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Vesicle exocytosis stimulated by α-latrotoxin is mediated by latrophilin and requires both external and stored Ca^{2+}

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α-Latrotoxin (LTX) stimulates massive neurotransmitter release by two mechanisms: Ca^{2+}-dependent and -independent. Our studies on norepinephrine secretion from nerve terminals now reveal the different molecular basis of these two actions. The Ca^{2+}-dependent LTX-evoked vesicle exocytosis (abolished by botulinum neurotoxins) is 10-fold more sensitive to external Ca^{2+} than secretion triggered by depolarization or A23187; it does not, however, depend on the cation entry into terminals but requires intracellular Ca^{2+} and is blocked by drugs depleting Ca^{2+} stores and by inhibitors of phospholipase C (PLC). These data, together with binding studies, prove that latrophilin, which is linked to G proteins and inositol polyphosphate production, is the major functional LTX receptor. The Ca^{2+}-independent LTX-stimulated release is not inhibited by botulinum neurotoxins or drugs interfering with Ca^{2+} metabolism and occurs via pores in the presynaptic membrane, large enough to allow efflux of neurotransmitters and other small molecules from the cytoplasm. Our results unite previously contradictory data about the toxin's effects and suggest that LTX-stimulated exocytosis depends upon the co-operative action of external and intracellular Ca^{2+} involving G proteins and PLC, whereas the Ca^{2+}-independent release is largely non-vesicular.

Keywords: Ca^{2+}/exocytosis/latrophilin/α-latrotoxin/norepinephrine release

Introduction

α-Latrotoxin (LTX) from Black Widow spider venom causes massive release of neurotransmitters from nerve endings (reviewed by Rosenthal and Meldolesi, 1989) and is a powerful tool to study neuroexocytosis. Experiments on the frog neuromuscular junction (NMJ) demonstrated the vesicular character of the toxin-stimulated secretion (Ceccarelli and Hurlbut, 1980). At the NMJ, LTX elicits exocytosis of only small synaptic vesicles (SV) (Matteoli *et al*., 1988), but can also evoke release from large dense-core vesicles (LDCV) in chromaffin cells (Barnett *et al*., 1996), pancreatic β-cells (Lang *et al*., 1998) and sensory neurones (De Potter *et al*., 1997), implicating a similar mechanism in exocytosis of both vesicle types. In addition, LTX triggers leakage of cytoplasmic glutamate and acetylcholine from nerve terminals (McMahon *et al*., 1990; Deri *et al*., 1993).

LTX has been demonstrated to form non-selective cation-permeable channels in lipid bilayers (Finkelstein *et al*., 1976) and to cause massive influx of Ca^{2+} into PC12 cells and synaptosomes (Grasso *et al*., 1980; Meldolesi *et al*., 1984). This suggested a plausible mechanism of action whereby the influx of Ca^{2+} through LTX channels triggers neurotransmitter release (Grasso *et al*., 1982; Nicholls *et al*., 1982). However, the toxin is able to open channels only in neuronal or neuroendocrine cells; these channels have different properties from those formed by LTX in lipid bilayers (Wanke *et al*., 1986). The substantial amount of release caused by LTX in the absence of extracellular Ca^{2+} (Ca^{2+}_e) cannot be satisfactorily explained by the channel-forming function of the toxin alone (Misler and Hurlbut, 1979; Meldolesi *et al*., 1983; Hurlbut *et al*., 1994). Thus, this Ca^{2+}-independent LTX mechanism remains unclear. Moreover, uncertainties also obscure the precise role of Ca^{2+}_e in the toxin's action. For example, external Ca^{2+} supports LTX-stimulated secretion from hippocampal neurones and chromaffin cells apparently without entering the cytoplasm (Capogna *et al*., 1996; Michelena *et al*., 1997).

In order to exert its action, LTX requires a cell-surface receptor. Two different receptor proteins have been found: neurexin and latrophilin (Petrenko *et al*., 1990; Ushkaryov *et al*., 1992; Davletov *et al*., 1996; Krasnoperov *et al*., 1996). Whereas soluble neurexin Iα construct binds LTX only in Ca^{2+}, latrophilin does not need the cation (Davletov *et al*., 1996) and may, therefore, mediate the toxin's action in all conditions. Latrophilin (also called CIRL) is a heptahelical receptor coupled to G proteins and to secretion (Krasnoperov *et al*., 1997; Lelianova *et al*., 1997). This finding implicates a G protein-based signalling cascade in the modulation of neurosecretion, but does not reveal the mechanism of LTX action. The relative contribution of the two receptors to the toxin's effects also remains unresolved because in neurexin Iα knockout mice, Ca^{2+} still enhances both LTX binding and action (Geppert *et al*., 1998). In addition, some pancreatic β-cells that express neurexin Iα remain unresponsive to LTX unless transfected with latrophilin (Lang *et al*., 1998).

To decipher the mechanisms underlying the dual effects of LTX and with the additional aim of rationalizing the existing conflicting data, we analysed separately the Ca^{2+}-dependent and -independent toxin's actions by studying release of a vesicular neurotransmitter, norepinephrine (NE) from rat synaptosomes (Nicholls *et al*., 1982;

