

Taipoxin Induces Synaptic Vesicle Exocytosis and Disrupts the Interaction of Synaptophysin I with VAMP2

Dario Bonanomi, Maria Pennuto, Michela Rigoni, Ornella Rossetto, Cesare Montecucco, and Flavia Valtorta

Department of Neuroscience, S. Raffaele Scientific Institute and "Vita-Salute" University, Milan, Italy (D.B., M.P., F.V.); and Department of Biomedical Sciences, University of Padova, Italy (M.R., O.R., C.M.)

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ABSTRACT

The application of the snake neurotoxin taipoxin to hippocampal neurons in culture induced Ca^{2+} -dependent synaptic vesicle (SV) exocytosis, with swelling of nerve terminals and redistribution of SV proteins to the axolemma. Using digital imaging videomicroscopy to measure fluorescence resonance energy transfer in live neurons, we also found that taipoxin modulates

the machinery for neurosecretion by causing dissociation of the SV proteins synaptobrevin 2 and synaptophysin I at a stage preceding taipoxin-induced facilitation of SV fusion. These early effects of the toxin are followed by severe impairment of SV exo-endocytosis, which might underlie the prevention of neurotransmitter release reported after intoxication by taipoxin.

Transfer of information in the brain occurs mainly through the exocytotic release of neurotransmitters that are contained in synaptic vesicles (SVs). SV exo-endocytosis is a multistep process that involves a highly regulated interplay of both soluble and membrane-associated proteins (Valtorta and Benfenati, 1995). A detailed molecular map of SVs has been generated, and ubiquitous homologs of SV proteins have been identified, leading to the notion that similar sets of proteins are involved in all membrane trafficking events in eukaryotes (Südhof, 2004). A large number of molecular interactions have been shown to occur in vitro among proteins involved in SV exo-endocytosis (Benfenati et al., 1999), but demonstration of their occurrence in intact cells and their significance has often proven difficult.

Synaptobrevin/vesicle-associated membrane protein 2 (VAMP2) plays a pivotal role in the process of SV fusion, because it is involved in the formation of a heterotrimeric complex (termed SNARE complex) with soluble *N*-ethylma-

leimide-sensitive factor attachment protein-25 (SNAP-25) and syntaxin, two proteins associated with the cytosolic face of the presynaptic membrane (Südhof, 2004). Tetanus toxin and certain serotypes of botulinum toxin cleave the cytosolic part of VAMP2 and block neurosecretion (for review, see Schiavo et al., 2000). Synaptophysin I (SypI) is involved in multiple, important aspects of SV exocytosis, including SV biogenesis and formation of the fusion pore initiating neurotransmitter release (Valtorta et al., 2004). In addition, studies with the isolated proteins have suggested that, when bound to SypI, VAMP2 is prevented from engaging in the SNARE complex (Calakos and Scheller, 1994; Edelman et al., 1995; Washbourne et al., 1995).

We have detected changes in protein-protein interactions that occur during stimulation of SV exocytosis by α -latrotoxin (α -Ltx) (Pennuto et al., 2002). Such changes were monitored by video-enhanced microscopy of hippocampal neurons transfected with fluorescent chimeras of the SV proteins SypI and VAMP2, which transfer fluorescence resonance energy between them depending on their distance. In resting nerve terminals, VAMP2 was found to be close to SypI, whereas α -Ltx stimulation induced the two proteins to dissociate from each other before SV exocytosis. These findings, obtained in intact neurons, are particularly relevant in view of the important role played by the two proteins in neurosecretion.

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ABBREVIATIONS: SV, synaptic vesicle; VAMP2, Synaptobrevin/vesicle-associated membrane protein 2; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP, soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein; SypI, Synaptophysin I; α -Ltx, α -latrotoxin; Tpx, taipoxin; NMJ, neuromuscular junction; PCR, polymerase chain reaction; DIV, days in vitro; PEI, polyethylenimine; FRET, fluorescence resonance energy transfer; KRH, Krieb's Ringers-HEPES; DIC, differential interference contrast; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein.

Animal and bacterial toxins are very useful research tools for dissecting the molecular steps involved in neuroexocytosis (Rappuoli and Montecucco, 1997). Indeed, thanks to continuous refinement in the course of evolution, some toxins are exquisitely specific for a selected target step(s) in the process. An important family of neurotoxins is constituted by snake neurotoxins endowed with phospholipase A₂ activity (Schiavo et al., 2000; Kini, 2003). One member of this family, taipoxin (Tpx), isolated from the venom of the snake *Oxyuranus scutellatus* (Kamenskaya and Thesleff, 1974; Fohlman et al., 1976), has been shown to act presynaptically using neuromuscular junction (NMJ) preparations. Its precise mechanism of action remains unknown. Tpx induces blockade of neurotransmitter release, which eventually results in the death of the poisoned animal by respiratory failure. However, under certain experimental conditions, the development of neurotransmission failure at NMJs is preceded by a phase of facilitation of release, raising the possibility that blockade occurs because of depletion of the store of SVs (Su and Chang, 1984). Indeed, electron microscopic pictures taken at late stages of intoxication show the presence of swollen and enlarged axon terminals virtually devoid of SVs (Cull-Candy et al., 1976; Harris et al., 2000).

The presynaptic membrane receptor for taipoxin has not been identified yet, although secreted neuronal pentraxins and an endoplasmic reticulum Ca²⁺-binding protein have been reported to bind taipoxin and to form multimers with an integral neuronal pentraxin receptor (Kirkpatrick et al., 2000). The toxin seems to bind specifically to neuronal plasma membranes, and its effects have been ascribed to the phospholipase A2 activity of the toxin, with the consequent production of fatty acids and lysophospholipids. It has been hypothesized that the changes in lipid composition induced by the phospholipase A2 activity, when occurring in the SV membrane, might increase its fusogenicity but hamper its retrieval from the axolemma (Montecucco and Rossetto, 2000).

