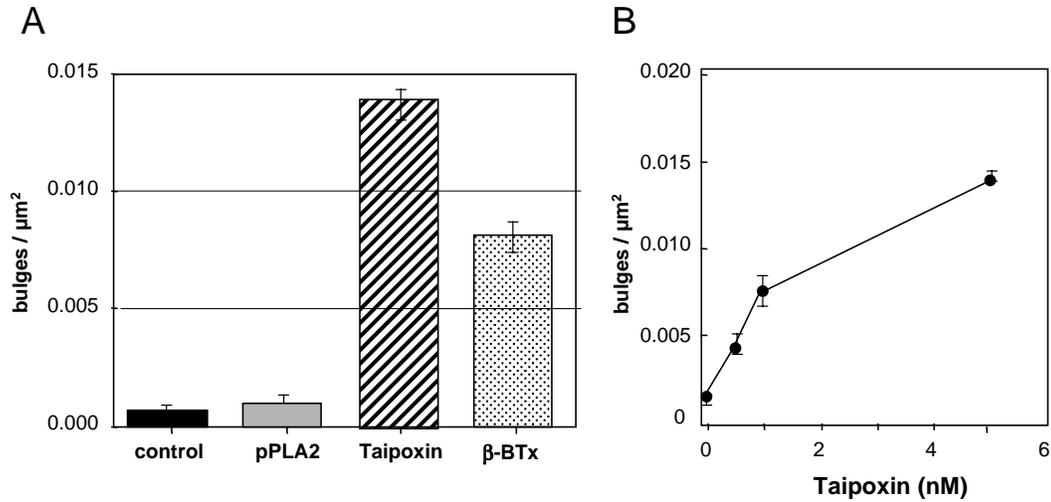
A

B

Fig. 4. Quantification of bulge density in CGNs by FESEM analysis. (A) Bulges were counted and expressed as bulge density (bulges μm^{-2}) in control cells or in cells treated for 1 hour with 200 nM pancreatic PLA2 or with 5 nM taipoxin or β-BTx. (B) Dose-dependent quantification of bulges in CGNs treated for 1 hour with taipoxin concentration 0.1-5.0 nM. Bars represent the data obtained from three different cell preparations ± s.d.

membrane not followed by vesicle endocytosis (Montecucco and Rossetto, 2000). This hypothesis predicts a swelling of synaptic sites owing to the incorporation in the plasma membrane of SV components, including SV membrane proteins exposing their intravesicular domain on the cell surface.

To investigate the origin of bulge formation, the spatial distribution of SypI and VAMP2, two integral SV membrane markers, was examined in CGNs, cortical and hippocampal neurons treated with the four SPANs for 1 hour at 37°C by confocal or epifluorescence microscopy. In intoxicated cells, the immunostaining of SypI and VAMP2 became much more

intense in correspondence to the bulges (Fig. 7). Fig. 7G,H show that, in neurons transfected with SypI-EYFP and ECFP-VAMP2, respectively, the SPANs also induce an accumulation of the vesicular components within the synaptic bulges. By contrast, the immunofluorescence staining of SNAP-25 and syntaxin, two synaptic t-SNAREs localized to the plasma membrane, did not change upon intoxication (not shown).