Thureson-Klein, 1983; De Potter *et al*., 1997). Herein, we report that the G protein-coupled LTX receptor, latrophilin, participates in both the Ca^{2+}-dependent and -independent activities of the toxin. By localizing LTX binding sites in the brain and expressing both receptors in COS cells, it is demonstrated, for the first time, that latrophilin constitutes the major functional LTX receptor. The use of botulinum neurotoxins revealed the different molecular nature of the two mechanisms of the toxin's action and showed that only secretion triggered by LTX in the presence of Ca^{2+}_e (but not in its absence) requires intact synaptobrevin, syntaxin and SNAP-25 (collectively called SNARE proteins). By further exploring the pharmacological and biochemical properties of each mechanism, it is found that: (i) LTX greatly increases the sensitivity of terminals to Ca^{2+}_e, probably by activating the G protein-coupled receptor; (ii) the Ca^{2+}-dependent LTX-induced exocytosis may proceed without Ca^{2+} influx into terminals but requires intracellular Ca^{2+} (Ca^{2+}_i); (iii) Ca^{2+}_e-dependent NE secretion induced by LTX is mediated by phospholipase C (PLC); (iv) Sr^{2+} can fully replace Ca^{2+} in supporting this LTX-triggered secretory mechanism, without the toxin binding to neurexin. In the absence of Ca^{2+}_e: (i) LTX-evoked NE release is much weaker and slower than in the presence of Ca^{2+} and occurs through non-selective toxin-induced pores permeable to small molecules; (ii) LTX opens these pores only in the presynaptic membrane; and (iii) the pores are intrinsic membrane components present only in some cells. Thus, our findings reveal the distinct molecular mechanisms of LTX activities and suggest that exocytosis can be regulated by a mechanism that requires both external and stored Ca^{2+} and involves a G protein-coupled receptor and PLC.

Results

G protein-coupled latrophilin is the main functional LTX receptor

To simplify the analysis of LTX action, we first attempted to find conditions under which the toxin would interact with only one of its two proposed receptors. Because neurexin and latrophilin have different cation requirements (Davletov *et al*., 1996), we compared Sr^{2+} and Ca^{2+} for their ability to support the toxin binding. As Figure 1A demonstrates, the binding of iodinated LTX ([^{125}I]LTX) to rat brain nerve terminals in the presence of these cations was very similar, being only 15% higher in Ca^{2+}. Scatchard analysis also reveals that Ca^{2+} augments only the maximal level but not the affinity of the toxin binding (Figure 1A, right). When we performed chromatography of solubilized synaptosomes on immobilized LTX in the presence of these cations, neurexin interacted with the LTX column only in the presence of Ca^{2+}, while latrophilin bound to the column in all cations (Figure 1B). To compare the interaction of LTX with cell surface-expressed receptors as opposed to solubilized receptors, we transfected COS cells with neurexin Iα and, for the first time, obtained functional expression of this protein on the surface of heterologous cells (Figure 1C). Neurexin-expressing cells bound LTX only in Ca^{2+} but not in Sr^{2+} or Mg^{2+} (Figure 1C, left), whilst latrophilin on the cell surface always interacted with the toxin, confirming the results of the chromatographic experiments. Expression in COS cells also allowed us to compare the contents of the two toxin receptors in the brain. Western blotting revealed that brain expresses 5-fold less neurexin than transfected COS cells, whereas the amount of latrophilin in brain is much higher (3-fold) than in transfected cells (Figure 1C, right). Since LTX binding to both transfected cells was quantitatively similar, we conclude that brain membranes contain ~10–15 times more latrophilin than neurexin, consistent with the small increase in the toxin binding brought about by the inclusion of Ca^{2+}.

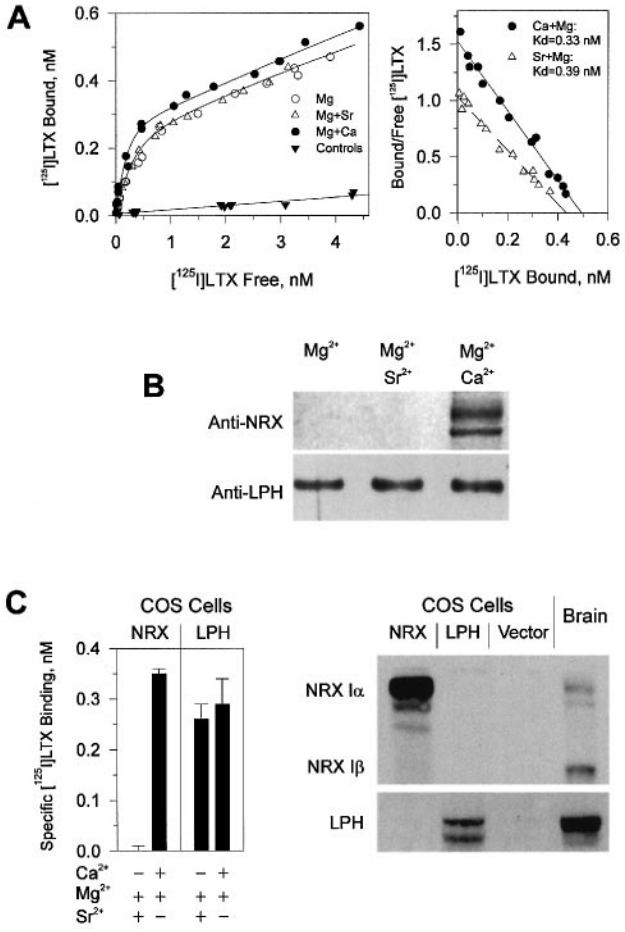

Fig. 1. The toxin LTX always interacts with latrophilin but binds neurexin only in Ca^{2+}. (**A**, left) The binding of the toxin to synaptosomes (1 mg/ml) was measured by filtration after incubation with [^{125}I]LTX in the presence of 2 mM Mg^{2+} alone or with 2 mM Sr^{2+} or Ca^{2+}. Non-specific toxin binding, measured in the presence of 100-fold excess of unlabelled LTX, was independent of cations present (controls). (**A**, right) Scatchard plot analysis of the binding data in the left panel. Note the almost identical K_d values and the small increase of the binding brought about by Ca^{2+}. (**B**) Affinity chromatography of solubilized synaptosomal membranes on immobilized LTX (see Materials and methods) was conducted in the presence of 2 mM divalent cations as indicated. Eluted fractions were analysed by SDS–PAGE and immunoblotting using antiserum against neurexin Iα (Anti-NRX) or latrophilin (Anti-LPH). (**C**) Expression of latrophilin and neurexin in COS cells. COS cells were transfected with 10 μg of the vector (pcDNA3) or latrophilin (LPH) or neurexin (NRX) constructs and grown for three days. The binding of 5 nM [^{125}I]LTX to both transfected cells (1 mg/ml) was measured (left). LTX bound to the neurexin-expressing cells only in the presence of Ca^{2+}. 50 μg of the cell membranes were then analysed by Western blotting in parallel with 50 μg of brain membranes, using antibodies as indicated (right). Note the higher contents of latrophilin and the lower contents of neurexin in the brain compared with the respective transfected cells that exhibit a similar LTX binding in Ca^{2+}.

