α -Latrotoxin Stimulates a Novel Pathway of Ca^{2+} -Dependent Synaptic Exocytosis Independent of the Classical Synaptic Fusion Machinery

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α -Latrotoxin induces neurotransmitter release by stimulating synaptic vesicle exocytosis via two mechanisms: (1) A Ca^{2+} -dependent mechanism with neurexins as receptors, in which α -latrotoxin acts like a Ca^{2+} ionophore, and (2) a Ca^{2+} -independent mechanism with CIRL/latrophilins as receptors, in which α -latrotoxin directly stimulates the transmitter release machinery. Here, we show that the Ca^{2+} -independent release mechanism by α -latrotoxin requires the synaptic SNARE-proteins synaptobrevin/VAMP and SNAP-25, and, at least partly, the synaptic active-zone protein Munc13-1. In contrast, the Ca^{2+} -dependent release mechanism induced by α -latrotoxin does not require any of these components of the classical synaptic release machinery. Nevertheless, this type of exocytotic neurotransmitter release appears to fully operate at synapses, and to stimulate exocytosis of the same synaptic vesicles that participate in physiological action potential-triggered release. Thus, synapses contain two parallel and independent pathways of Ca^{2+} -triggered exocytosis, a classical, physiological pathway that operates at the active zone, and a novel reserve pathway that is recruited only when Ca^{2+} floods the synaptic terminal.

Introduction

At a synapse, neurotransmitters are released by fusion of synaptic vesicles with the presynaptic plasma membrane. Synaptic vesicle fusion is mediated by a core membrane fusion machinery comprised of the SNARE-proteins synaptobrevin/VAMP, SNAP-25, and syntaxin-1, and the SM-protein Munc18-1 (Rizo and Rosenmund, 2008). When an action potential enters a nerve terminal, synaptic vesicle fusion is triggered by Ca^{2+} influx via voltage-gated Ca^{2+} channels and Ca^{2+} binding to synaptotagmin, the major Ca^{2+} sensor for exocytosis (Geppert et al., 1994; Südhof, 2004). In addition, synaptic vesicle fusion is triggered by at least two nonphysiological mechanisms: application of hypertonic sucrose solutions (Fatt and Katz, 1952; Furshpan, 1956; Rosenmund and Stevens, 1996), and addition of the neurotoxin α -latrotoxin (Schiavo et al., 2000; Südhof, 2001). Deletion of the synaptic SNARE-proteins synaptobrevin-2 or SNAP-25 almost completely eliminates Ca^{2+} -evoked synaptic vesicle fusion, and

greatly reduces spontaneous and sucrose-stimulated synaptic fusion (Schoch et al., 2001; Washbourne et al., 2002; Deák et al., 2004, 2006a; Bronk et al., 2007), consistent with the hypothesis that synaptic neurotransmitter release requires the classical synaptic vesicle fusion machinery (Südhof, 2004; Rizo and Rosenmund, 2008).

α -Latrotoxin is a large protein composed of an N-terminal region containing disulfide bonds, and a C-terminal region containing 22 ankyrin repeats (Südhof, 2001; Ushkaryov et al., 2008). α -Latrotoxin binds to two, and possibly three, cell-surface receptors with high affinity: neurexins (Ushkaryov et al., 1992; Geppert et al., 1998; Sugita et al., 1999), CIRL/Latrophilins (Davletov et al., 1996; Krasnoperov et al., 1997; Lelanova et al., 1997; Sugita et al., 1998), and protein-tyrosine phosphatase- σ (Krasnoperov et al., 2002; Lajus and Lang, 2006). On receptor binding, α -latrotoxin stimulates synaptic and neuroendocrine exocytosis by two independent mechanisms: A Ca^{2+} -independent mechanism that only operates in synapses, and a Ca^{2+} -dependent mechanism that works not only in synapses, but also in neuroendocrine cells (Gorio et al., 1978; Rosenthal et al., 1990; Ádám-Vizi et al., 1993; Capogna et al., 1996; Sugita et al., 1999; Khvotchev et al., 2000). The second mechanism involves insertion of α -latrotoxin into the plasma membrane where it forms Ca^{2+} -permeable pores (Chanturiya and Nikoloshina, 1994; Khvotchev and Südhof, 2000; Van Renterghem et al., 2000). α -Latrotoxin forms homotetramers with a central, nonselective cation-conducting pore (Orlova et al., 2000) that is blocked by the N4C-

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mutation of α -latrotoxin which contains a four-residue insertion before the first ankyrin repeat (Ichtchenko et al., 1998; Capogna et al., 2003; Volynski et al., 2003; Li et al., 2005). The Ca^{2+} -dependent mechanism of α -latrotoxin mimics Ca^{2+} influx via voltage-gated Ca^{2+} channels during an action potential, whereas the nature of the Ca^{2+} -independent mechanism remains unclear.