To monitor SV recycling in intoxicated neurons, we used a well-established assay based on the use of antibodies specific for the lumenal domain of the SV protein synaptotagmin I (SytI-ecto Abs), which were shown to bind SytI in intact neurons following depolarization and to recycle with the SV
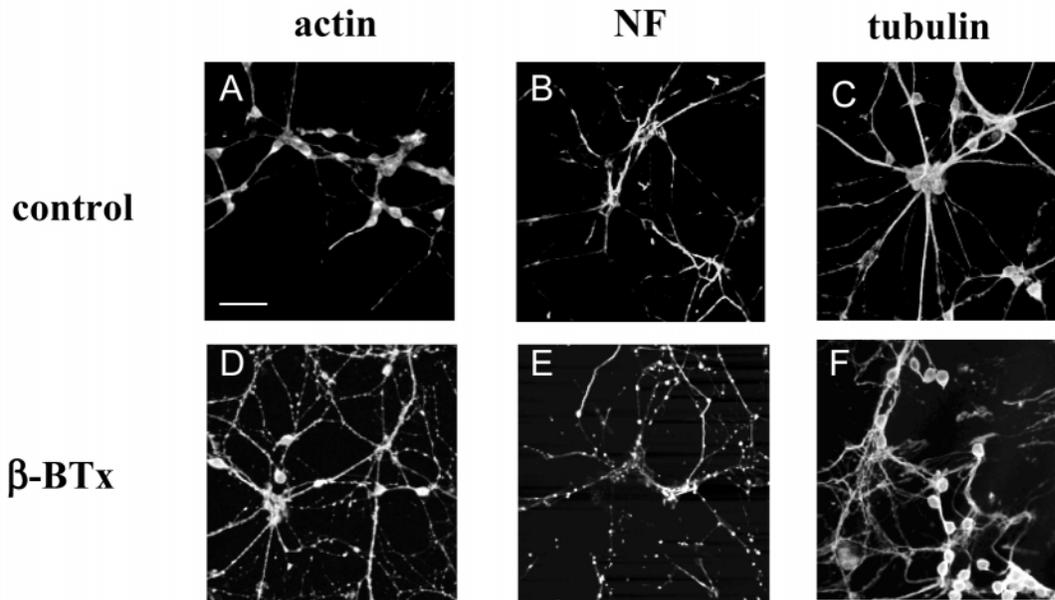

Fig. 5. Effect of SPANs on the distribution of cytoskeletal markers. Untreated CGNs (A-C) were compared with CGNs treated for 1 hour with β-BTx (D-F). The distribution of F-actin (A,D) and of neuron-specific neurofilaments (NF) (B,E) changed from filamentous structures to dotted aggregates, whereas the appearance of tubulin (C,F) remained unaltered. Scale bar, 50 μm.
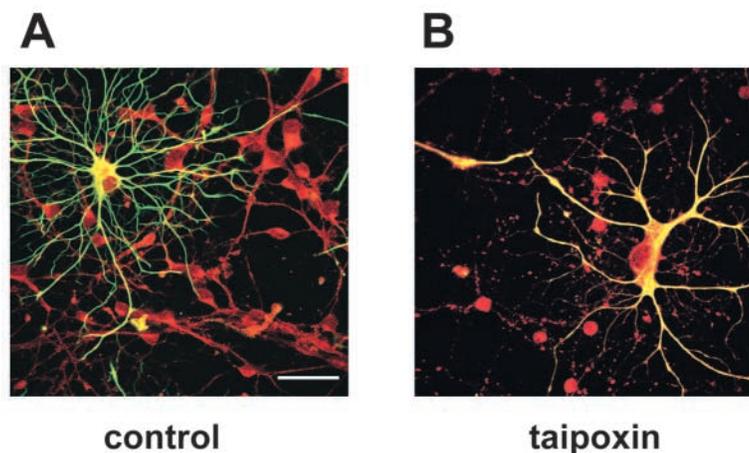
Fig. 6. Cellular effects of SPANs are restricted to neuronal cells. Control CGNs (A) were compared with cells that were treated for 1 hour with taipoxin (B). Distribution of actin labelled with phalloidin (red) changed from filamentous structures to dots in intoxicated neurons, whereas the actin appearance of GFAP-positive astrocytes was unaltered (yellow). Scale bar, 50 μm.

(Matteoli et al., 1992; Verderio et al., 1999). When anti-SytI-ecto antibodies were applied to SPAN-treated neurons before fixation, a bright, selective staining of bulges was observed without permeabilization (Fig. 8). By contrast, the intravesicular domain of SytI required permeabilization to