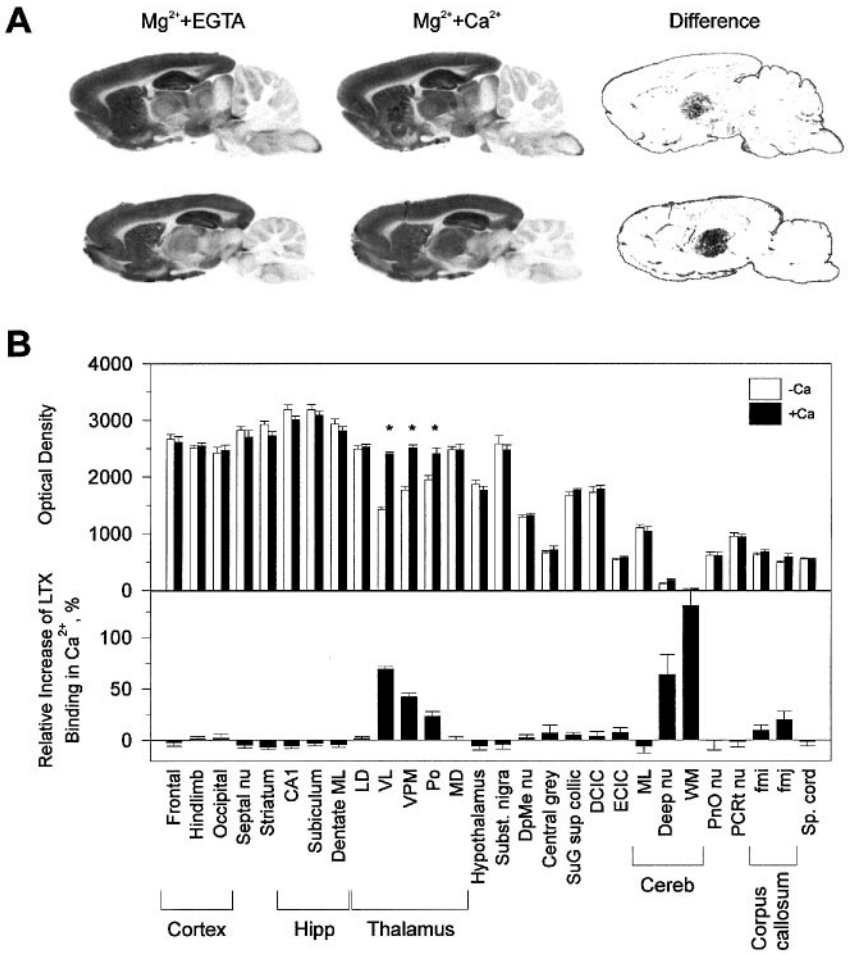

Fig. 2. Latrophilin is the major LTX receptor. (**A**) Autoradiographic images illustrating the distribution of the Ca^{2+}-dependent and -independent toxin receptors in rat brain. Adjacent sections of rat brains (two different brains are shown) were incubated with 1 nM [^{125}I]LTX in the presence of 2 mM Mg^{2+} and 2 mM Ca^{2+} or 10 mM EGTA, then washed, fixed and exposed to film. Images, shown in the left and middle panels, were subtracted from each other to produce the differential images (right panels), in which the brain structures that bind more [^{125}I]LTX in Ca^{2+} appear as darker spots. (**B**) Quantitative comparison of the toxin binding to brain regions (top) and relative increase of the binding in the presence of Ca^{2+} (bottom). One hundred and twenty-six sections from seven brains were processed as in (A), and the optical density of images was determined in areas corresponding to anatomical brain structures, using computer-assisted densitometry. Mean optical densities (±SEM) are shown in arbitrary units. DCIC, dorsal cortex of inferior colliculus; DpMe, deep mesencephalic nu; ECIC, external cortex of inferior colliculus; fmi, fmj, forceps minor and major of corpus callosum; ML, molecular layer; nu, nuclei; PCRt, parvocellular reticular nu; PnO, pontine reticular nu, oral; SuG, superficial grey layer of superior colliculus; WM, white matter. Note that Ca^{2+} does not increase the binding of LTX to the cerebellar cortex (ML), brain cortex or hippocampus (Hipp), areas of high level of neurexin Iα mRNA. The regions demonstrating an increased binding in Ca^{2+} are ventrolateral (VL), ventral posteromedial (VPM) and posterior (Po), but not laterodorsal (LD) or mediodorsal (MD) thalamic nuclei. Deep cerebellar nuclei and white matter in cerebellum and forebrain (WM, fmi, fmj) also show moderate to high relative increase in binding. *$P<0.0001$, $n=18$.

Thus, the replacement of Ca^{2+} with Sr^{2+} abolished the toxin binding to neurexins and provided a convenient way to test latrophilin separately for its role in the toxin-evoked secretion (see below).