The recent demonstration that Tpx is active also on neurons derived from the central nervous system, where it induces the formation of bulges along neurites with redistribution of SV proteins within those bulges (Rigoni et al., 2004), prompted us to investigate in live hippocampal neurons the relationship between bulges formation and SV exocytosis, and the correlation between Tpx-induced exocytosis and the dynamics of SytI-VAMP2 interactions.

Materials and Methods

Generation of the Chimeric Fluorescent Proteins. Rat synaptophysin I (SytI) full-length cDNA (921 bp) cloned into the pBlueScript vector (Stratagene, La Jolla, CA) was provided by Dr. R. Leube (University of Mainz, Germany). SytI cDNA was amplified by PCR with the following oligonucleotides: forward, 5'-GGGGGAAGCTTCAGCAGCAATGGACGTG-3'; reverse, 5'-GGGGGGATCCGCTGCTGTAGTAGCAGTAGGTCTTGGGCTCCACGCCCTTCATCTGATTGGAGAAAGGAGGTGG-3'. HindIII and BamHI restriction sites, introduced with the forward and reverse primers, respectively, are underlined. The reverse primer was designed to remove the stop codon and, in addition, to introduce a linker of 13 amino acids (KGVEPKTYCYSS) (Nakata et al., 1998) at the COOH-terminal end of SytI cDNA. The resultant HindIII/BamHI PCR fragment was inserted into the corresponding sites of pECFP-N3 and pEYFP-N3 vectors (BD Biosciences Clontech, Palo Alto, CA).

VAMP2 full-length cDNA (351 bp) cloned into pBlueScript K⁺ (Rossetto et al., 1996) was amplified by PCR with the following oligonucleotides: forward, 5'-GGGGTGTACAAGATGTCGGCTACCGCTGCCAC-3'; reverse, 5'-GGGGGCGGCCGCTTAAGTGCTGAAGTAAAC-3'. BsrGI and NotI restriction sites, introduced with the forward and reverse primers, respectively, are underlined. The resultant BsrGI/NotI PCR fragment was inserted into the corresponding sites of pECFP-N3 and pEYFP-N3.

Cell Cultures and Transfections. Low-density primary cultures of hippocampal neurons were prepared from Sprague-Dawley rat embryos (embryonic day 18; Charles River Italiana, Calco, Italy) as described previously (Banker and Cowan, 1977). Neurons were transfected at 3 days in vitro (DIV) using 25-kDa polyethylenimine (PEI 25) (Sigma-Aldrich, Steinheim, Germany). Fresh medium was applied to cell cultures 1 h before starting the procedure. Then, PEI 25 (28 nmol/dish) and plasmid DNA (2.5 µg/dish) were diluted in 50 µl of 150 mM NaCl in separate tubes. The solution containing PEI was added to that containing the DNA, and the mixture was vortexed four times within 12 min before addition to the cells. Coverslips were placed in a clean 35-mm Petri dish and cells were rinsed with minimal essential medium supplemented with 10% horse serum, 2 mM glutamine, and 3.3 mM glucose. The medium was removed and cells were incubated for 2 h at 37°C in a 5% CO₂ humidified atmosphere with 1 ml of the same medium containing the 100 µl PEI/DNA solution. Coverslips were then repositioned above astrocyte monolayers in the original dishes and kept in culture for 15 to 18 days. Transfection efficiency varied from 0.1 to 1%.

Taipoxin was purchased from Venom Supplies (Tanunda, South Australia). Purity was checked by SDS-polyacrylamide gel electrophoresis. α-Latrotoxin (α-Ltx) was a kind gift of Dr. Alexander Petrenko (New York University, New York, NY).

FM4-64 Assay. FM4-64 (10 µM; Molecular Probes, Eugene, OR) was loaded into recycling SVs of 15-DIV hippocampal neurons using a depolarizing solution containing KRH supplemented with 45 mM KCl. The incubation was carried out for 60 s at room temperature and was followed by rinsing for 15 min with a 2 ml/min flow of KRH containing 10 µM 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (Tocris, Ellisville, MO) and 1 µM tetrodotoxin (Tocris, Ellisville, MO). After the washing protocol, FM4-64 staining was imaged using a bandpass filter for excitation at 530 to 595 nm, a long-pass filter for emission at 615 nm, and a 63× oil immersion objective. Corresponding differential interference contrast (DIC) images were used to identify the swollen boutons in the Tpx-treated neurons. The intensity of FM4-64 fluorescence at single synapses was measured before and after treatment.

Fluorescence Resonance Energy Transfer Analysis. Expression vectors encoding fluorescent proteins were cotransfected at a ratio of 1:2 or 1:4 (donor/acceptor). Cells (15–18 DIV) were washed once with KRH/EGTA and incubated in the same solution in the presence or absence of either 0.1 nM α-Ltx or 6 nM Tpx for 30 min at 37°C in 5% CO₂; the cells were then washed twice with KRH/EGTA. Images were acquired within 30 to 45 min after treatment of the cells. The specimen was irradiated at the wavelength of 436 ± 10 nm and a time-lapse series of images of the donor fluorescence was recorded at the wavelength of 480 ± 30 nm during continuous illumination. From the first image of the series, a binary mask was prepared, in which each spot corresponded to a synaptic bouton. Fluorescent spots that moved quickly along the axon (presumably representing traveling packets) were excluded from the analysis. The time-series data for each pixel position within a bouton were fit to an exponential decay function to determine decay constants of photobleaching.