Here, we show that the Ca^{2+} -dependent mechanism of α -latrotoxin-induced release uses a novel pathway of membrane fusion that does not require the classical synaptic fusion machinery, and thus differs from the physiological action potential-induced, Ca^{2+} -triggered release pathway. In contrast, the Ca^{2+} -independent mechanism of α -latrotoxin-induced release does require the classical fusion machinery, indicating that despite its Ca^{2+} -independence, this pathway operates directly on this machinery. Our data characterize two independently Ca^{2+} -triggered pathways of synaptic vesicle fusion at central synapses that likely perform distinct physiological functions.

Materials and Methods

Mouse husbandry, culture of hippocampal neurons, and infection with recombinant lentiviruses. Mice heterozygous for synaptobrevin-2, Munc13-1, or SNAP-25 were bred as described (Augustin et al., 1999; Schoch et al., 2001; Washbourne et al., 2002; Deák et al., 2006b; Bronk et al., 2007). Hippocampal neurons were cultured from synaptobrevin-2 and SNAP-25 KO mice at embryonic day 18, and cortical neurons from newborn munc13-1 KO mice. Neurons were cultured on 12 mm coverslips coated with Matrigel essentially as described (Deák et al., 2004, 2006a). Neurons were always cultured in parallel from homozygous mutant and littermate wild-type or heterozygous mice, and analyzed after 12–25 d *in vitro*. Lentivirus expressing wild-type or mutant synaptobrevin-2 containing a 12 aa insertion between the SNARE motif and transmembrane region and tagged N-terminally with enhanced cyan fluorescent protein (eCFP) were produced and used for rescue experiments as described (Dittgen et al., 2004; Deák et al., 2006a).

α -Latrotoxin was purified from black widow spider venom by standard procedures (Frontali et al., 1976; Khvotchev et al., 2000). Recombinant wild-type and N4C-mutant α -latrotoxin containing a four-residue insertion—VPRG—N-terminal to the first ankyrin repeat were produced in bacteria as described (Li et al., 2005).

Fluorescence imaging. Modified Tyrode's bath solution was used in all experiments [in mM: 150 NaCl, 4 KCl, 2 MgCl_2 , 10 glucose, 10 HEPES-NaOH, pH 7.4, and 2 CaCl_2 (~310 mOsm)], and was supplemented with 10 μM CNQX and 50 μM AP-5 to block recurrent neuronal network activity. Neurons were incubated with 0.4 nM α -latrotoxin for 5 min, after which synaptic boutons were loaded by addition of 0.4 mM *N*-(3-triethylammoniumpropyl)-4-(4-(diethylamino)styryl)pyridinium dibromide (FM2-10; Invitrogen, Carlsbad, CA) for 1.5 min, and then washed for 10 min in dye-free Tyrode's solution lacking Ca^{2+} to minimize transmitter release. In all experiments, isolated boutons (~1 μm^2) were selected for analysis. Synaptic vesicle fusion was induced by gravity perfusion (~4 ml/min) of hypertonic 0.5 M sucrose in Ca^{2+} -free Tyrode's solution for 30 s. Subsequently, after 1 min perfusion with Ca^{2+} -free Tyrode's solution, full destaining was achieved by a 1 min perfusion with Tyrode's solution containing 2 mM Ca^{2+} and 90 mM KCl (substituted for NaCl), followed by a 1 min perfusion with Ca^{2+} -free Tyrode's solution again and repeated this way four more times. Fluorescence images were acquired with a cooled CCD camera (Roper Scientific, Trenton, NJ) during illumination (1 Hz and 200 ms) at 480 ± 20 nm (505 dichroic long pass and 534 ± 25 bandpass) via an optical switch (Sutter Instruments, Novato, CA) and analyzed using Metafluor Software (Universal Imaging, Downingtown, PA). Fluorescence levels remaining after sucrose stimulation and after five consecutive rounds of high K^+ application were subtracted as background from all fluorescence images.

Electron microscopy. Cultured neurons from littermate synaptobrevin-2 KO and wild-type control mice were treated for 10 min with 0.2 nM α -latrotoxin in Tyrode's solution containing 2 mM Ca^{2+} , followed by a 20

min perfusion with Tyrode's solution containing either 2 mM Ca^{2+} or 4 mM EGTA. Coverslips were rinsed and fixed for 30 min at 37°C with 2% glutaraldehyde in 0.1 M Na-phosphate buffer pH 7.4, rinsed twice again, and incubated in 1% OsO_4 for 30 min at room temperature. After rinsing with distilled water, specimens were stained *en bloc* with 2% aqueous uranyl acetate for 15 min, dehydrated in ethanol, and embedded in Poly/Bed 812 for 24 h. Thin sections (60 nm) were collected on copper grids, post-stained at room temperature first with 5% uranyl acetate solution for 20 min and then with 1.5% lead citrate for 5 min, and viewed with a JEOL 1200 EX transmission microscope. Quantifications of ultrastructural parameters were done on random images of random sections in a double blind setting in which neither the person doing the EM nor the person analyzing the pictures knew the identity of the sample examined. The images were analyzed in groups, and only after quantifications group codes and genotype were revealed. As random sections and not serial sections were examined, the active zones imaged could be in any orientation and section plane — i.e., the average numbers obtained do not reflect the average size of the active zone, but only the average observed size.