The Western blotting described above could not provide precise quantification of the relative contribution of neurexin Iα and latrophilin to the toxin's binding and, consequently, effect in the presence of Ca^{2+}. To assess the qualitative and quantitative aspects of neurexin's involvement in LTX binding to neurones, we performed autoradiography of rat brain sections, a very sensitive, well-established procedure used to study the distribution of toxin receptors *in situ* (Malgaroli *et al*., 1989; Awan and Dolly, 1991). When incubated with [^{125}I]LTX in the presence of Ca^{2+} or EGTA, the sections specifically bound the toxin but inclusion of Ca^{2+} did not significantly affect the distribution of LTX-binding sites (Figure 2A). Statistical analysis carried out on 126 sections from seven rat brains demonstrated that the total amount of bound LTX in most brain areas remained unchanged in the presence of Ca^{2+}, except some thalamic nuclei where Ca^{2+} enhanced LTX binding by 25–70% (Figure 2B). Subtraction of the two sets of images unambiguously illustrates that only the thalamus expresses a substantial number of Ca^{2+}-dependent LTX receptors (Figure 2A, right), which may be neurexins. Surprisingly, there was no increase in toxin binding to the brain cortex, hippocampus and, most notably, cerebellar molecular layer, despite the fact that these structures contain higher levels of the neurexin Iα mRNA (Ushkaryov *et al*., 1992; Ullrich *et al*., 1995) (Figure 2B, top). Instead, in addition to thalamus, white matter tracts of the forebrain (*corpus callosum*) and cerebellum bound 20–150% more LTX in the presence of Ca^{2+} (Figure 2B, bottom). This is consistent with the

known abundance of neurexin in axons (Russell and Carlson, 1997). We interpret these results as evidence of a limited involvement of neurexins in toxin binding at the neuronal cell surface even when Ca^{2+} is present. Therefore, NE release stimulated by LTX, with or without Ca^{2+}, in the majority of brain synapses is probably mediated by only one receptor, latrophilin.

Dependence of LTX activity on external Ca^{2+}

To study the effect of LTX on nerve terminals, we measured secretion from synaptosomes of accumulated exogenous NE after stimulation by depolarization (30 mM KCl) or LTX in the presence or absence of Ca^{2+}_e. LTX is able to trigger NE release under all conditions tested. However, the addition of Ca^{2+}_e increases the response of synaptosomes to the toxin by 2.5-fold (Figure 3A). Interestingly, the LTX-stimulated secretion appears to be ~10× more sensitive to external Ca^{2+} than that triggered by K^+ (Figure 3A). The LTX effect approaches a maximum at 100 μM Ca^{2+}; at this cation level, K^+ depolarization elicits only a fraction of its maximal effect. Note that Ca^{2+}_e above 1 mM partially inhibits the toxin-evoked release, whilst even at 10 mM (not shown) the cation does not suppress exocytosis stimulated by high K^+.

In Sr^{2+}, when LTX does not interact with neurexin, it still stimulates the same maximal level of NE release (Figure 3A) as in Ca^{2+}. The sensitivity of terminals to Sr^{2+} is also shifted by LTX to μM cation concentrations, similar to the toxin effect in Ca^{2+}. Partial inhibition of secretion at mM Sr^{2+} concentrations is evident too, consistent with the same mechanism of toxin action both in Sr^{2+} and in Ca^{2+}.

Is this increased sensitivity of terminals to divalent cations due to an ionophore-like effect of LTX? When Ca^{2+} was introduced into synaptosomes using a Ca^{2+} ionophore, A23187, the cation sensitivity (Figure 3B) was lower than that in LTX but similar to that observed with a high $[K^+]$ and in electrophysiological experiments (Figure 3A; Heidelberger *et al*., 1994). This suggests that the toxin augments the divalent cation-sensitivity of the release machinery via an intracellular signalling mechanism rather than by simply increasing the membrane permeability to cations. Based on the above observations, 100 μM Ca^{2+} was used in further experiments in order to distinguish the Ca^{2+}-dependent LTX effect from that of high $[Ca^{2+}]_i$ arising from entry of external cation through LTX-induced channels (Grasso *et al*., 1980; see also below).

The dose-dependence curves for LTX with or without Ca^{2+} (Figure 3C) demonstrate the same EC_{50} values (1–2 nM), while reaching different maximal levels, consistent with the idea of one receptor mediating both toxin's actions, despite their different underlying mechanisms.

LTX action requires intracellular Ca^{2+}

The Ca^{2+}_e-dependent component of the LTX-induced NE secretion was examined next. To address the question of whether Ca^{2+} is required for LTX action inside the terminals, we used a cell-permeable Ca^{2+}-buffer, BAPTA-AM (1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester). Intracellular BAPTA-AM dose-dependently inhibited up to 80% of the LTX-triggered

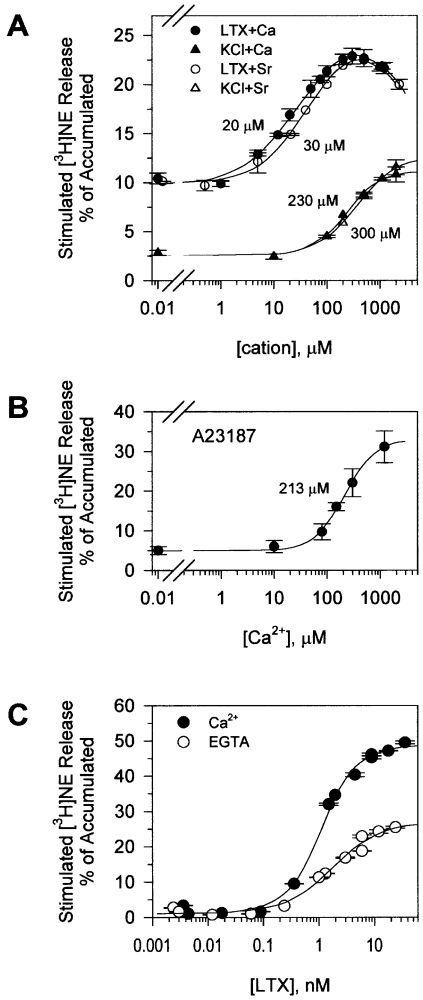

Fig. 3. LTX increases the sensitivity of NE release to extracellular Ca^{2+} and Sr^{2+}. (**A**) Synaptosomes (1 mg of protein/ml) were preloaded with [^{3}H]NE (see Materials and methods) and stimulated for 5 min with 1 nM LTX or 30 mM KCl in a buffer containing 0–2 mM free Ca^{2+} or Sr^{2+} and 2 mM Mg^{2+}. The toxin-evoked NE secretion is more sensitive to Ca^{2+} than the K^+-stimulated release (EC_{50} values are given above the curves for Ca^{2+} and below for Sr^{2+}). In Sr^{2+}, LTX produces a similar effect to that in Ca^{2+} without interacting with neurexin. (**B**) The sensitivity of terminals to Ca^{2+} is low, compared with LTX, when a Ca^{2+}-ionophore, A23187 (2.5 μM), is used to stimulate NE release (EC_{50} for Ca^{2+} is indicated). (**C**) Dose–response curves for LTX in the presence of 100 μM Ca^{2+}_e or 100 μM EGTA. Experiments were carried out as in (A), except that the Ca^{2+} concentration was kept constant and the toxin concentration varied. Both toxin actions are likely mediated by one receptor (note the same EC_{50} values).