When FRET occurs between donor and acceptor fluorophores, the time constant for donor photobleaching increases (Jovin and Arndt-Jovin, 1989). Thus, the efficiency (*E*) of FRET was calculated as the percentage change in the average time constant of donor photobleaching measured in specimens transfected with the SV-located acceptor fluorescent proteins (τ_{SV+cyt}), with respect to that measured

in specimens transfected with cytosolic EYFP acceptor (τ_{sv*cyt}): $E = 1 - (\tau_{sv*cyt}/\tau_{sv*sv})$.

One of the advantages of this method for measuring FRET is that the measurements do not depend on absolute values of fluorescence. Indeed, we found no significant correlation between initial intensities of fluorescence and photobleaching rates ($R \approx 0.4$). The photobleaching time constants were found to have skewed distributions that became normal after logarithmic transformation. Therefore, data were analyzed using the natural logarithms of the photobleaching time constants, and efficiencies and statistics were derived by retransformation of the pertinent values. Where indicated, one-tailed t tests were performed to estimate the significance of differences between mean FRET efficiencies. To estimate the probability that a given mean FRET efficiency was statistically different from zero, the mean value normalized by the standard deviation of the mean was compared with a one-tailed Z distribution (Pennuto et al., 2002).

Results

Effect of Taipoxin on Synaptic Boutons. Hippocampal neurons were prepared from rat embryos (embryonic day 17) and kept in culture until 15–18 DIV, which corresponds to their full maturation and the establishment of a synaptic network with surrounding cells (Valtorta and Leoni, 1999). The neurons were then treated with 6 nM purified Tpx for 30 min in Ca^{2+} -containing medium (KRH). Video analysis showed that, after a delay of a few minutes, the morphology of the axons changed progressively, and with time it assumed a characteristic bead-shaped structure, with the formation of discrete bulges (Fig. 1, top). When higher concentrations of Tpx or longer incubation times were used, the number of bulges increased accordingly (see below). Virtually no changes in the morphology of cell bodies were ever observed.

Similar bulges were recently reported to be induced by various snake presynaptic neurotoxins with phospholipase A_2 activity in several types of neurons (Rigoni et al., 2004), and they resemble the nerve terminal swelling induced by α -Ltx as a consequence of stimulation of massive exocytosis paralleled by impairment of endocytosis (Ceccarelli and Hurlbut, 1980; Valtorta et al., 1988; Pennuto et al., 2002). However, whereas α -Ltx was able to induce nerve terminal swelling when applied in the absence of extracellular Ca^{2+} , Tpx required the presence of extracellular Ca^{2+} to produce this effect (Fig. 1, bottom).

Hippocampal neurons were transfected at 3 DIV with ex-

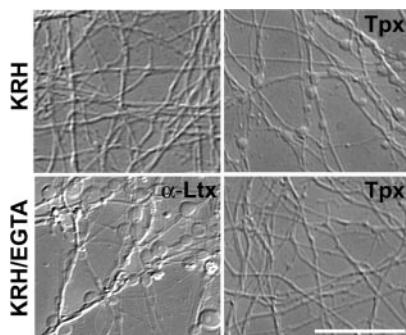


Fig. 1. Ca^{2+} -dependence of the effects of Tpx and α -Ltx. DIC images of hippocampal neurons incubated for 30 min in either Ca^{2+} -containing (KRH) or Ca^{2+} -free (KRH/EGTA) medium in the presence or absence of either 6 nM Tpx or 0.1 nM α -Ltx. Nerve terminal swelling is induced by Tpx exclusively in the presence of Ca^{2+} and by α -Ltx in the absence of Ca^{2+} . Scale bar, 10 μ m.

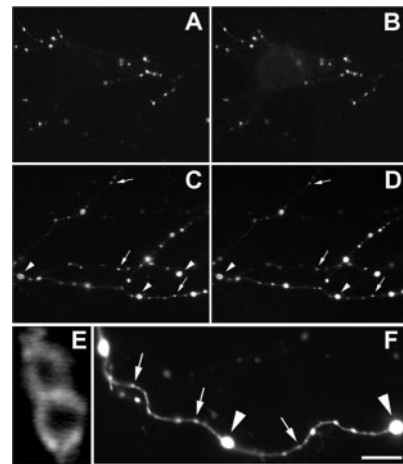


Fig. 2. Redistribution of synaptic vesicle proteins upon treatment with Tpx. Hippocampal neurons cotransfected with vectors encoding Sypl-EYFP (A and C) and ECFP-VAMP2 (B and D) were incubated for 30 min in KRH in either the absence (A and B) or presence (C and D) of 6 nM Tpx. Sypl and VAMP2 fluorescent chimeras largely colocalize and are present exclusively at synapses. Two distinct classes of synaptic boutons are present in the Tpx-treated sample: one with diameters in the range of those observed in control samples (arrows) and the other with considerably larger dimensions (arrowheads). E, high magnification confocal image of Tpx-induced swollen boutons from a Sypl-EYFP-expressing neuron. The peripheral ring of fluorescence is a result of the insertion of the chimera into the plasma membrane after exhaustive Tpx-induced SV exocytosis. F, neurons expressing soluble EYFP treated with 6 nM Tpx. Both small (arrows) and swollen (arrowheads) terminals are associated with the axon of an individual neuron. Scale bar: A–D, 10 μ m; E, 1.8 μ m; F, 3.8 μ m.

