Calcium Influx and Mitochondrial Alterations at Synapses Exposed to Snake Neurotoxins or Their Phospholipid Hydrolysis Products

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Snake presynaptic phospholipase A2 neurotoxins (SPANs) bind to the presynaptic membrane and hydrolyze phosphatidycholine with generation of lysophosphatidylcholine (LysoPC) and fatty acid (FA). The LysoPC + FA mixture promotes membrane fusion, inducing the exocytosis of the ready-to-release synaptic vesicles. However, also the reserve pool of synaptic vesicles disappears from nerve terminals intoxicated with SPAN or LysoPC + FA. Here, we show that LysoPC + FA and SPANs cause a large influx of extracellular calcium into swollen nerve terminals, which accounts for the extensive synaptic vesicle release. This is paralleled by the change of morphology and the collapse of membrane potential of mitochondria within nerve bulges. These results complete the picture of events occurring at nerve terminals intoxicated by SPANs and define the LysoPC + FA lipid mixture as a novel and effective agonist of synaptic vesicle release.

Toxins in general, and neurotoxins in particular, are invaluable tools in the molecular analysis of specific cellular processes, from the activation of G protein-coupled receptors to the characterization of the events controlling regulated exocytosis (1). Much attention has been recently dedicated to a class of neurotoxins with phospholipase A2 (PLA2) activity that are produced by different families of poisonous snakes (SPANs), whose precise biochemical and cellular mode of action has long remained elusive (2). A hallmark of their action in vivo and in vitro is the induction of enlargement of nerve terminals with large depletion of their content of synaptic vesicles (SV) (3–7).

We have recently shown that SPANs hydrolyze phospholipids of cultured neurons with generation of lysophosphatidylcholine (LysoPC) and fatty acids (FA) (8). This leads to a massive release of SV, with their incorporation into the presynaptic plasma membrane and consequent surface exposure of SV luminal epitopes (8–10). These and other experiments performed with models of SNARE-mediated membrane fusion provided evidence for the involvement of hemifusion lipid intermediates in exocytosis (11–17). The presence of LysoPC on the external leaflet of the presynaptic plasma membrane and of FA on both sides, caused by SPANs or by the addition of LysoPC and FA mixture (LysoPC + FA), promotes the formation of the hemifusion intermediate and its transition to an open pore. At the same time, this change in lipid composition of the membrane inhibits the reverse process (i.e. the fission and retrieval of SV). A balanced SV exocytosis-endocytosis cycle is at the basis of synaptic transmission at nerve terminals (18–20). SPANs promote exocytosis and inhibit endocytosis and, therefore, disrupt this finely tuned balance, causing the fusion of SV and formation of nerve terminal bulges decorated with the luminal domain(s) of SV proteins on their surface (9, 10). EM analysis reveals that nerve terminals are almost completely depleted of SV, with disappearance of both the “ready-releasable” pool and of the much larger “reserve” SV pool (3–8). Whereas depletion of the already docked SV was expected (16), more surprising is the depletion of the “reserve” SV pool (21), since the membrane changes induced by LysoPC + FA are predicted to act predominantly on SV bound to the presynaptic membrane or which can rapidly enter in contact with its cytosolic leaflet. These SV have been defined as rapidly releasable vesicles, to be distinguished from the reserve pool of vesicles whose release is caused by the rise of the nerve terminal cytosolic Ca\(^{2+}\) concentration, which follows an extensive stimulation (21–23).

Here we have investigated the mechanism by which SPANs and the LysoPC + FA lipid mixture cause a massive SV release. Using primary cultures of different types of neurons, we found that the synaptic bulging induced by SPANs and LysoPC + FA is followed by a sustained increase in [Ca\(^{2+}\)]. At the same time, the mitochondrial membrane potential collapses. Upon SPAN or lipid mixture incubation, mitochondria change shape and appear to accumulate within bulges characterized by high calcium. Based on these findings, we present here a general model of nerve terminal blockade induced by SPANs or by the LysoPC +
FA mixture that explains the release of both the recycling and the reserve pools of SV.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Rat cerebellar granular neurons (CGNs) were prepared from 6- to 8-day-old Wistar rats as previously described (9, 24) and used 6–8 days after plating. Primary rat spinal motor neurons (SMCs) were isolated from Sprague-Dawley (embryonic day 14) rat embryos and cultured following previously described protocols (25, 26). All experiments were performed using SMCs differentiated for 5–8 days in vitro.