Electrophysiology. Synaptic responses were recorded in the whole-cell patch configuration from pyramidal cells in modified Tyrode's solution supplemented with 50 μM picrotoxin and 1 μM tetrodotoxin to block action potentials and IPSCs. Data were acquired with an Axopatch 200B amplifier and Clampex 8.0 software (Molecular Devices, Union City, CA), filtered at 2 kHz, and sampled at 200 μs . The internal pipette solution included the following (in mM): 115 Cs-MeSO₃, 10 CsCl, 5 NaCl, 0.1 CaCl_2 , 10 HEPES, 4 Cs-BAPTA, 20 tetraethylammonium-Cl, 4 Mg-ATP, 0.3 mM $\text{Na}_2\text{-GTP}$, and 10 lidocaine *N*-ethyl-bromide, pH 7.35 (300 mOsm). Both spontaneous and α -latrotoxin induced synaptic excitatory currents were blocked after the application of 10 μM CNQX and 50 μM AP-5 (AMPA and NMDA receptor inhibitors, respectively, data not shown). Data were analyzed by Clampfit 9.0 (Molecular Devices, Union City, CA) and Mini Analysis5 (Synaptosoft, Decatur, GA) software.

Miscellaneous. Data are shown as means $\pm$ SEMs. Paired Student's *t* test or variance analysis was used to determine significance.

Results

α -Latrotoxin induces neurotransmitter release in the absence of synaptobrevin-2

At a synapse, deletion of synaptobrevin-2 (a.k.a. VAMP2) strongly reduces the frequency of spontaneous miniature EPSCs (mEPSCs), and the magnitude of EPSCs induced by action potentials or application of hypertonic sucrose (Schoch et al., 2001). It was thus a major surprise that application of a low concentration of α -latrotoxin (0.2 nM) in Ca^{2+} -containing extracellular medium triggered a massive increase of neurotransmitter release in synaptobrevin-2 KO neurons (Fig. 1A). However, as soon as extracellular Ca^{2+} was removed by chelation with 4 mM EGTA, release was suppressed in synaptobrevin-2 KO neurons, to be reinitiated on readdition of Ca^{2+} . In wild-type neurons, in contrast, removal of Ca^{2+} did not decrease release triggered by α -latrotoxin (Fig. 1A; also see below).

To quantify the amount of transmitter release triggered by α -latrotoxin in synaptobrevin-2 KO neurons, we monitored mEPSCs induced by 0.2 nM α -latrotoxin in the presence of 50 μM picrotoxin and 1 μM tetrodotoxin, which block action potentials and IPSCs. We determined the mEPSC frequency consecutively under three conditions: (1) in 2 mM Ca^{2+} ; (2) after application of 0.2 nM α -latrotoxin in the same 2 mM Ca^{2+} medium, and (3) after removal of Ca^{2+} by addition of 4 mM EGTA in the continued presence of α -latrotoxin. Sample traces for these measurements are shown in Figure 1B, and summary graphs from multiple experiments in Figure 1C.

Deletion of synaptobrevin-2 had no effect on the ability of α -latrotoxin to stimulate release in the presence of Ca^{2+} , demonstrating that synaptobrevin-2 is not required for release triggered by α -latrotoxin induced Ca^{2+} influx (Fig. 1C). In contrast, deletion of

Figure 1. Synaptobrevin-2 is required for Ca^{2+} -independent but not Ca^{2+} -dependent exocytosis induced by α -latrotoxin. **A**, Representative traces of mEPSCs recorded in wild-type and synaptobrevin-2 KO neurons before and after application of 0.2 nM α -latrotoxin in the presence of 2 mM Ca^{2+} , followed by removal of Ca^{2+} with 4 mM EGTA, and subsequent readdition of Ca^{2+} . Note the compressed timescale. **B**, Representative mEPSC traces monitored in neurons cultured from littermate wild-type (WT) and synaptobrevin-2 KO (Syb2 KO) mice. mEPSCs were obtained under control conditions in 2 mM Ca^{2+} (spontaneous), after addition of 0.2 nM α -latrotoxin in the same Ca^{2+} -containing medium, and after further addition of 4 mM EGTA as indicated. The inset below illustrates the kinetics of individual synaptic events from wild-type and KO neurons. **C**, Summary graphs of the mean mEPSC frequency (left, note logarithmic scale), mEPSC amplitude (center), and mEPSC rise-time (right) during the three conditions shown in **B**: spontaneous mEPSCs before addition of α -latrotoxin (Spontaneous), after addition of α -latrotoxin in Ca^{2+} (α -Ltx + 2 mM Ca^{2+}), and after further addition of 4 mM EGTA (α -Ltx + 4 mM EGTA; $n = 9$ synaptobrevin-2 KO, $n = 14$ WT neurons from 5 cultures). Data shown are means $\pm$ SEMs; calibration bars apply to all traces immediately above them. Asterisks denote significant differences between wild-type and synaptobrevin-2 KO neurons as assessed by Student's t test (* $p < 0.05$; ** $p < 0.01$).

synaptobrevin-2 greatly diminished release stimulated by α -latrotoxin in the absence of extracellular Ca^{2+} . During α -latrotoxin-induced, Ca^{2+} -triggered release, mEPSCs exhibited the same average amplitude and rise times in synaptobrevin-2 KO and

control neurons (Fig. 1C). Since extrasynaptic release would be expected to decrease the amplitude and increase the rise time of mEPSCs, the latter result indicates that the synaptobrevin-independent α -latrotoxin-triggered release was synaptic.