become labelled in mock-treated or KCl-treated samples. No immunostaining was observed with antibodies specific for synapsin I, a protein associated with the cytosolic face of the SV membrane, demonstrating that these toxins do not cause permeabilization of the plasma membrane to antibodies (not shown). These data suggest that bulges originate, at least partially, from the fusion of SV with the plasma membrane, which is uncoupled to a proportionate membrane retrieval as observed during physiological SV recycling.
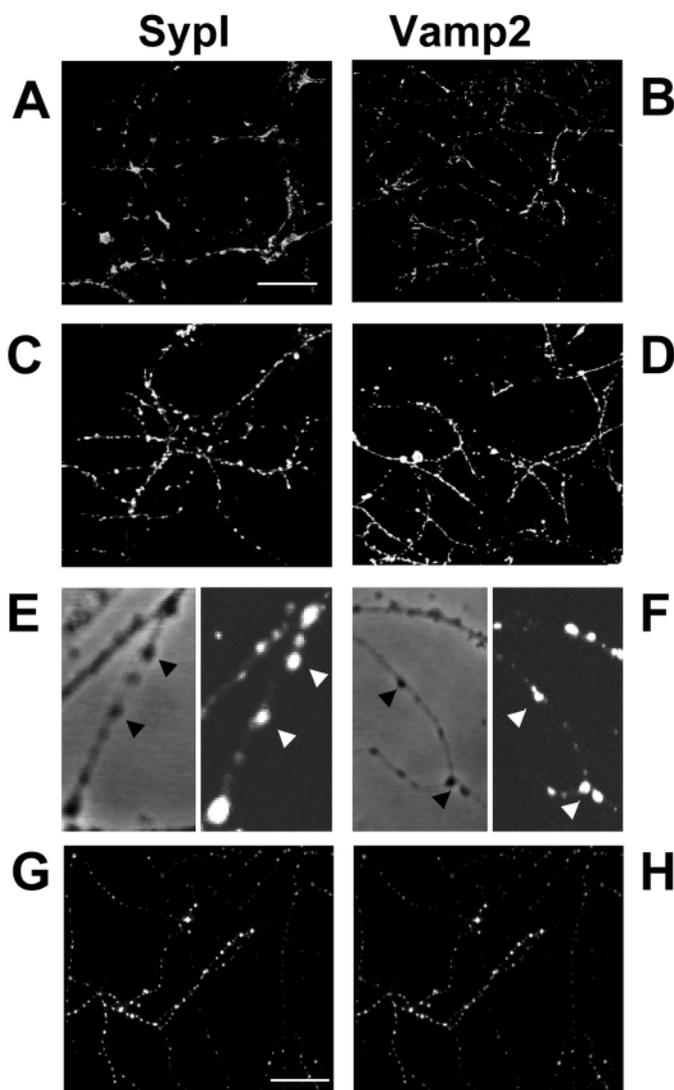
Glutamate release from intoxicated CGNs

The major neurotransmitter present in CGNs is glutamate, which is partially stored in SVs. Based on the findings described above, glutamate is expected to be released into the medium following treatment of neurons to SPANs. To test this hypothesis, we used the method established in CGNs by Graham and Burgoyne (Graham and Burgoyne, 1995). We found that all the four SPANs were able to stimulate glutamate release from CGNs; as an example, 5 nM taipoxin induced the release of 2.3±0.57% (n=3) of the total glutamate of CGNs after 30 minutes and of 5.1±0.74% (n=3) after 60 minutes, compared with 8.8±0.85% (n=5) induced by depolarization with 90 mM KCl. These results parallel the SPAN-induced release of acetylcholine from NMJ (Table 1) and the reported stimulation of neurotransmitters release from synaptosomes (Fletcher and Rosenberg, 1997). This toxin-induced glutamate release strongly supports the proposal that synaptic bulging in cultured neurons caused by SPANs is determined by the exocytosis of neurotransmitter-containing SV.