**Fura2-AM Loading and Image Acquisition**—Neurons grown on 24 mm coverslips were incubated in complete medium with 3 μM Fura-2/AM and 0.02% pluronic (Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C and then washed. To prevent Fura-2 leakage and sequestration, 250 μM sulfipyrazone (Sigma) was present throughout the loading procedure and [Ca2+]i measurements. After loading, cells were bathed in Krebs-Ringer buffer (KRH, in the case of CGNs) or E4 medium (Extra-4, in the case of SMCs). KRH composition was as follows: 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. E4 composition was as follows: 120 mM NaCl, 3 mM KCl, 2 mM MgSO4, 2 mM CaCl2, 10 mM glucose, and 10 mM HEPES, pH 7.4. Coverslips were mounted on a thermostated chamber (Medical System Corp.), placed on the stage of an inverted epifluorescence microscope (Axiovert 100 TV; Zeiss), equipped for single cell fluorescence measurements and imaging analysis (27). Samples were alternatively illuminated at 340 and 380 nm (every 15 s for 20–30 min after SPAN addition and every 10 s for 15–20 min after lipid exposure) through a ×40 oil immersion objective (numerical aperture 1.30; Zeiss), exposure times of 100 ms. Data were analyzed with MATLAB software (The MathWorks, Natick, MA) and ImageJ version 1.35.

**Lipid Mixture Preparation, Neurotoxins, and Inhibitors**—The Lipid Mixture Preparation, Neurotoxins, and Inhibitors section describes the preparation of lipid mixtures and the use of neurotoxins and inhibitors in the experiments. lipase activity was measured with the Cayman secretory PLA2 assay kit.

**Electron Microscopy**—CGNs were plated onto 13-mm poly-l-lysine-coated Thermoflow coverslips (Nunc) and, after 6 days in culture, exposed to either SPANs (6 nM for 1 h) or mLysoPC + OA (25 μM for 15 min). Samples were then fixed for 1 h at room temperature in 2.5% glutaraldehyde (EM grade; Applichem) in phosphate buffer. Cells were then washed repeatedly with phosphate buffer and subjected to secondary fixation with 1% osmium tetroxide for 30 min, followed by extensive washes. Samples were then dehydrated with ascending grades of ethanol, araldite-embedded for 2 days, and stained with aqueous uranyl acetate and lead citrate. Sections were imaged in a Jeol 1010 electron microscope equipped with a 2,000 × 2,000-pixel digital camera (GATAN).

**RESULTS**

Cytosolic [Ca2+]i Increases in Nerve Terminals Exposed to Snake Presynaptic PLA2 Neurotoxins—A rise in intrasynaptic Ca2+, such as that caused by prolonged nerve stimulation, is known to trigger SV mobilization from the reserve pool (21–23). We thus hypothesized that the extensive SPAN-induced

**Mitochondrial Imaging**—SCMs or CGNs were loaded with tetramethylrhodamine methyl ester (TMRE) (10 nM; Molecular Probes) in medium supplemented with the multidrug resistance pump inhibitor CsH (1.6 μM final concentration) (28) for 30 min at 37 °C. Fluorescence images were acquired with a Leica AD MIRE3 inverted microscope, equipped with a Leica DC500 CCD camera, ×63 oil immersion objective (NA 1.4), using an exposure time of 50 ms. Data were collected using Leica FW4000 software and analyzed with Leica Deblur and ImageJ version 1.35. The average fluorescence of isolated mitochondria exposed to SPANs or the lipid mixture was recorded as a function of time. Intensity variations were expressed as a percentage of the initial value. Data represent the average of at least five regions of interest. For morphological staining of mitochondria, CGNs were incubated with 300 nM Mitotracker Red (Molecular Probes) for 15 min at 37 °C and then washed; images were acquired as above.

**Measurement of Cellular ATP**—CGNs (300,000/13-mm coverslip) were transfected at 5 days in vitro with cytotoxic luciferase with a standard Lipofectamine procedure, using 1.5 μg of DNA. Measurements of cell luminescence were performed 24 h after transfection in cells pretreated or not with 25 nm taipoxin or the mLysoPC + OA lipid mixture (25 μM for 15 min; n = 4 for each condition) as described (29). Cells were constantly perfused with a modified Krebs-Ringer buffer (mKRB: 125 mM NaCl, 5 mM KCl, 1 mM Na3PO4, 1 mM MgCl2, 1 mM CaCl2, 20 mM HEPES, 5.5 mM glucose, pH 7.4, at 37 °C) with 20 μM luciferin. Complete equilibration in the chamber with the new medium was obtained in 5 s. Under these conditions, the light output of a coverslip of transfected cells was in the range of 5,000–10,000 counts/s versus a background lower than 100 counts/s.