Figure 2. α -Latrotoxin stimulates synaptic exocytosis in synaptobrevin-2 KO synapses by mediating Ca^{2+} influx. **A, B**, Representative traces (**A**) and summary graphs of the mean frequency (**B**) of mEPSCs monitored in wild-type (WT) and synaptobrevin-2 KO (Syb2 KO) neurons before (Spontaneous) and after addition of N4C-mutant of α -latrotoxin in which 4 aa are inserted between the N-terminal cysteine-rich domain and the C-terminal ankyrin repeats of α -latrotoxin [$0.4 \text{ nM } \alpha\text{-Ltx}^{\text{N4C}} + 2 \text{ mM } \text{Ca}^{2+}$] (Ichtchenko et al., 1998; WT, $n = 3$; Syb2 KO, $n = 4$ from 3 cultures). **C, D**, Representative traces (**C**) and summary graphs of the mean frequency (**D**) of mEPSCs monitored in WT and synaptobrevin-2 KO (Syb2 KO) neurons before (Spontaneous) and after addition of α -latrotoxin in $2 \text{ mM } \text{Ca}^{2+}$, and after further addition of either $0.2 \text{ mM } \text{Cd}^{2+}$ ($\alpha\text{-Ltx} + \text{Cd}^{2+}$) or $0.2 \text{ mM } \text{La}^{3+}$ ($\alpha\text{-Ltx} + \text{La}^{3+}$) (WT, $n = 3$; Syb2 KO, $n = 4$ from 3 cultures). Both Cd^{2+} and La^{3+} block the Ca^{2+} -conducting pore produced by α -latrotoxin (Chanturiya and Nikoloshina, 1994; Hurlbut et al., 1994). Data shown are means $\pm$ SEMs; calibration bars apply to all traces immediately above them. Asterisks denote significant differences between wild-type and synaptobrevin-2 KO neurons as assessed by Student's t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

We next examined whether Ca^{2+} -dependent exocytosis induced by α -latrotoxin in the absence of synaptobrevin-2 was caused by Ca^{2+} -permeable pores formed by α -latrotoxin, as expected based on previous studies (Khvotchev et al., 2000; Orlova et al., 2000). If Ca^{2+} influx is necessary for the α -latrotoxin effect in synaptobrevin-2 KO neurons, any interference with the pore function should attenuate the resulting synaptic activity. To test this hypothesis, we used a non-pore-forming mutant of α -latrotoxin, called α -latrotoxin^{N4C} (Ichtchenko et al., 1998). α -Latrotoxin^{N4C} carries a four-residue insertion—VPRG—between the N-terminal cysteine-rich domain of α -latrotoxin, and its C-terminal ankyrin repeats, which disables pore formation by α -latrotoxin (Khvotchev and Südhof, 2000; Volynski et al., 2003; Li et al., 2005).

α -Latrotoxin^{N4C} effectively stimulated release in wild-type neurons, but not in synaptobrevin-2 KO neurons (Fig. 2A,B).

In a second test of the hypothesis that Ca^{2+} influx via α -latrotoxin induced pores mediates exocytosis in synaptobrevin-2 KO neurons, we measured the effects of Cd^{2+} or La^{3+} on α -latrotoxin induced release because both metal ions are thought to block the pores produced by α -latrotoxin. Cd^{2+} or La^{3+} (both applied at 0.2 mM) potentially blocked α -latrotoxin induced exocytosis in synaptobrevin-2 KO neurons, but not in control wild-type neurons (Fig. 2C,D), providing further evidence for the conclusion that α -latrotoxin induces exocytosis in synaptobrevin-2 KO neurons by gating massive Ca^{2+} influx.

Figure 3. The Ca^{2+} ionophore ionomycin potently stimulates synaptic exocytosis in synaptobrevin-2 KO neurons. **A**, **B**, Representative traces (**A**) and mean frequency (**B**) of mEPSC monitored in neurons from littermate wild-type (WT) and synaptobrevin-2 KO (Syb2 KO) mice obtained in 2 mM Ca^{2+} before (Spontaneous) and after addition of 0.1 mM ionomycin (Ionom. + 2 Ca^{2+}), and after further addition of 4 mM EGTA (Ionom. + 4 EGTA; wild-type, $n = 4$; synaptobrevin-2 KO, $n = 3$ from 2 cultures). Calibration bars apply to all traces above them. Data shown are means $\pm$ SEMs; asterisks denote significant differences between wild-type and synaptobrevin-2 KO neurons as assessed by Student's t test ($*p < 0.05$).