exocytosis in Ca^{2+}-containing medium, reducing release to the level stimulated in the absence of Ca^{2+} (Figure 4A). The latter release, however, remained unchanged, indicating that it is independent of both Ca^{2+}_e and Ca^{2+}_i. Pre-loading of synaptosomes with BAPTA-AM also blocked the K^+-evoked secretion in control experiments (Figure 4A). Thus, an increase in the cytoplasmic $[Ca^{2+}]$ is required for secretion stimulated by LTX in the presence of Ca^{2+}_e. Is this rise in cytoplasmic $[Ca^{2+}]$ due to the cation influx?

Among other methods, radioactive Ca^{2+} tracer has been used (Grasso *et al*., 1980) to follow the uptake of the cation by PC12 cells upon stimulation with the toxin.

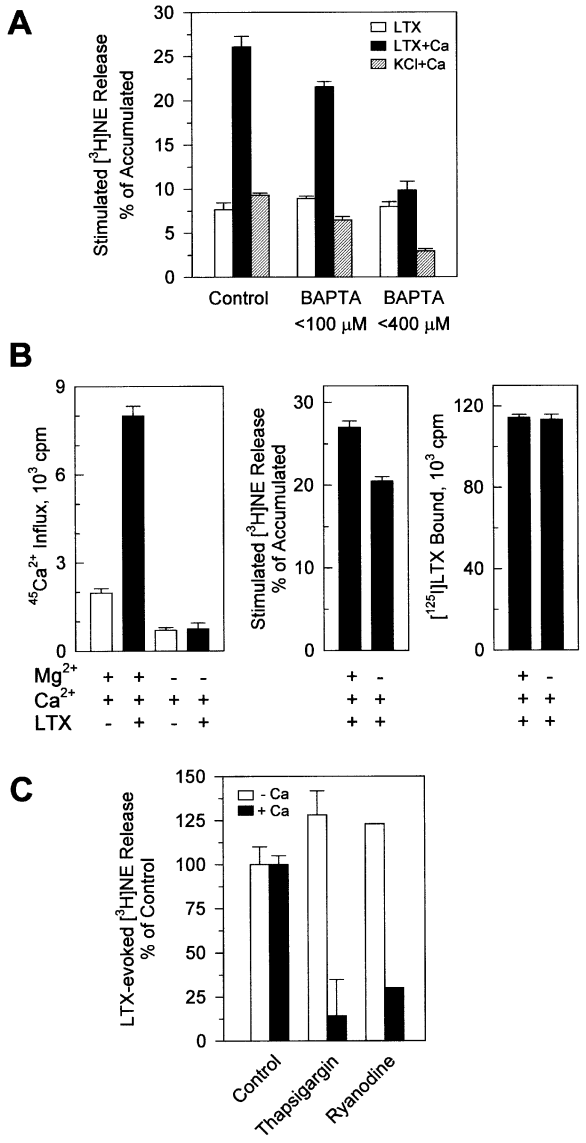

Fig. 4. LTX action depends on intracellular Ca^{2+}. (**A**) Ca^{2+} is required inside nerve terminals for the Ca^{2+}_e-dependent LTX effect. Synaptosomes were preloaded with [^{3}H]NE, then with BAPTA-AM at indicated concentrations, washed and stimulated for 5 min by 1 nM LTX in the presence of 200 μM EGTA or Ca^{2+} or by 30 mM KCl and 2 mM Ca^{2+}. Plotted is the secretion above basal under respective conditions. (**B**) Ca^{2+}_e does not need to enter nerve terminals to support the Ca^{2+}_e-dependent toxin activity. The influx of Ca^{2+} into synaptosomes (left) was measured at 2 min, [^{3}H]NE release (middle) and [^{125}I]LTX binding (right) at 5 min after the application of 1 nM toxin and 100 μM Ca^{2+} ± 2 mM Mg^{2+}. For the influx measurements, the solution also contained 1 μM $^{45}Ca^{2+}$. (**C**) Drugs depleting intracellular Ca^{2+} stores inhibit the Ca^{2+}_e-dependent toxin action. Synaptosomes, preloaded with [^{3}H]NE, were treated with 10 μM thapsigargin or 50 μM ryanodine and stimulated with 5 nM LTX for 5 min in the presence of 100 μM Ca^{2+} or EGTA. NE release is expressed as percentage of respective controls.

Using the same technique, we demonstrate that Ca^{2+} quickly enters nerve terminals (Figure 4B), followed by a delayed release of NE (see Figure 7C). Surprisingly, the removal of Mg^{2+} from the medium abolished the LTX-induced influx of $^{45}Ca^{2+}$ into terminals at all times measured. However, the Ca^{2+}_e-dependent NE release elicited by LTX in the absence of Mg^{2+} was not substantially reduced, nor was the toxin binding (Figure 4B). This blockade of Ca^{2+} entry by the lack of Mg^{2+} persisted at all [Ca^{2+}] tested ($\leqslant$1.2 mM).