In addition, the cellular ATP content was determined in CGNs, plated in a Petri dish (2 × 106 cells), at 7 days in vitro by a luciferin luciferase assay (ATP Bioluminescent Assay kit CLS II; Roche Applied Science) with an LKB Wallac 1250 luminometer. After treatment (mKRB, 25 μM lipid mixture for 15 min or 25 nm taipoxin for 60 min), cells were diluted in Tyrode buffer medium (145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.5 mM Na3PO4, 5 mM glucose, 15 mM HEPES, pH 7.7), and ATP was extracted by adding 0.3 M perchloroacetic acid. Samples were kept on ice for 5 min and centrifuged at 18,000 × g for 10 min. The pH of the supernatant was adjusted to 7.7 ± 0.1 with a buffer solution of KHCO3 (1 M) and KOH (1 M); the suspension was centrifuged at 18,000 × g for 5 min, and the supernatant was used for the analysis. The ATP content of each sample was estimated using the internal standard method in which 25 pmol of ATP were added twice to the assay. Analysis was repeated on triplicates for each condition. In both procedures for ATP content assay, the number of cells analyzed in each condition was equal, as determined by protein concentration measurement.

**RESULTS**

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release of SV, leading to a complete impairment of the nerve terminal, could indeed be caused by a rise in [Ca\(^{2+}\)]. To test this possibility, we have used primary cultures of rat cerebellar granular cells and spinal cord motor neurons. Cells were loaded with the intracellular calcium indicator Fura-2 and then treated with either SPANs or LysoPC + FA mixture. Fig. 1A shows three video images of Fura-2 loaded CGNs taken at different time points after application of 25 nm taipoxin, a SPAN isolated from the venom of Oxyuranus scutellatus scutellatus. The images are pseudocolor-coded and report the ratio of the probe emission intensity at 510 nm following alternative excitation at 340 and 380 nm. The 340/380 ratio is a function of the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]), whereby an increase in [Ca\(^{2+}\)], is encoded as a shift of the pseudocolors from blue to red (video provided as supplemental Movie 1). A significant increase of the 340/380 ratio was observed in the bulges already at 10 min after toxin application, and the rise continued with time. In contrast, [Ca\(^{2+}\)] was unchanged in cell bodies. This selected localization of the Ca\(^{2+}\) rise is likely to result from the specific binding, and therefore action, of the toxin at nerve terminals. The larger surface to volume ratio of nerve terminals compared with the cell body could also be influenced by the different surface/volume ratio of the two compartments. Any modification of the homeostatic mechanisms controlling Ca\(^{2+}\) at the plasma membrane is expected to cause substantial variations in the tiny cytosolic rim of the presynaptic membrane, whereas the same modification should take longer to affect the bulk [Ca\(^{2+}\)], in the cell body.

Supplemental Fig. S2 shows that the component of the LysoPC + FA lipid mixture most effective in raising the intracellular Ca\(^{2+}\) concentration is LysoPC and that FA, when added alone, had no effect. However, the two molecules clearly synergize in order to produce the effect shown by the mixture in Fig. 2C and in supplemental Fig. 2A. This finding parallels the neuromuscular effects that the lipid mixture and the two lipids alone have on the neuromuscular junction of the hemidiaphragm preparation (8).

**Mechanisms of [Ca\(^{2+}\)] Increase inside Nerve Terminals Poisoned with Lysophosphatidylcholine/Fatty Acid Mixtures—**These findings raise the question as to whether the Ca\(^{2+}\) increase triggered by the toxins or by the lipid mixture derives from Ca\(^{2+}\) influx from the extracellular medium or depends on Ca\(^{2+}\) mobilization from intracellular stores, or both. To address this question, the experiments shown in Fig. 2 were repeated in CGNs incubated with medium without CaCl\(_2\) and supplemented with 150 μM EGTA (Ca\(^{2+}\)-free buffer) (Fig. 3B). SPANs could not be used in these tests as Ca\(^{2+}\) is strictly required for their phospholipase activity (2). Under these conditions, the lipid mixture caused a very small Ca\(^{2+}\) rise and at very late time points (Fig. 3, compare A and B). This Ca\(^{2+}\) increase is small and must derive from intracellular stores. In order to distinguish in our primary neuronal cultures the different Ca\(^{2+}\)-containing compartments on the basis of their luminal pH, we used
**Ca²⁺ Entry in Poisoned Nerve Terminals**