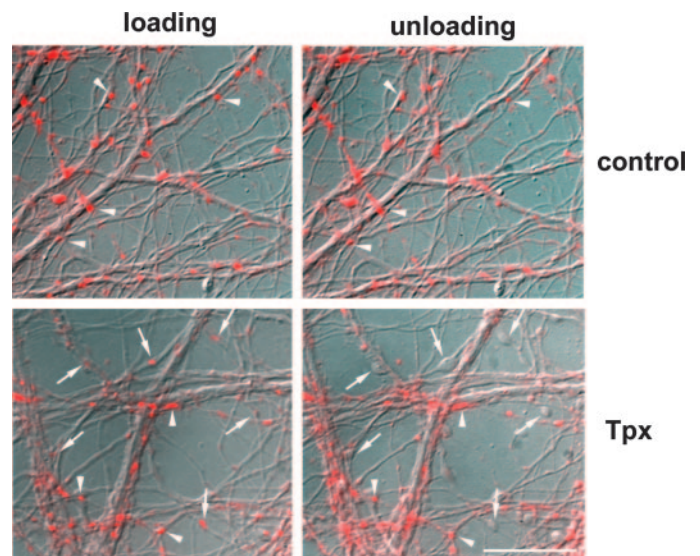


Fig. 3. The size of synaptic boutons reflects the extent of Tpx-induced exocytosis. Hippocampal neurons were loaded with FM4-64 during a 60-s incubation in depolarizing solution. DIC and FM4-64 images were recorded at time 0 (loading) and after a 30-min incubation in KRH in either the presence or absence of 6 nM Tpx (unloading). The merge between DIC and FM4-64 (red) images is shown. After toxin treatment, some of the synaptic boutons appeared swollen (arrows, lower right). The swollen boutons had released virtually all the dye (compare arrows, left and right bottom), whereas the dye had remained in the small synaptic boutons (compare arrowheads, left and right bottom), which were similar in size and brightness to those of the untreated sample (compare arrowheads, left and right top). Scale bar, 10 μ m.

pression vectors encoding for, SypI-EYFP and ECFP-VAMP2, the fluorescent chimeras of the SV proteins SypI and VAMP2, and kept in culture until 15 to 18 DIV. As reported previously (Pennuto et al., 2002, 2003), the chimeras showed a high degree of colocalization at synaptic boutons (Fig. 2, A and B), with a fluorescent pattern virtually overlapping with the immunolabeling for endogenous SV proteins (data not shown).

After exposure to Tpx, two distinct classes of synaptic boutons were present: a class of boutons virtually indistinguishable from those observed in untreated samples and a class of boutons considerably larger in size (Fig. 2, C and D). Observation of the samples by DIC microscopy confirmed that the swollen boutons corresponded to the axonal bulges described above (data not shown). Exposure to increasing concentrations of Tpx produced a dose-dependent increase in the percentage of swollen terminals [$25 \pm 6\%$ (6 nM Tpx), $60 \pm 3\%$ (12 nM Tpx), and $88 \pm 3\%$ (24 nM Tpx) of the total boutons; $n = 950$ per each condition].

When swollen terminals were imaged at high magnification by confocal microscopy, the fluorescent signal for the SV protein chimeras was found to be concentrated along a peripheral ring as a result of the insertion of the SV membrane into the plasma membrane upon induction of exhaustive exocytosis by Tpx (Fig. 2E). Expression of soluble EYFP was exploited to identify the projections belonging to a single neuron. After treatment with 6 nM Tpx for 30 min, both normally sized and swollen synaptic boutons were found along individual EYFP-positive axons, indicating that the existence of two classes of synaptic boutons reflects a property of individual terminals rather than a property of different neuronal populations (Fig. 2F). The differential effect of

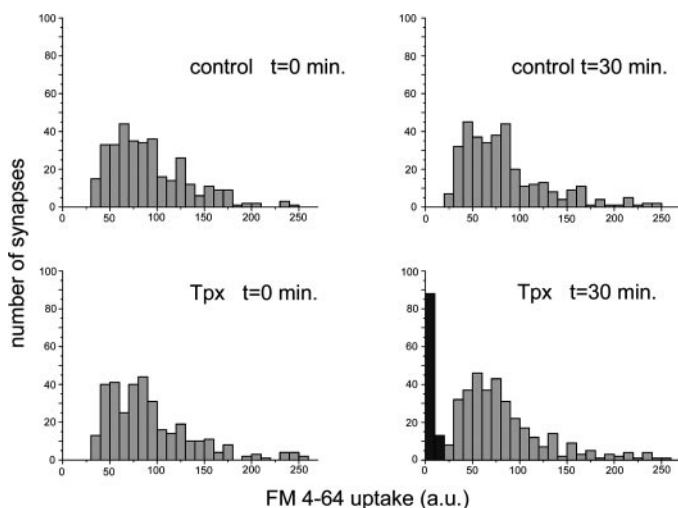


Fig. 4. Tpx induces exhaustive SV exocytosis in a population of synaptic boutons. Distribution of classes of FM4-64 fluorescence. Neurons were loaded with FM4-64 during a 60-s incubation in depolarizing solution. The intensity of dye uptake was measured in single synaptic boutons at time 0 (left) and after a 30-min incubation in KRH in either the presence (right, bottom) or absence (right, top) of 6 nM Tpx. FM4-64 fluorescence was measured separately for the two populations of swollen (dark gray) and normally sized (light gray) synaptic boutons. The amount of dye retained in the normally sized boutons of the Tpx-treated sample was comparable with the amount found in the resting terminals. On the contrary, after Tpx treatment, the dye content of swollen synaptic boutons was in the lowest classes of intensity (which were not represented in the control samples), indicating that approximately all of the dye had been released from these terminals.