Fig. 7. Effect of SPANs on the distribution of synaptophysin I and VAMP2. Control CGNs (A,B) and neurons treated for 1 hour with 5 nM taipoxin (C-F) were fixed, permeabilized, counterstained for SypI (A,C,E) or for VAMP2 (B,D,F) and analysed by fluorescence confocal microscopy. The two vesicular membrane proteins changed distribution, being more intense in correspondence to the bulges of intoxicated neurons. (E,F) Higher magnifications of phase-contrast and fluorescence images of intoxicated neurons showing the intense staining of the two SV proteins in correspondence of the bulges (arrow). (G,H) Hippocampal neurons were co-transfected with expression vectors containing SypI-EYFP (G) and ECFP-VAMP2 (H), exposed to 5 nM taipoxin for 1 hour at 37°C and then analysed without fixation by epifluorescence microscopy. Scale bar, 50 μm (A-D), 10 μm (G-H).
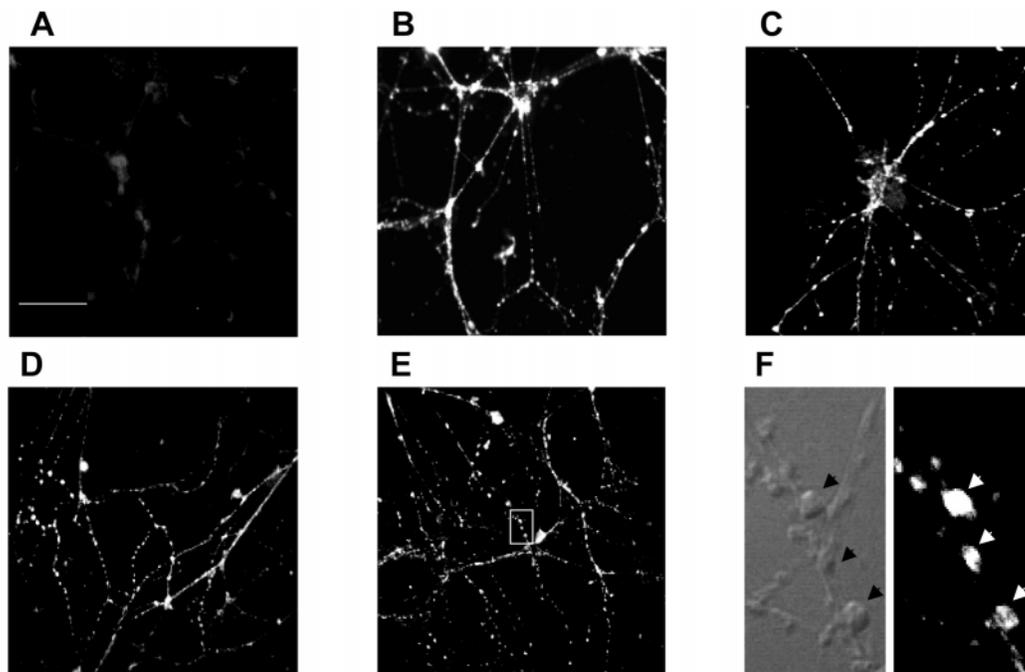
Fig. 8. SPANs induce SV exocytosis without proportionate recycling. Control CGNs (A) and cells treated for 1 hour at 37°C with notexin (B), β-BTx (C), textilotoxin (D) or taipoxin (E) were incubated for 5 minutes at 37°C with anti-SytI-ecto antibodies before fixation and then processed for indirect immunofluorescence. (F) Higher magnification of the area indicated by the square in (E), which highlights the bright selective staining of bulges in the intoxicated sample (arrows). Scale bar, 50 μm (A-E).

Discussion

Despite the number of studies on the presynaptic activity of snake PLA2 neurotoxins carried out with different experimental approaches, their molecular mechanism(s) of action is still ill-defined. β-BTx has been more widely used than other SPANs and, in several cases, at concentrations (150-750 nM) far exceeding the mice LD_{50} values. To obtain results of physiological relevance and, of more general interest, we have here used SPANs of different structural complexity at low concentrations and have studied their effects on different types of primary cultures of neurons.

Such an experimental approach led to the finding that SPANs act very similarly, rapidly inducing a defined swelling of synaptic sites with formation of bulges. This appears to be the earliest detectable morphological sign of cell intoxication. In this respect, the four neurotoxins used here behave identically, suggesting that they might act via the same mechanism. In the belief that the study of the initial events induced by SPANs in neurons might provide novel and relevant information about their molecular mode of action, we have analysed this phenotype in detail employing a range of techniques.