A simple procedure (31). Cells were first treated with ionomycin in Ca²⁺-free buffer. This Ca²⁺ ionophore exchanges Ca²⁺ for 2H⁺ and can very effectively release the cation but only from organelles with neutral/alkaline pH lumina (primarily ER, mitochondria) (32). A very small and transient rise in [Ca²⁺], was observed in control cells under these conditions (Fig. 3C). The acidic pH of other organelles (Golgi, secretory vesicles, and lysosomes) was then collapsed by the addition of monensin, and this induced a much larger increase in [Ca²⁺], indicating that most Ca²⁺ stores in these cells have an acidic luminal pH (Fig. 3C). Monensin was ineffective without ionomycin (not shown). The experiments presented in Fig. 3, D and E, demonstrate that the residual late rise of [Ca²⁺], shown in Fig. 3B derives mainly from intracellular acidic compartments. Indeed, in the experiments presented in Fig. 3D, neurons were first preincubated with 1 μM ionomycin in Ca²⁺-free buffer and then exposed to 25 μM mLysoPC + OA. Under these conditions, the lipid mixture still elicited a late Ca²⁺ rise, indicating that it acted on stores insensitive to ionomycin. A complete suppression of the Ca²⁺ rise by mLysoPC + OA was achieved by the concomitant pretreatment with ionomycin and monensin (Fig. 3E).

Last, but not least, it should be noted that neurons exposed to the lipid mixture in Ca²⁺-free buffer showed a higher response to ionomycin with respect to control neurons, and most bulges did not respond at all to monensin (Fig. 3, compare F with C). This latter observation suggests that, following exposure to the lipid mixture, there is an intracellular partition of fatty acids, which are known to act similarly to monensin and to quench transmembrane proton gradients (33). Intracellular acidic compartments, that under normal conditions cannot be emptied by ionomycin, become thus available to its action. Indeed, the same result was obtained when neurons were pre-treated in Ca²⁺-free buffer with the protonophore carbonylcyanide p-trifluoromethoxyphenyl hydrazone, a well known quencher of transmembrane pH gradients (not shown).

In order to investigate the nature of Ca²⁺ influx through the plasma membrane, neurons were incubated with the P/Q and N-type voltage-gated Ca²⁺ channel (VDCC) inhibitor ω-conotoxin MVIIIC and with the L-channel inhibitor nimodipine. This treatment did not prevent the rise of [Ca²⁺], caused by the lipid mixture, although a very small reduction in the rate of the Ca²⁺ increase was observed in some neurons treated with these VDCC inhibitors (Fig. 4, compare A and B). Using isolated neuromuscular junction preparations, whose VDCCs are well characterized...
and known to be effectively inhibited by ω-conotoxin MVIIIC (34), we did not observe any change in the paralysis time induced by SPANs (not shown). Control experiments performed with neurons treated with these channel inhibitors and depolarized with 55 mM KCl showed about 50% reduction in the calcium influx (Fig. 4, compare A and B). Taken together, these results indicate that these synaptic VDCCs may contribute to the rise of \([\text{Ca}^{2+}]\) induced by SPANs in primary cultures of neurons but are clearly dispensable. One possible explanation is that LysoPC + OA induces in cultured neurons a nonspecific increase in the membrane permeability for small molecules, as it was shown to occur for cultured vascular smooth muscle and endothelial cells (35, 36) and in lymphoma cells (37). These leaks must be small or specific, since we detected no leakage of Fura-2 (756 Da) during treatment with SPANs or LysoPC + OA. Alternative possibilities are considered below.

**Ca**\(^{2+}\) Efflux Is Inhibited in Nerve Terminals Exposed to Lyso-phosphatidylcholine/Fatty Acid Mixtures—A sustained rise of \([\text{Ca}^{2+}]\), could additionally, or alternatively, be dependent on the impairment of the \(\text{Ca}^{2+}\) efflux mechanisms. Given that the steady-state \([\text{Ca}^{2+}]\), at rest is the result of the kinetic equilibrium between the rate of \(\text{Ca}^{2+}\) leak through the plasma membrane and the rate of \(\text{Ca}^{2+}\) efflux mediated by plasma membrane \(\text{Ca}^{2+}\) pumps and \(\text{Na}^{+}/\text{Ca}^{2+}\) exchangers, a reduction in the extrusion efficiency inevitably results in a rise of \([\text{Ca}^{2+}]\). In order to test this possibility, cells were incubated with the LysoPC + FA lipid mixture for 15 min, and then the extracellular \(\text{Ca}^{2+}\) was rapidly chelated by EGTA. The rate of \([\text{Ca}^{2+}]\), decrease, which is indicative of the rate of \(\text{Ca}^{2+}\) efflux, under these conditions was very slow and unable to bring the \([\text{Ca}^{2+}]\), to the resting level (Fig. 5A, arrow). At variance, in control cells