Tpx on small and swollen boutons could not be ascribed to differences in toxin binding, because incubation of neurons with Alexa568-conjugated Tpx produced a similar fluorescent signal for both classes of terminals (data not shown).

Optical Analysis of Tpx-Induced SV Exocytosis. To estimate the fraction of vesicles that underwent exocytosis in both swollen and small boutons, the fluorescent styryl dye FM4-64 (Betz et al., 1996) was loaded into SVs of 15-DIV hippocampal neurons using high K^+ depolarization in a well established protocol that labels the entire pool of recycling vesicles (Pyle et al., 2000). The amount of FM4-64 loaded in single synaptic boutons was compared with the amount remaining in the bouton after a 30-min incubation of the neurons in Ca^{2+} -containing medium in either the absence or presence of Tpx (Fig. 3).

After 30 min, the dye content measured in the untreated terminals was comparable with the amount retained in the class of normally sized boutons of the Tpx-treated sample (mean intensity $\pm$ S.D., 82.9 ± 44.4 in control synapses, 82.4 ± 45.5 in small Tpx-treated terminals, $n = 342$), indicating that only a small fraction of the dye was released during the 30-min incubation compared with the amount initially loaded (mean intensity $\pm$ S.D., 91.1 ± 43.4 , $n = 684$). In contrast, the swollen synaptic boutons had released virtually the whole of the loaded dye (mean intensity $\pm$ S.D., 5.3 ± 3.1 , $n = 101$), implying that exhaustive SV fusion had oc-

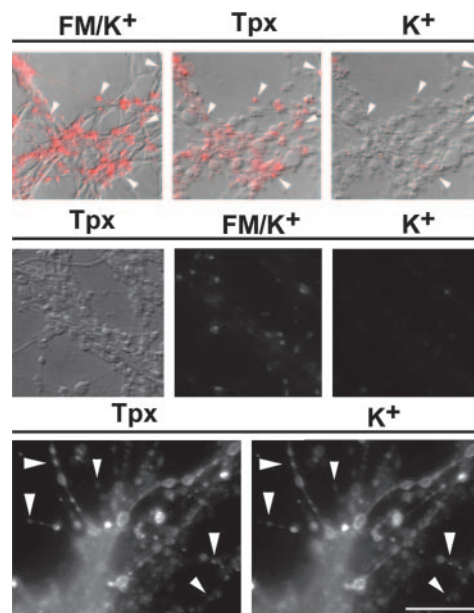


Fig. 5. Tpx does not prevent exo-endocytosis in normally sized synaptic boutons. Top, neurons were loaded with FM4-64 during a 60-s incubation in depolarizing solution. DIC and FM4-64 images were recorded at time 0 (left), after a 30-min incubation with 12 nM Tpx in KRH (middle) and after a further depolarizing stimulus (right). The merge between DIC and FM4-64 (red) images is shown. The dye retained by the normally sized terminals after Tpx treatment was released after depolarization (arrowheads). Middle panel, neurons treated with 12 nM Tpx for 30 min (left, DIC image) were loaded with FM4-64 during a 60-s incubation in depolarizing solution (middle) and subsequently subjected to a further depolarizing stimulus (right). FM4-64 was internalized by the normally sized terminals after Tpx treatment and released after depolarization. Bottom panel, neurons expressing SypI-EYFP were treated with 12 nM Tpx for 30 min and imaged before (left) and after (right) incubation in a depolarizing solution for 3 min. The size of both small (arrowheads) and swollen terminals was not affected by depolarization. Scale bars: top, 10 μ m; middle, 6.5 μ m; bottom, 7.5 μ m.

curred in response to Tpx treatment in this class of nerve terminals. Indeed, analysis of the distribution of fluorescence intensity showed that in the taipoxin-treated samples, a population of synaptic boutons with minimal fluorescence intensity was present. This population, which was absent in the control samples, could be entirely accounted for by swollen terminals (Fig. 4).

In some instances, after loading with FM4-64 during a 1 min-depolarization with high K^+ and successive incubation with 12 nM Tpx for 30 min, neurons were exposed to an additional round of depolarization for 1 min in the absence of FM4-64. Such treatment effectively induced release of the previously loaded dye from the small synaptic boutons, which was not accompanied by swelling of the boutons. Thus, in this class of terminals, treatment with Tpx did not induce a general blockade of exo-endocytosis (Fig. 5, top). In a different set of experiments, neurons expressing Sypl-EYFP were incubated in a depolarizing solution for 3 min after being exposed to 12 nM Tpx for 30 min. Sypl-EYFP-positive small synaptic boutons did not undergo swelling after intense depolarization, suggesting the presence of an active process of exocytic retrieval (Fig. 5, bottom).

In addition, high K^+ depolarization was able to induce loading of FM4-64 in the normally sized boutons of Tpx-intoxicated neurons but not in the swollen synaptic boutons,

further indicating that endocytosis had been blocked by Tpx in the latter but not in the former class of terminals. FM4-64 was unloaded from the small terminals upon the application of a second depolarizing stimulus (Fig. 5, middle).

FRET Analysis of Sypl-VAMP2 Interaction during Tpx-Induced SV Exocytosis. The *in vivo* study of the molecular interactions between the SV proteins Sypl and VAMP2 was carried out by measuring FRET in transfected neurons. Neurons (3 DIV) were cotransfected with the fluorescent fusion proteins ECFP-VAMP2 (donor protein) and Sypl-EYFP (acceptor protein), and FRET was measured at 15–18 DIV as donor photobleaching using time-lapse video-digital imaging, as previously described in detail (Pennuto et al., 2002).