Thus, in the presence of Ca^{2+}_e, LTX action depends upon the elevation of cytoplasmic Ca^{2+} but does not absolutely require the entry of the cation into terminals. This suggested that in the presence, but not in the absence of Ca^{2+}_e, the toxin could induce mobilization of stored Ca^{2+}. In order to test this hypothesis, we used thapsigargin and ryanodine, drugs that deplete Ca^{2+}_i stores (Thastrup *et al*., 1990; McPherson *et al*., 1991). Treatment of nerve terminals with either substance inhibited NE secretion stimulated by LTX in the presence of Ca^{2+}_e (Figure 4C), indicating that 100 μM Ca^{2+}_e alone is not sufficient for the toxin to trigger exocytosis when internal cation stores are empty. Increasing the $[Ca^{2+}]_e$ partially reversed the effect of thapsigargin (not shown). At the same time, the drugs did not inhibit but actually enhanced the Ca^{2+}_e-independent NE release evoked by LTX, probably due to the presence of a higher proportion of neurotransmitter in the cytoplasm (see below).

PLC is involved in LTX-stimulated release

These results and the fact that latrophilin is a G protein-coupled receptor suggested that LTX action could be based on the release of Ca^{2+} from intracellular pools, a process that can be regulated by PLC. Therefore, we tested aminosteroid U73122 (1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione), which irreversibly blocks PLC (Smith *et al*., 1990) and/or interferes with G protein coupling to PLC (Thompson *et al*., 1991). Whilst U73122 did not affect the Ca^{2+}-independent LTX-induced release, this drug dose-dependently and completely inhibited the Ca^{2+}_e-dependent LTX-triggered exocytosis (Figure 5A). The EC_{50} of the U73122 effect on LTX action was 8 μM, whereas an analogue of this drug, U73343 (1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-2,5-pyrrolydinedione), which does not inhibit PLC, failed to alter release stimulated by LTX (Figure 5A). It was possible that U73122 interfered with Ca^{2+} entry, which could result in an inhibition of NE release similar to that observed (Figure 5A). However, as shown in Figure 5B, Ca^{2+} at concentrations of 0.1–1.2 mM still enters terminals treated with 20 μM U73122 without reversing the effect of the aminosteroid. These results suggest that under conditions of PLC inhibition Ca^{2+} influx alone is not sufficient to trigger the Ca^{2+}-dependent LTX-induced exocytosis. This is consistent with the stimulation by LTX of a PLC-based intracellular signalling cascade.

Botulinum neurotoxins block only the Ca^{2+}_e-dependent LTX action

Our experiments suggested that the two components of the toxin secretory action (Ca^{2+}_e-dependent and -independent) have different underlying mechanisms. It was, therefore, logical to examine the exocytotic nature of these two processes. SNARE proteins have been implicated in vesicular exocytosis, and their cleavage by Clostridial neurotoxins perturbs this type of secretion (Sollner *et al*., 1993). In this study, we used botulinum neurotoxin (BoNT) C, which proteolyses both syntaxin and SNAP-25 (Blasi *et al*., 1993; Foran *et al*., 1996), and BoNT D, which cleaves synaptobrevin (Schiavo *et al*., 1993).

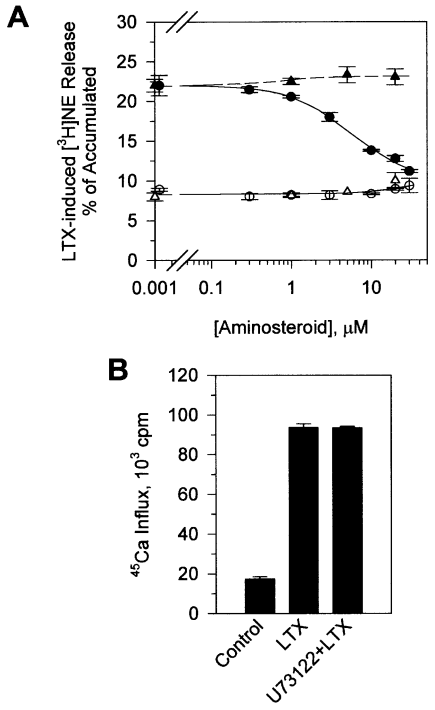

Fig. 5. Phospholipase C inhibitor, U73122, blocks only the $Ca^{2+}{}_e$-dependent stimulation by LTX of NE release. (**A**) Synaptosomes were loaded with [^{3}H]NE, then treated with increasing concentrations of U73122 (circles) or its inactive derivative, U73343 (triangles), for 10 min at 37°C, and stimulated with 1 nM LTX for 5 min. The buffer used during stimulation contained either 100 μM Ca^{2+} (solid symbols) or 100 μM EGTA (open symbols). (**B**) Aminosteroid U73122 does not prevent the LTX-induced entry of Ca^{2+} into synaptosomes. Synaptosomes, untreated or pre-treated with 20 μM U73122, were stimulated as in (A) in the presence of 100 μM free Ca^{2+} and 10 μM $^{45}Ca^{2+}$; then the suspension was quickly filtered, and the radioactivity of filters determined in duplicate independent experiments.