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\text{[Ca}^{2+}]_{i} = \text{[Ca}^{2+}]_{o} 
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where \([\text{Ca}^{2+}]\), was increased to similar levels by depolarization, the efflux rate was much higher as shown by the rapid return of \([\text{Ca}^{2+}]\), to basal level upon the addition of EGTA (Fig. 5B, arrow).

One possible cause of the inhibition of \(\text{Ca}^{2+}\) efflux in the presence of LysoPC + FA is a reduction of the cytosolic ATP by blockade of its mitochondrial synthesis (e.g. upon the addition of the membrane-permeable mitochondrial uncoupler carbonylcyanide-p-trifluoromethoxyphenyl hydrazone). Interestingly, β-bungarotoxin is known to induce mitochondrial depolarization in brain synaptosomes, which in turn would result in an inhibition of ATP synthesis (38–40). We measured the ATP content of CGNs with two different methods: transfected luciferase in live neurons (29) and the traditional luciferin-luciferase assay performed on lysed cells. The first method indicates a reduction of 21% in ATP content in lipid mixture-treated cells compared with controls (6750 ± 440 versus 5350 ± 300, average counts/s ± S.E., \(n = 4\)) and of 20% in the case of taipoxin-treated cells (6850 ± 560 versus 5548 ± 400, average counts/s ± S.E., \(n = 4\)). With the luciferin-luciferase assay, a reduction of 34% in ATP content in both lipid mixture and taipoxin-treated cells was found with respect to controls (180 ± 9 pmol of ATP/10\(^6\) control cells; 120 ± 2.4 pmol of ATP/10\(^6\) lipid mixture-treated cells; 120 ± 11 pmol of ATP/10\(^6\) taipoxin-treated cells; average value ± S.E., \(n = 3\)). A lower ATP decrease is detected by the luciferase transfection methods, because the intracellular luciferase response to ATP is nonlinear. Considering that neuronal projections and terminals are mainly affected by the SPANs and by the LysoPC + OA mixture (Figs. 1 and 2), these decreases indicate that the local decrease of the ATP concentration may be more dramatic.

Taken together, these findings suggest changes in mitochondrial function in nerve terminals poisoned by SPANs or treated with the LysoPC + OA lipid mixture.

**Mitochondrial Changes Induced by Snake PLA2 Presynaptic Neurotoxins and Lyso-phosphatidylcholine/Fatty Acid Mixtures**—We then analyzed the effects of SPANs and LysoPC + FA on single mitochondria in CGNs and SCMs. Neurons were loaded with the potentiometric dye TMRE and then treated with SPANs or with the lipid mixture (Fig.
TMRE is accumulated within the mitochondrial matrix due to the negative membrane potential across the inner membrane of the organelles. Both treatments cause a progressive mitochondrial loss of dye, indicating that incubation of neurons with SPANs and LysoPC + FA results in a major reduction of the mitochondrial membrane potential (Fig. 6 and data not shown).

A loss of TMRE from mitochondria may derive from a decrease of the plasma membrane potential and only a secondary loss of mitochondrial dye without uncoupling (41). This is, however, not the case in the experiment of Fig. 6, because a collapse of plasma membrane potential caused by KCl results in a decrease of the TMRE signal much smaller than that triggered by the lipid mixture (data not shown). The mitochondrial TMRE signal disappears first in the neurite processes and later in the cell body. Fig. 7 clearly shows that the shape of mitochondria is altered as well. It is noteworthy that we noticed an accumulation of mitochondria within bulges (Fig. 7, panels B and C and panels E and F). Mitochondria appear swollen and rounded in treated cells, as compared with control neurons imaged by EM (compare D, E, and F of Fig. 7) and fluorescence microscopy (Fig. 7, G and H).