Under resting conditions, the distribution of the time constants of donor photobleaching (τ_{bl}) in the synaptic boutons of samples coexpressing ECFP-VAMP2 and Sypl-EYFP was shifted toward a slower time constant with respect to the distribution observed in samples coexpressing ECFP-VAMP2 and soluble EYFP, indicating the occurrence of FRET between the chimeras of the two SV proteins. In contrast, in samples treated with Tpx, the curves of distribution of τ_{bl} were largely superimposable (Fig. 6).

To discriminate FRET on a synapse-by-synapse basis, the average time constants of donor photobleaching were visual-

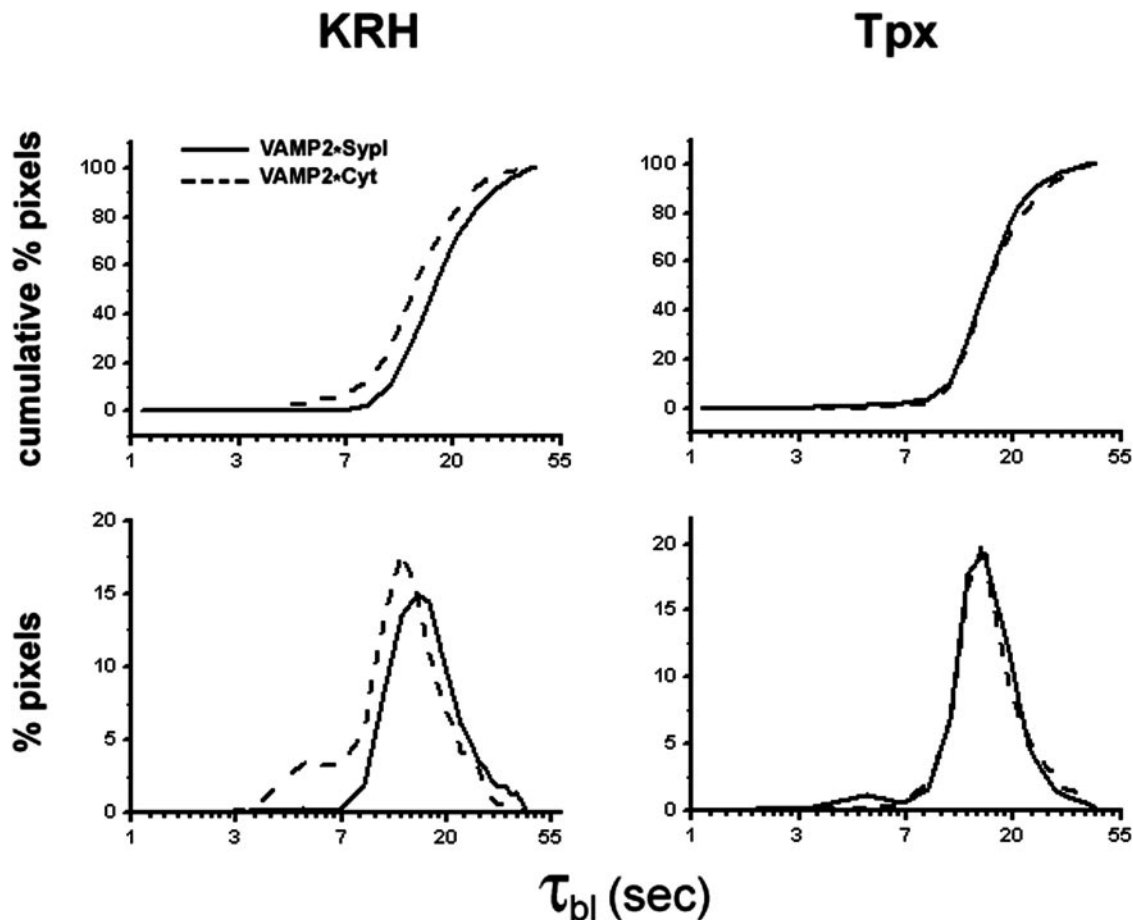


Fig. 6. FRET analysis of Sypl-VAMP2 interaction during exocytosis. The time constants of donor photobleaching (τ_{bl}) were measured in hippocampal neurons coexpressing either ECFP-VAMP2 and Sypl-EYFP (VAMP2*Sypl) or ECFP-VAMP2 and cytosolic EYFP (VAMP2*Cyt) after a 30-min incubation in Ca^{2+} -containing medium in either the absence (KRH) or the presence (Tpx) of 6 nM Tpx. The results from a representative experiment are shown. The changes in the distribution of the time constants of photobleaching indicate that VAMP2 binds to Sypl on the SV membrane and dissociation of VAMP2 from Sypl occurs upon stimulation by taipoxin.

ized using a pseudocolor scale (Fig. 7A). Under resting conditions, the time constants were essentially between 19 and 36 s for all single-pixel values within all synaptic boutons. After exposure to Tpx, the large majority of pixels in both small and large boutons displayed time constants in the 1- to 18-s range.

When small and large boutons of Tpx-treated samples were separately analyzed, for both types of boutons, the curves of the distribution of τ_{bl} were similar for samples transfected with ECFP-VAMP2 and Sypl-EYFP or with ECFP-VAMP2 and soluble EYFP (Fig. 7B). In synaptic boutons of untreated samples, the FRET efficiency was calculated to be $17.64 \pm 0.5\%$, indicating that in living neurons, the two proteins were near each other on the SV membrane. At variance, in Tpx-treated samples, FRET efficiencies were $0.2 \pm 0.7\%$ for small synaptic boutons and $-6.49 \pm 0.4\%$ for large boutons. The negligible FRET efficiency observed in both swollen and small boutons implies that VAMP2 dissociates from Sypl before Tpx-induced vesicle fusion, as previously reported for α -Ltx (Pennuto et al., 2002). The negative FRET efficiency observed in large boutons indicates that, under these conditions, a somewhat better transfer occurs between ECFP-VAMP2 and soluble EYFP than between ECFP-VAMP2 and Sypl-EYFP.