Figure 4. Vesicle pools monitored by FM2-10 staining in wild-type and synaptobrevin-2 KO synapses. **A**, Representative fluorescence images from wild-type and synaptobrevin-2 KO hippocampal cultures. Synapses were stained with FM2-10 by α -latrotoxin stimulation (see Materials and Methods for details), and washed for 10 min. Neurons were then stimulated by application of 0.5 M sucrose, and destaining was monitored by fluorescence microscopy. Background fluorescence remaining after an exhaustive stimulation with five applications of 90 mM K^{+} -containing Tyrode's solution was subtracted (Scale bar, 10 μm). **B**, Mean FM2-10 destaining time course on application of 0.5 M sucrose to neurons loaded with FM2-10 by α -latrotoxin stimulation. Destaining was measured by fluorescence microscopy in wild-type synapses (WT; $n = 6$, 352 synapses) and synaptobrevin-2 KO synapses (Syb2 KO; $n = 5$, 251 synapses from 3 cultures). **C**, Bar graph depicting the total amount of FM2-10 fluorescence loaded into synapses during α -latrotoxin stimulation (left; WT: 247 ± 37 vs Syb2 KO: 216 ± 33 A.U.), and destained on application of 0.5 M sucrose (right; WT: 57 ± 19 vs Syb2 KO: 34 ± 4.8 A.U.). Data shown are means $\pm$ SEMs; asterisks denote significant differences between wild-type and synaptobrevin-2 KO neurons as assessed by Student's t test ($*p < 0.05$). Quantitations in **B** and **C** were performed after background subtraction as described in **A**.

The Ca^{2+} ionophore ionomycin effectively stimulates release in synaptobrevin-2 KO neurons

If α -latrotoxin triggers release in synaptobrevin-2 KO neurons simply by gating Ca^{2+} influx, i.e., acts like a Ca^{2+} ionophore, other Ca^{2+} ionophores should mediate the same effect. To test this, we applied ionomycin to neurons cultured from littermate

wild-type and synaptobrevin-2 KO mice. Strikingly, although spontaneous mEPSCs were greatly depressed by deletion of synaptobrevin-2, ionomycin caused the same massive increase in mEPSC frequency in wild-type and synaptobrevin-2 KO neurons (Fig. 3). After removal of extracellular Ca^{2+} by addition of EGTA, the mEPSC frequency dropped as expected, and the difference between wild-type and synaptobrevin-2 KO neurons was restored. Thus, nonlocalized Ca^{2+} influx mediated by α -latrotoxin and by ionomycin is sufficient to cause massive synaptic vesicle exocytosis that is independent of the v-SNARE synaptobrevin-2.

α -Latrotoxin stimulates synaptic exocytosis without depleting vesicles or impairing synaptic integrity

A potential concern is that massive Ca^{2+} influx mediated by an ionophore such as α -latrotoxin or ionomycin might simply make holes in a nerve terminal, thereby damaging it. To address this possibility, we performed two types of experiments: (1) we examined whether vesicles whose exocytosis was triggered by α -latrotoxin recycle can subsequently be stimulated to undergo exocytosis again by stimulation with hypertonic sucrose (Fig. 4), and (2) we investigated the effect of α -latrotoxin on nerve terminals by electron microscopy (Fig. 5).

We incubated wild-type and synaptobrevin-2 KO neurons with 0.2 nM α -latrotoxin for 4 min, and then added FM2-10 for an additional 1.5 min. Neurons were then washed in Ca^{2+} -free medium for 10 min with continuous quantitative fluorescence imaging. Thereafter, we applied 0.5 M sucrose in Ca^{2+} -free medium to stimulate synaptic exocytosis which was imaged as a loss of punctate FM2-10 fluorescence, and finally synapses were exhaustively stimulated in Ca^{2+} -containing medium by multiple rounds of hyperkalemic stimulation to determine the nonspecific background fluorescence in the cells. Sucrose stimulation was chosen for the second step to allow stimulation of exocytosis in the absence of Ca^{2+} ; by using this secretagogue in synaptobrevin-2 KO neurons, we were able to test whether the same vesicles whose exocytosis was induced by α -latrotoxin could then be stimulated to undergo exocytosis again by a different synaptic secretagogue.

Analysis of the imaging data revealed that approximately the same amount of FM2-10 loading by α -latrotoxin stimulation was observed in wild-type and synaptobrevin-2 KO neurons (Fig. 4). This result confirms the electrophysiological conclusion (Fig. 1), and demonstrates that vesicles released by α -latrotoxin recycle. Subsequent sucrose stimulation released a fraction of the

FM12–10 labeled vesicles in wild-type and synaptobrevin-2 KO neurons (Fig. 4). Consistent with the fact that sucrose-stimulated release is partially abolished in synaptobrevin-2 KO neurons (Schoch et al., 2001), relatively fewer vesicles were released by sucrose in the mutant synapses, but clearly the vesicles whose exocytosis was stimulated by α -latrotoxin in synaptobrevin-2 KO synapses are partly sensitive to sucrose.