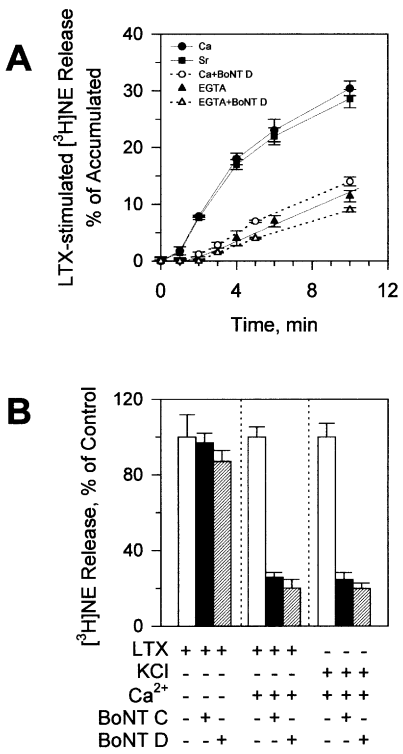

Fig. 6. Botulinum neurotoxins C and D block only the $Ca^{2+}{}_e$-dependent effect of LTX. (**A**) The rate of [^{3}H]NE release elicited by LTX from control synaptosomes or those pre-treated with 100 nM of BoNT D. Synaptosomes were stimulated by 1 nM LTX in the presence of 2 mM Mg^{2+} and 100 μM free Ca^{2+}, Sr^{2+} or EGTA. Release from non-stimulated synaptosomes was subtracted and the resulting stimulated [^{3}H]NE secretion expressed as percentage of the total accumulated [^{3}H]NE under each condition. (**B**) [^{3}H]NE release stimulated by LTX or by K^+ from synaptosomes pre-treated with 100 nM BoNT C or D. A 5-min stimulation protocol employed either 1 nM LTX with or without 100 μM Ca^{2+} or 30 mM KCl with 2 mM Ca^{2+}. The values of stimulated release from BoNT-treated terminals are expressed as percentage of control (evoked release from terminals not treated with BoNTs).

Pre-treatment with either BoNT abolished K^+-evoked exocytosis from synaptosomes (Figure 6). Similarly, these neurotoxins potently reduced both the total amount and the rate of the $Ca^{2+}{}_e$-dependent LTX-stimulated NE secretion, bringing it down to the level of the Ca^{2+}-independent release. On the other hand, the cleavage of SNARE proteins left the rate and amount of Ca^{2+}-independent LTX release largely unchanged. These findings suggest that the secretion stimulated by LTX occurs via vesicular exocytosis in the presence of $Ca^{2+}{}_e$. However, when external Ca^{2+} is removed, the release is SNARE-independent and may be non-vesicular.

LTX stimulates Ca^{2+}-independent non-vesicular leakage

The rates of NE release stimulated by LTX with and without $Ca^{2+}{}_e$ differ dramatically. Ca^{2+} (and Sr^{2+}) support fast exocytosis (Figure 6), which occurs after a short delay and within ~4 min parallels the Ca^{2+}-independent LTX-evoked release. The latter has a substantially longer lag time and does not level off even within 20 min of stimulation with LTX (Figure 6A). Since the $Ca^{2+}{}_e$-independent release could be non-vesicular, we tested whether neurotransmitter can leak through the pores which are known to be induced by the toxin in the plasma membrane. In that case, the first substances to leak out would be cytoplasmic constituents. Indeed, in the absence of Ca^{2+}, LTX-stimulated release of a cytoplasmic amino acid, D-aspartate, was much faster and stronger than secretion of NE (Figure 7A).

The slow rate of NE release could be due to a delayed leakage of this neurotransmitter from vesicles into the cytoplasm (probably as a result of ATP depletion; McMahon *et al*., 1990) followed by its escape into the extracellular milieu. If this is the case, then inhibiting vesicular uptake of NE should further increase its concentration in the cytoplasm and, subsequently, augment the leakage component of release in the presence of LTX. We tested this suggestion by using reserpine, a selective inhibitor of the vesicular NE transporter (Kruk and Pycock, 1993). As expected, reserpine decreased the $Ca^{2+}{}_e$-dependent LTX-stimulated exocytosis from terminals but enhanced both the amount (Figure 7B) and the rate (not shown) of the $Ca^{2+}{}_e$-independent NE release. This result is consistent with the leakage hypothesis because when neurotransmitter is excluded from the vesicles, its efflux does not depend on $Ca^{2+}{}_e$ and follows the kinetics of cytoplasmic leakage.

The toxin-dependent pores are not large since they do not let through synaptic vesicles or indeed cytoplasmic proteins (e.g. lactate dehydrogenase, not shown). To