Discussion

The mechanism of action of taipoxin has been studied mainly at the NMJ, where it causes depletion of transmitter and SVs from motor nerve terminals (Su and Chang, 1984; Cull-Candy et al., 1976; Harris et al., 2000). NMJ are not well suited for biochemical and molecular investigations; recently,

however, taipoxin was shown to be active on cultured neurons isolated from various regions of the central nervous system, where it induces release of neurotransmitter with the formation of bulges along the axon (Rigoni et al., 2004). To better understand the mechanism of action of this toxin and to study its influence on protein-protein interactions occurring during the process of neurotransmitter release, we have now studied the effects of taipoxin on rat embryonic hippocampal neurons in culture, using an experimental set-up that we have developed and validated (Pennuto et al., 2002; Rigoni et al., 2004).

Taipoxin at nanomolar concentrations induces the formation of bulges on axonal neurites. These bulges can be identified as swollen nerve terminals, because they correspond to areas in which SV proteins are concentrated. The taipoxin-induced swelling of synaptic boutons was shown here to be accompanied by massive SV exocytosis, as indicated by the complete discharge of the lipophilic styryl dye FM4-64, which in contrast is retained in boutons that do not undergo swelling. In addition, fluorescent chimeras of two SV membrane proteins were redistributed along the peripheral rim of the swollen boutons, suggesting that, in these boutons, the SV membrane has become incorporated into the plasma membrane. Moreover, SV endocytosis was impaired in the swollen boutons, as indicated by the absence of depolarization-induced loading of FM4-64.

Taken together, these results indicate that taipoxin, when applied in the presence of extracellular Ca^{2+} , induces massive SV exocytosis not followed by a proportionate SV membrane retrieval, with the permanent incorporation of the SV membrane into the axolemma. Because the phospholipase A2

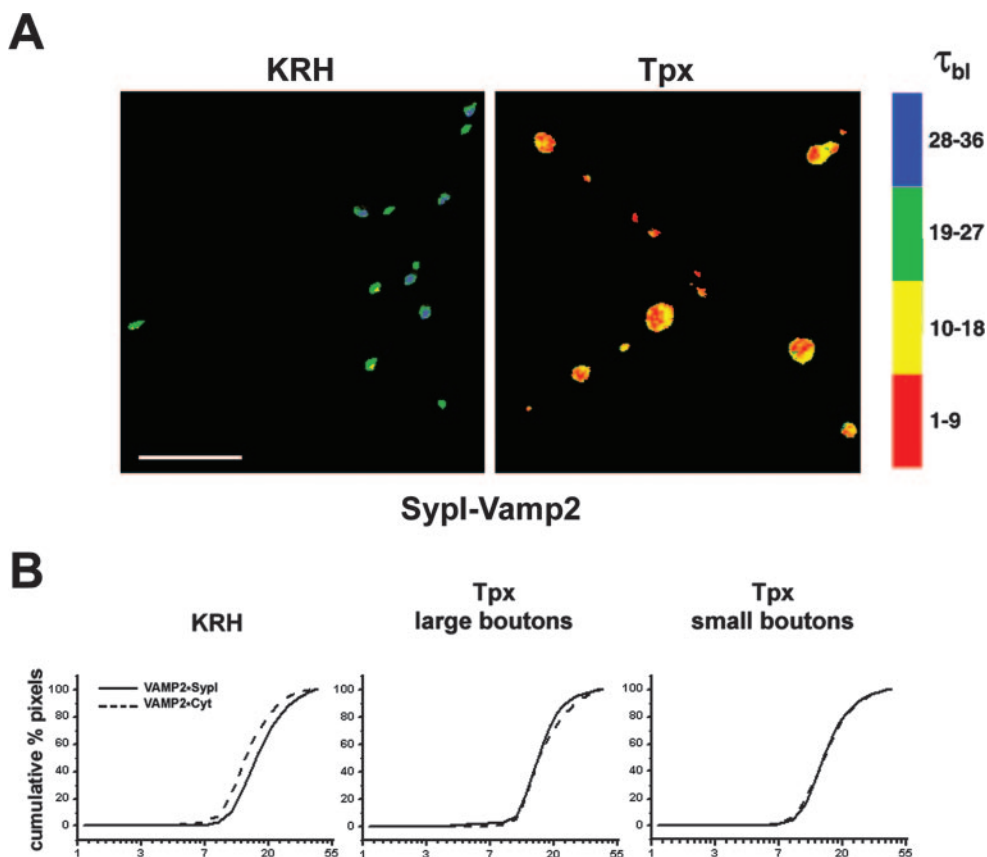


Fig. 7. Spatial and temporal dynamics of Sypl-VAMP2 interaction during exocytosis. Pseudocolor representation of the distribution of the time constants of donor photobleaching (τ_{bl}) measured in hippocampal neurons cotransfected with the expression vectors encoding for ECFP-VAMP2 and Sypl-EYFP. Under resting conditions, the τ_{bl} values ranged from 36 to 19 s, whereas after Tpx treatment, they decreased (ranging from 1 to 18) in both normally sized and swollen synaptic boutons. B, cumulative distribution of the time constants of donor photobleaching from the same experiment showed in A. In the Tpx-treated samples, normally sized and swollen synaptic boutons were analyzed separately. The similarities of the curves obtained for the two types of boutons confirms that Sypl and VAMP2 interact under resting condition and dissociate after Tpx-stimulation in both small and swollen (large) boutons. Scale bar, 5 μm .

activity of the toxin has been shown to be Ca^{2+} -dependent, our findings that Tpx induces exocytosis and nerve terminal swelling exclusively in the presence of extracellular Ca^{2+} support a role for the enzymatic activity of the toxin in its mechanism of action.