We next examined the effect of α -latrotoxin on the structure of synaptic nerve terminals. We applied 0.2 nM α -latrotoxin to cultured neurons from littermate synaptobrevin-2 KO and wild-type control mice for 10 min in Ca^{2+} -containing medium, followed by a 20 min superfusion with either Ca^{2+} - or EGTA-containing medium. Afterward, neurons were fixed and analyzed by electron microscopy (Fig. 5A). We observed no major changes in α -latrotoxin treated terminals as a function of Ca^{2+} . Especially, different from what has been reported for the frog neuromuscular junction (Frontali et al., 1976; Ceccarelli and Hurlbut, 1980; Henkel and Betz, 1995), we detected no depletion of synaptic vesicles in the presence as well as in the absence of extracellular Ca^{2+} (Fig. 5B). Interestingly, we found that α -latrotoxin stimulated neurons from synaptobrevin-2 KO mice exhibited a small but significant relative increase in docked vesicles in the presence of Ca^{2+} , and a relative decrease in the absence of Ca^{2+} (Fig. 5C), without a change in active-zone size (Fig. 5D). We conclude that at low (0.2 nM) α -latrotoxin concentrations, the structural integrity of synapses is maintained.

Ca^{2+} -dependent and Ca^{2+} -independent, α -latrotoxin induced release exhibit differential requirements for the synaptic fusion machinery

Based on our results, we hypothesize that α -latrotoxin stimulates synaptic exocytosis by a Ca^{2+} -independent mechanism that involves the classical synaptic fusion machinery, and by a second Ca^{2+} -dependent mechanism that does not. To test this hypothesis, we analyzed the involvement of additional components of the classical synaptic fusion machinery in synaptic exocytosis triggered by α -latrotoxin.

First, we monitored mEPSCs in neurons cultured from littermate wild-type and SNAP-25 KO mice which exhibit a phenotype similar to that of synaptobrevin-2 KO neurons (Washbourne et al., 2002; Bronk et al., 2007). Essentially, the same pattern of release was observed as in synaptobrevin-2 KO neurons: In the presence of Ca^{2+} , deletion of SNAP-25 had only a small effect on release triggered by

Figure 5. α -Latrotoxin treatment does not impair synaptic terminal integrity. **A**, Representative electron micrographs of wild-type and synaptobrevin-2 KO synapses after a 10 min application of 0.2 nM α -latrotoxin in Tyrode's solution containing either 2 mM Ca^{2+} , followed by a change to Tyrode's solution with the same Ca^{2+} concentration, or 4 mM EGTA. Cells were fixed after 20 min in these solutions. Calibration bar applies to all images. **B–D**, Bar graphs depicting the mean synaptic vesicle number per nerve terminal (**B**), docked synaptic vesicle number per active zone (**C**), and size of the active zone (**D**; $n = 11$ –15 synapses per condition). Data shown are means $\pm$ SEMs. Asterisks denote significant differences between wild-type and synaptobrevin-2 KO neurons as assessed by Student's t test (** $p < 0.01$). Other abbreviations are as defined in the legend to Figure 1.

α -latrotoxin, whereas removal of Ca^{2+} greatly dampened release in SNAP-25 deficient but not in wild-type synapses (Fig. 6A,B). Thus, neither the v-SNARE synaptobrevin nor the t-SNARE SNAP-25 are required for Ca^{2+} -triggered exocytosis mediated by the ionophore action of α -latrotoxin, but both are required for Ca^{2+} -independent exocytosis induced by α -latrotoxin.

Figure 6. Role of the SNARE-protein SNAP-25 in the active-zone protein Munc13-1 in Ca^{2+} -dependent and Ca^{2+} -independent α -latrotoxin-triggered release. **A, B**, Representative traces (**A**) and summary graphs of the mean frequency (**B**) of mEPSCs monitored in wild-type (WT) and SNAP-25 KO neurons. mEPSCs were obtained under control conditions in 2 mM Ca^{2+} (Spontaneous), after addition of 0.2 nM α -latrotoxin in Ca^{2+} (α -Ltx + 2 mM Ca^{2+}), and after further addition of 4 mM EGTA (α -Ltx + 4 mM EGTA; $n = 3$ for both WT and SNAP25 KO from 3 cultures). Note the logarithmic ordinate. **C, D**, Same as **A** and **B**, respectively, except that wild-type and Munc13-1 KO neurons were compared (WT, $n = 4$; Munc13-1 KO, $n = 5$ from 3 cultures). Data shown are means $\pm$ SEMs; calibration bars apply to all traces immediately above them. Asterisks denote significant differences between wild-type and synaptobrevin-2 KO neurons as assessed by Student's *t* test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Next, we investigated the role of the active-zone protein Munc13-1 that is essential for most action potential- and sucrose-triggered release (Brose et al., 1995; Augustin et al., 1999). Here, the results were less clear-cut as the Munc13-1 KO by itself had only a minor effect on mEPSC frequency (Fig. 6C,D). In the presence of Ca^{2+} , the Munc13-1 KO did not impair the ability of α -latrotoxin to stimulate release; on Ca^{2+} -removal with EGTA, the Munc13-1 deletion decreased release ~ 5 -fold compared with wild-type synapses (Fig. 6C,D). This result supports the notion that this active-zone protein is part of the fusion machinery stimulated by the Ca^{2+} -independent mechanism of α -latrotoxin action, but is not required for its Ca^{2+} -dependent mechanism. The relatively lesser effect of the Munc13-1 deletion than that of the synaptobrevin-2 and SNAP-25 deletions is that Munc13-2 is still expressed in the neurons used here (Augustin et al., 1999), whereas other synaptobrevin isoforms (synaptobrevin-1 and cellubrevin) and SNAP-25 variants are not (Schoch et al., 2001; Washbourne et al., 2002).