**DISCUSSION**

Here, we have documented that SPANs and a lipid mixture composed of LysoPC and OA cause a sustained rise of \([\text{Ca}^{2+}]_i\) in nerve terminals with similar time course and extent. This \([\text{Ca}^{2+}]_i\) rise mainly derives from the extracellular medium with a little, but significant, contribution from intracellular acidic compartments. Voltage-gated \(\text{Ca}^{2+}\) channels inhibited by \(\omega\)-conotoxin MVIIIC and nimodipine contribute little, if any, to this \(\text{Ca}^{2+}\) influx. On the other hand, lysophospholipids have been shown to activate some other types of plasma membrane calcium channels for which there are no specific inhibitors (42, 43) and to cause the formation of transient nonprotein calcium pores in lymphoma cells (37). Whatever the exact pathway of \(\text{Ca}^{2+}\) entry, a defined role in the intracellular \(\text{Ca}^{2+}\) rise is also played by a reduced \(\text{Ca}^{2+}\) efflux, which follows the inhibition of mitochondrial function elicited by SPANs and LysoPC + OA. A number of evidence implicates mitochondria in this reduced \(\text{Ca}^{2+}\) efflux, including the inability of lipid mixture-treated neurons to bring the increased \([\text{Ca}^{2+}]_i\), to resting levels upon EGTA addition (Fig. 5A). This is observed even after a few minutes of treatment, when the \([\text{Ca}^{2+}]_i\) increase is small (data not shown). Moreover, the shift of intracellular acidic compartments toward neutral pH values after lipid mixture exposure in the presence of extracellular \(\text{Ca}^{2+}\) (Fig. 3F), might be due to a decreased
ATP level, measured both in living cells using recombinant luciferase (29) or in cell extracts.

Different factors appear to contribute to this mitochondrial impairment: (i) oleic acid and in general fatty acids, which are known to partition into intracellular membranes and to act as mitochondrial uncouplers (33); (ii) an accumulation of Ca\(^{2+}\) inside the mitochondrial matrix driven by the electrochemical potential, which is well known to occur as [Ca\(^{2+}\)]\(_i\), increases and results in mitochondrial damage (44). Taken together, our findings assign a major role to mitochondria damage in the mechanism of SPAN poisoning of nerve terminals and are in agreement with previous  \textit{in vitro}  studies performed with \(\beta\)-bungarotoxin on synaptosomes (38–40).

Although the morphological and electrophysiological aspects of the paralysis of peripheral nerve terminals caused by SPANs were defined long ago, the underlying molecular mechanisms at the basis of the synaptic inhibition remained unexplained. Recently, we found that the physiological effectors of SPANs are the lipids generated by their hydrolytic activity on membrane phospholipids, which progressively accumulate within the membrane (8). This activity can be mimicked to a large extent by the extracellular addition of an equimolar mixture of LysoPC and fatty acids (8). However, there are intrinsic differences between the action of a SPAN and that of the added lipid mixture that should be recalled here. They derive from SPANs binding to the presynaptic membrane where they display their enzymatic hydrolytic activity. As a result of this SPAN localization, the released LysoPC and fatty acid are well localized within the presynaptic portion of the plasma membrane, at least shortly after SPAN treatment. On the contrary, added lipids have to partition from the water phase into the membrane, and they can do so at any place of the cell surface, included the cell body. This account for a less localized calcium increase (compare Figs. 1 and 2). Nonetheless, previous (8) and present data indicate that, whichever read-out we use, snare neurotoxins and lipid mixture addition lead to very similar final results.

Lysoospholipids and FA alter the curvature of the membrane and facilitate exocytosis by promoting the opening of lipidic pores between SV and the plasma membrane. At the same time, they inhibit the reverse process of SV fission (16). The data presented here allow us to complete the picture of events taking place during synaptic intoxication by SPANs. Lysoospholipids and FA, released by SPANs, cause a sustained increase of [Ca\(^{2+}\)]\(_i\), within nerve terminals, which in turn induces the fusion of most SV with the presynaptic membrane. This latter phenomenon has been documented to take place following tetanic stimulation of nerve terminals (22) or latrotoxin treatment (23). This leads to the enlargement of nerve terminals, a feature that establishes one further parallelism between tetanic stimulation and SPAN action, since the tetanized neuromuscular junction is enlarged (22) as well as neurons exposed to SPANs  \textit{in vivo}  (4) and  \textit{in vitro}  (8, 9). This analysis strongly supports our previous proposal (7, 8) that lysoospholipids and FA, generated by the PLA2 activity of SPANs, are the biochemical effectors of the poisoning action. Moreover, it further supports the notion that the LysoPC + FA mixture is a useful tool for the study and modulation of neuro-exocytosis.

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