At the doses of taipoxin employed here, swelling occurs only in a fraction of nerve terminals. However, at higher doses, most synaptic boutons appear swollen. The dose dependence of Tpx-induced swelling and its binding to both normally sized and swollen synaptic boutons suggest that a different sensitivity of individual terminals to Tpx action may account for the existence of the two classes of synaptic boutons. Different degrees of responsiveness of synaptic terminals to Tpx may derive from a differential expression of the protein(s) involved in its binding and/or internalization. On the other hand, if Tpx gains access to the vesicle lumen via SV endocytosis, as proposed previously (Montecucco and Rossetto, 2000), synapses displaying lower levels of basal SV exo-endocytosis will require higher doses of Tpx, or longer incubation times, to become effectively intoxicated and to undergo swelling. The association of both small and swollen synaptic boutons with the same axon indicates that the different sensitivity to Tpx reflects a property of individual terminals rather than a property of different neurons.

At variance with the swollen terminals, SV exo-endocytosis is not altered in the normally sized synaptic boutons. The extent of FM4-64 release in this class of terminals during incubation with Tpx is comparable with that measured in nonintoxicated synapses. In addition, depolarization-induced loading and unloading of FM4-64 can be observed in small boutons after Tpx treatment. The induction of exocytosis from small boutons of Tpx-intoxicated neurons is consistently not accompanied by swelling of the terminals, implying that it is followed by a proportionate endocytosis. Thus, small and swollen boutons are likely to represent different stages of the alteration of the synaptic compartment, which occurs in the course of intoxication by Tpx. From an experimental perspective, the existence of two classes of boutons offers the possibility to discriminate between early and late effects of Tpx, although the precise kinetics of the intoxication process remains unknown.

It is remarkable that FRET analysis of the Sypl/VAMP2 interactions in living neurons stimulated with Tpx at physiological $[\text{Ca}^{2+}]_{\text{out}}$ revealed that the two proteins interact on the SV membrane under resting conditions and dissociate after stimulation in both the normally sized and swollen synaptic boutons. This may be taken as an indication that the disruption of the Sypl-VAMP2 interaction by Tpx precedes the enhancement of exocytotic fusion and the subsequent inhibition of endocytosis, which lead to swelling of the terminals. Although our data, obtained in live neurons, do not formally prove that disruption of the Sypl/VAMP2 interaction and enhancement of exocytosis induced by Tpx are mechanistically connected, they are in keeping with previous *in vitro* studies (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995; Reisinger et al., 2004) supporting the modulatory role of the Sypl/VAMP2 complex at synapses. Release of VAMP2 from Sypl seems to precede fusion and might be a prerequisite to make SVs competent for exocytosis (see also Reisinger et al., 2004). However, disruption of the Sypl/VAMP2 interaction by Tpx is not sufficient per se to promote SV exocytosis, because it was also

observed in those terminals in which SV had not undergone massive fusion (i.e., the small synaptic boutons).

We propose that Tpx induces a change in lipid composition of the synaptic terminal that causes dissociation of Sypl-VAMP2 complex and then facilitates the exocytosis of SVs but hampers their retrieval from the axolemma. To explain how Tpx might favor the dissociation of the Sypl-VAMP2 interaction, it is tempting to speculate that its phospholipase A2 activity produces an immediate change in the lipid composition of the SV membrane sufficient to cause disruption of the complex, whereas the fusogenicity of SVs is increased only when the hydrolysis of membrane phospholipids proceeds to a certain critical level. It is interesting that the Sypl/VAMP2 interaction was shown to depend on a high cholesterol content in the SV membrane (Mitter et al., 2003), highlighting the importance of the lipid environment in determining the stability of this complex. Moreover, VAMP2 binds phospholipids on the SV membrane and this interaction prevents SNARE complex assembly (Hu et al., 2002; Quetglas et al., 2002). Another possibility is that changes in presynaptic ionic currents induced by Tpx (Fossier et al., 1995) activate molecular events leading to the dissociation of the Sypl-VAMP2 complex.

It is interesting that the effects of Tpx on the presynaptic compartment closely resemble those observed with α -Ltx (Fig. 1; Pennuto et al., 2002). However, at variance with α -Ltx, taipoxin induces nerve terminal swelling exclusively when applied in the presence of extracellular Ca^{2+} . Moreover, the secretagogue activity of α -Ltx has been linked both to the opening of cation channels and to the coupling with large G proteins and the subsequent activation of other signal transduction pathways (e.g., phosphoinositide hydrolysis; for review, see Schiavo et al., 2000). However, the different molecular modes of action of α -Ltx and of taipoxin eventually impinge on the same machinery for exocytosis (Sudhof, 2004; Valtorta et al., 2004), and the availability of two neurotoxins that act via different mechanisms but produce similar and profound effects on SV recycling opens the possibility of using them as tools for the study of the dynamics of protein-protein interactions occurring during SV exocytosis.

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Address correspondence to: Flavia Valtorta, DIBIT 3A3, San Raffaele Scientific Institute, via Olgettina 58, 20132 Milan, Italy. E-mail: valtorta.flavia@hsr.it