These data thus confirm the hypothesis that the Ca^{2+} -dependent mechanism of release stimulated by α -latrotoxin does not involve the classical synaptic fusion machinery, whereas the Ca^{2+} -independent mechanism requires this machinery. To further test this hypothesis with yet another approach, we measured the ability of wild-type and mutant synaptobrevin-2 to rescue the impairment of Ca^{2+} -independent α -latrotoxin induced release. We used a mutant of synaptobrevin-2 in which 12 aa are inserted between the SNARE motif and the transmembrane region. In synaptobrevin-2 KO neurons, this mutant (referred to as Syb^{12ins}) rescues spontaneous mini release, but not evoked release (Deák et al., 2006a), indicating a difference in the energetic barrier between spontaneous and evoked release. However, the Syb^{12ins} mutant was unable to rescue Ca^{2+} -independent release in synaptobrevin-2 KO neurons stimulated with α -latrotoxin, whereas wild-type synaptobrevin-2 did rescue this type of release (Fig. 7). This result confirms the

requirement for the classical fusion machinery in Ca^{2+} -independent release induced by α -latrotoxin.

Discussion

This study originated with the unexpected finding that α -latrotoxin triggered apparently normal neurotransmitter release in synaptobrevin-2 KO neurons, as long as Ca^{2+} was present (Fig. 1A). Previous studies showed that KO of synaptobrevin-2 dramatically reduced spontaneous and evoked neurotransmitter release (Schoch et al., 2001), with the small amount of residual release observed in synaptobrevin-2 KO neurons being ascribed to the presence of an unidentified, partially redundant other v-SNARE-protein. We now demonstrate that in contrast to their massively impaired release evoked by action potentials, synaptobrevin-2 KO neurons can be triggered to exhibit normal amounts of release by the Ca^{2+} ionophore actions of α -latrotoxin, and this novel type of release is independent of three elements of the classical synaptic fusion machinery—the v-SNARE synaptobrevin-2, the t-SNARE SNAP-25, and the active-zone protein Munc13-1. Moreover, our results demonstrate that the second mode of action of α -latrotoxin described in earlier studies, its Ca^{2+} -independent stimulation of synaptic exocytosis, does involve the classical fusion machinery, different from its Ca^{2+} -dependent stimulation mechanism of synaptic exocytosis.

Thus, synapses exhibit a second, previously unrecognized Ca^{2+} -dependent exocytosis pathway that operates in parallel to, but independent of, the normal transmitter release machinery. This pathway is not normally activated by Ca^{2+} influx via voltage-gated channels as SNAP-25 KO does not respond to hyperkalemic stimulation (Bronk et al., 2007), but is stimulated when a Ca^{2+} ionophore floods the nerve terminal with Ca^{2+} . The amplitudes and rise times of mEPSCs induced by this pathway are normal, indicating that it is truly synaptic and vesicular in origin (Fig. 1). The FM2-10 staining/destaining data suggest that the vesicle pool stimulated by this Ca^{2+} -dependent pathway is similar in size in synaptic terminals, independent of whether synaptobrevin-2 is lacking or present, and also operates as part of the classical transmitter release pathway, as defined by the release seen with the Ca^{2+} -independent actions of hypertonic sucrose (Fig. 4). Moreover, electron microscopy demonstrates that the Ca^{2+} -dependent release induced by α -latrotoxin via the second pathway does not consist of nerve terminal damage or reorganization, but only leads to relatively subtle structural changes (Fig. 5), and thus consists of true exocytosis. Finally, the finding that in

Figure 7. Synaptobrevin-2 with a 12-residue insertion between the SNARE motif and the membrane is unable to mediate Ca^{2+} -independent α -latrotoxin-triggered release in synaptobrevin-2 KO neurons. **A**, Schematic illustration of eCFP-tagged synaptobrevin-2 and its mutant version with a 12-residue insertion between the SNARE motif and transmembrane region. **B**, **C**, Representative traces of mEPSCs (**A**) and summary graphs of the mean mEPSC frequency (**B**) monitored in wild-type (WT) and synaptobrevin-2 KO neurons that were infected with lentivirus expressing wild-type synaptobrevin-2 (Syb KO + Syb) or mutant synaptobrevin 2 with the 12-residue insertion (Syb KO + Syb^{12ins}). mEPSCs were monitored under control conditions in 2 mM Ca^{2+} (Spontaneous), after addition of 0.2 nM α -latrotoxin in Ca^{2+} (α -Ltx + 2 mM Ca^{2+}), and after further addition of 4 mM EGTA (α -Ltx + 4 mM EGTA; WT, $n = 13$; Syb KO + Syb^{12ins}, $n = 6$ from 5 cultures). Data shown are means $\pm$ SEMs; calibration bars apply to all traces immediately above them. Asterisks denote significant differences between wild-type and synaptobrevin-2 KO neurons as assessed by Student's t test (*** $p < 0.001$).