Such bulges originate mainly from the toxin-induced fusion of SVs with the plasma membrane not followed by a proportionate retrieval, with consequent increase in the extension of the plasma membrane. Such interpretation is suggested by the following concomitant toxin-induced effects, which were first disclosed by the present work: (i) SV membrane proteins concentrate on the bulges; (ii) markers of the SV lumen become exposed on the neuronal surface in correspondence to the neurite enlargements; and (iii) SPANs induce the release of glutamate contained within SV.

Membrane bulging was found to be dose dependent and did not appear under conditions inhibiting the PLA2 activity of SPANs, nor in the presence of high concentrations of the non-neurotoxic pancreatic PLA2. Interestingly, these findings closely match those seen by electron microscopy at intoxicated NMJs, which appear swollen, depleted of SVs and enriched in clathrin-coated invaginations of the plasma membrane representing incomplete SV retrieval (Chen and Lee, 1970; Cull-Candy et al., 1976; Gopalakrishnakone and Hawgood, 1984). It was recently proposed that fusion of synaptic vesicles could be induced by a vesicle-based PLA2 activity of the SPANs (Montecucco and Rossetto, 2000). Although there is some evidence for an intracellular site of action of SPANs (Krizaj and Gubensek, 2000; Herkert et al., 2001; Neco et al., 2003; Sribar et al., 2003), this site(s) remains to be identified. Clearly, a PLA2 activity per se is not sufficient to cause the phenotype described here, because very high amounts of the non-neurotoxic pancreatic PLA2 did not induce any bulging. Therefore, the enzymatic activity of the SPANs has to be localized within defined neuronal sites, which can be reached following a neurotoxin nerve binding that takes place rather rapidly. A lack of binding accounts for the nontoxicity of pancreatic PLA2 and might also explain the lack of effect of SPANs on the glial cells present in our primary neuronal cultures. Their interaction with the neuronal cell surface is likely to be determined by the specific structure of each SPAN (Simpson et al., 1993). Different modes of binding could account for our finding that CGNs are more sensitive to β-BTx, taipoxin and textilotoxin than to notexin, which in turn is more effective on cortical neurons.

The addition of membrane brought about by synaptic vesicle

exocytosis occurring at defined sites is a prerequisite for neurite swelling and bulging, but it is not sufficient. A local increase of osmotic pressure is required. The local production of lysophospholipids and of fatty acids by SPAN-mediated hydrolysis causes an increase in membrane permeability to ions (Lee and Chan, 1977) and, as such, could provide the increase in osmotic pressure that would drive neurite swelling. The present validation of in vitro neuronal models amenable to further imaging analysis and biochemical studies should greatly facilitate the unravelling of the mechanism(s) of interaction of the SPANs with the neuronal surface and of the biochemical steps involved in their facilitation of neurexocytosis and inhibition of vesicle retrieval.

Another important finding described here is that SPANs induce a fragmentation of the distribution of both actin and neurofilaments in neurons. This result follows the recent description of the loss of the ring-like distribution of F-actin induced by taipoxin in chromaffin cells (Neco et al., 2003). In these neuroendocrine cells, taipoxin was suggested to promote the release of secretory granules through the alteration of the F-actin barrier. Synaptic actin plays a major role in controlling the size and mobilization of secretory vesicles belonging to the reserve pool (Benfenati et al., 1991) and in multiple steps of SV recycling (Benfenati et al., 1991; Morales et al., 2000; Slepnev and De Camilli, 2000; Shupliakov et al., 2002; Sankaranarayanan et al., 2003). This effect of SPANs on F-actin could, along with phospholipid hydrolysis, contribute to the facilitation of neurotransmitter release not followed by SV replenishment within nerve endings. A scenario can be envisaged whereby SPANs block the nerve terminal by means of two co-operative actions: first at the level of phospholipid hydrolysis, owing to their PLA2 activity; and, second, by altering the integrity and distribution of F-actin at the synapse. Thus, SPANs might be important and potentially very useful tools for exploring different aspects of the basic mechanism responsible for neurotransmitter release and membrane dynamics at the nerve terminal.

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