synaptobrevin-2 KO neurons, the α -latrotoxin effect can be reversible switched off by removal of Ca^{2+} and reactivated when Ca^{2+} is reintroduced confirms the integrity of the nerve terminal after α -latrotoxin action. Our data seemingly contradict earlier reports on loss of synaptic vesicles from α -latrotoxin treated nerve terminals (Frontali et al., 1976; Ceccarelli and Hurlbut, 1980; Van der Kloot and Molgó, 1994; Henkel and Betz, 1995).

However, the earlier studies were conducted on neuromuscular junction and used higher concentrations of α -latrotoxin for prolonged periods, whereas we studied central synapses and used subnanomolar α -latrotoxin concentrations. Although most of our experiments were for shorter time periods, in occasional experiments we observed an unmitigated stimulation of release by α -latrotoxin for up to 90 min, again arguing against vesicle depletion (data not shown).

The physiological role of the novel Ca^{2+} -triggered pathway described here remains unknown. This pathway may become physiologically activated during massive stimulation, and may represent a new type of reserve exocytosis that is activated when the classical pathway is shut down by regulatory mechanisms. It is possible that the facilitation observed in synaptobrevin-2 KO neurons on massive prolonged action-potential stimulation (Deák et al., 2006b) is due to the activation of this novel Ca^{2+} -triggered pathway. Please note, however, that this pathway is distinct from asynchronous release because asynchronous release is completely dependent on SNARE-proteins, similar to synchronous release (Schoch et al., 2001; Bronk et al., 2007). It is likely that the novel pathway also operates via SNARE-mediated fusion because all vesicular fusion in cells uses this mechanism (Südhof and Rothman, 2009), except that the specific SNARE-proteins involved are probably different from those mediating classical synaptic fusion. Indeed, it was suggested that some more distantly related v-SNAREs may substitute for synaptobrevin/VAMP isoforms in some types of synaptic function (Fasshauer et al., 1999; Ossig et al., 2000; Deák et al., 2006a). However, we have shown that neither synaptobrevin-1, nor cellubrevin/VAMP-3, the closest VAMP isoforms to synaptobrevin-2, are expressed at detectable levels in primary cultures of hippocampal neurons (Schoch et al., 2001). Furthermore, transmitter release in double knock-out mice lacking both synaptobrevin-2 and cellubrevin was indistinguishable from that in single synaptobrevin-2 KO, arguing against a role of this synaptobrevin isoform in hippocampal transmitter release (Deák et al., 2006a). Thus, synaptobrevin-1 and cellubrevin are unlikely to be responsible for Ca^{2+} -dependent release induced by α -latrotoxin, as is also evidenced by the impairment of Ca^{2+} -independent release induced by α -latrotoxin in synaptobrevin-2 KO neurons. At a more practical level, our data suggest that the use of ionomycin to probe the classical synaptic fusion machinery, as we (Maximov and Südhof, 2005) and others have done, is probably misleading. More importantly, it is possible that this pathway is also activated by Ca^{2+} -uncaging, although this fact would not necessarily lead to an overestimation of the vesicle pool size, since the new pathway described here operates on the same pool of vesicles as the classical pathway of exocytosis.

Equally interesting as the Ca^{2+} -dependent novel exocytosis pathway stimulated by α -latrotoxin is its previously described Ca^{2+} -independent release mechanism (Capogna et al., 1996, 1997; Khvotchev et al., 2000). Our results, consistent with earlier studies (Ichtchenko et al., 1998; Volynski et al., 2003; Li et al., 2005), show that this mechanism requires the classical synaptic fusion machinery but is independent of the Ca^{2+} ionophore action of α -latrotoxin. The evidence for this conclusion resides in the observations that all of the SNARE and active-zone protein mutations examined here impair Ca^{2+} -independent release triggered by α -latrotoxin (Figs. 1, 2, 6). Furthermore, Ca^{2+} -independent release required similar energetic mechanisms as evoked release, as uncovered in the rescue experiments with the synaptobrevin mutant (Fig. 7). Thus, Ca^{2+} -independent release triggered by α -latrotoxin resembles release triggered by hypertonic sucrose which is also dependent on the same proteins

(Schoch et al., 2001; Deák et al., 2006a; Bronk et al., 2007). This similarity suggests the possibility that α -latrotoxin induced release, just like sucrose-induced release, operates by a mechanical principle, i.e., by an effect on the tension of the presynaptic plasma membrane (Rosenmund and Stevens, 1996).

In summary, our results extend and confirm the notion that α -latrotoxin acts by two independent mechanisms, and demonstrate that one of these mechanisms consists of a simple Ca^{2+} ionophore action that stimulates a previously undescribed Ca^{2+} -dependent pathway of exocytosis, whereas the other mechanism Ca^{2+} -independently stimulates the classical synaptic fusion machinery directly.

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