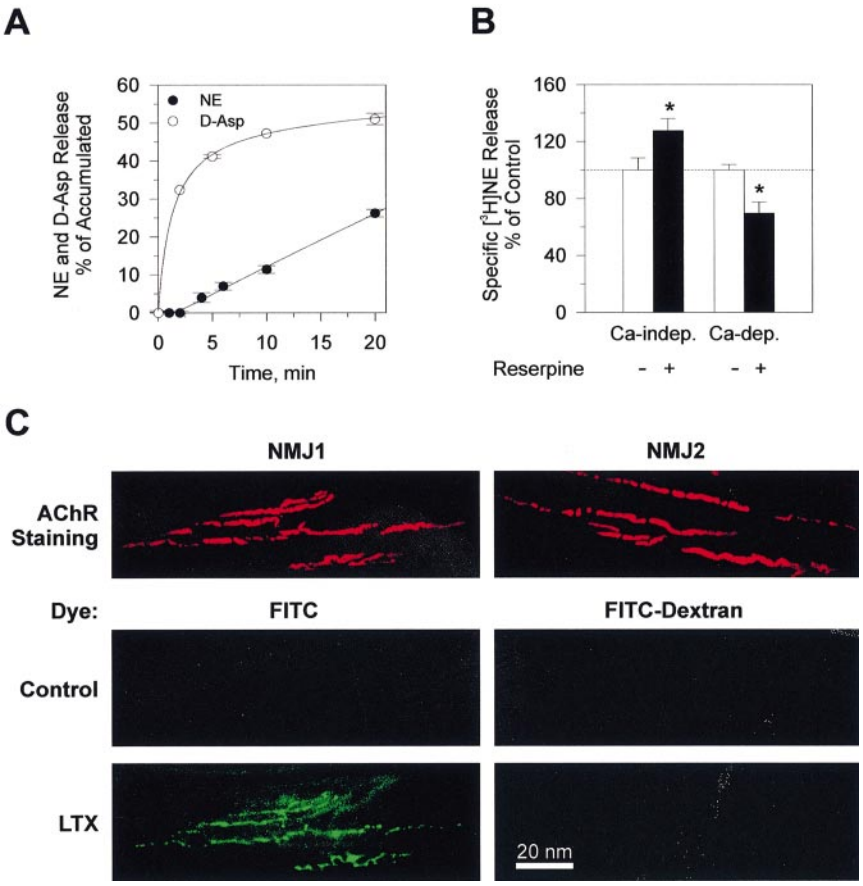

Fig. 7. The Ca^{2+}-independent LTX-evoked release of NE occurs via membrane pores. (**A**) Secretion rate of cytoplasmic D-[^{3}H]aspartate is much faster than that of vesicular [^{3}H]NE when triggered by LTX in the absence of Ca^{2+}_e. Terminals were loaded with each substance (as in Materials and methods) and stimulated with 1 nM LTX in 100 μM EGTA. The release of each substance is expressed as percentage of the total accumulated amount of that substance. (**B**) Treatment of synaptosomes with reserpine increases the Ca^{2+}-independent NE leakage but decreases the Ca^{2+}_e-dependent vesicular exocytosis evoked by LTX. Terminals pre-loaded with [^{3}H]NE were incubated for 20 min with 50 μM reserpine and stimulated for 5 min with 1 nM LTX in the presence or absence of 100 μM Ca^{2+}_e. Release of [^{3}H]NE is expressed as percentage of control under respective conditions (LTX-evoked secretion from untreated synaptosomes). (**C**) Pores induced by LTX in the neuronal plasma membrane are permeable to FITC. Nerve muscle preparations were pre-incubated for 90 min in a buffer containing 2 mM EGTA and labelled with rhodamine-conjugated α-bungarotoxin to localize NMJs (top panels). The addition of 5 μM FITC or FITC–dextran (M_r 4400) was followed by a 10 min incubation, extensive washes and acquisition of the image (middle panels). Then the incubation of the same terminals with the dyes was repeated in the presence of 1 nM LTX (bottom). Results shown are representative of nine independent experiments. Note that LTX stimulates the diffusion of FITC but not dextran only into nerve terminals and not into muscle cells.

characterize these pores further, we tested whether extracellular substances, such as fluorescent dyes, could enter nerve terminals through the pores. Fluorescent microscopy was used to visualize the diffusion of fluorescein isothiocyanate (FITC) isomer I into nerve terminals of frog NMJs. Nerve muscle preparations were incubated in a Ca^{2+}-free medium supplemented with 2 mM EGTA. Under these conditions, the vesicle recycling is totally blocked (Ceccarelli and Hurlbut, 1980; Henkel and Betz, 1995) and any terminal labelling would be due to diffusion of the dye rather than endocytosis. Postsynaptic nicotinic acetylcholine receptors were stained using rhodamine-conjugated α-bungarotoxin in order to localize NMJs (Figure 7C). When FITC was incubated with neuromuscular preparations without stimulation, followed by an extensive wash, no entry of the dye into cells was detectable. However, within the first 10 min after the addition of 1 nM LTX to the same preparation, the cytoplasm of the neuronal (but not the muscle) cell became extensively labelled (Figure 7C, bottom panel, left). This presynaptic labelling was cytoplasmic rather than vesicular because it quickly dissipated, closely following the kinetics of the efflux of cytoplasmic D-aspartate (Figure 7A and data not shown); ~50% of the FITC fluorescence disappeared in the first 15 min. This was not, however, due to physical quenching of the dye, which never exceeded 2–3% within that time. Large FITC-conjugated molecules, such as dextran (M_r 4400) (Figure 7C, right panels), could not permeate the toxin-dependent pores. These results suggest that the LTX-stimulated opening of small pores, rather than endocytosis or 'kiss-and-run' vesicular uptake, allows FITC to enter nerve terminals.

We also attempted to address the nature of the toxin-induced pores, which are often thought to be the same as the channels formed by LTX in artificial lipid bilayers. For this purpose, the concentrations of synaptosomes and latrophilin-transfected COS cells were adjusted to produce a similar level of toxin binding. Then LTX was used to stimulate $^{45}Ca^{2+}$ uptake in both preparations. Although synaptosomes responded to the toxin application by a massive $^{45}Ca^{2+}$ influx, no such effect was detectable in transfected cells (Figure 8), indicating that the pores are intrinsic

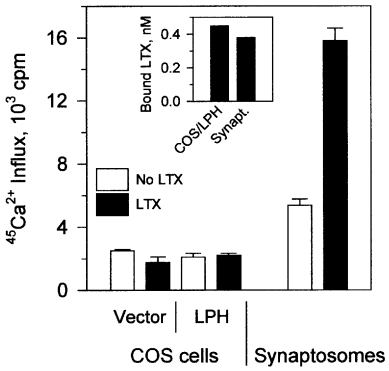

Fig. 8. The non-selective pores induced by LTX are cell-specific membrane components. COS cells were transfected with the latrophilin construct or an empty vector, grown for 3 days and tested for the toxin-stimulated influx of Ca^{2+}, in parallel with rat brain synaptosomes. The measurements were carried out in duplicate with 0.1–2 mM cation supplemented with 1–20 μM $^{45}Ca^{2+}$. Inset, binding of iodinated LTX (3 nM) to transfected cells and synaptosomes performed in order to normalize both preparations with respect to receptor contents.

membrane components distinct from the toxin and its receptor.

Discussion

The action of LTX depends on the presence of a membrane receptor intimately coupled to the release machinery (Rosenthal and Meldolesi, 1989). Two potential receptors have been purified from brain: neurexin and latrophilin. Neurexins are neuronal cell-surface proteins implicated in cell recognition and adhesion, whose participation in signal transduction is unclear (Missler and Sudhof, 1998). Presynaptic targeting of neurexin Iα has not been definitively shown; in fact, the protein is found on axons but not on terminals (Russell and Carlson, 1997) of the ray electric organ, in which LTX causes a presynaptic effect (Linial *et al*., 1995). Latrophilin, conversely, is expressed presynaptically at the frog NMJ and in the rat brain (Y.A.Ushkaryov, in preparation) and, being a G protein-coupled receptor, is the likely candidate to transduce signals into the presynaptic neurone. Our experiments unequivocally show that neurexin contributes very little to the binding of LTX in Ca^{2+} even in areas of apparently high expression of neurexin Iα mRNA (Figure 2; Ushkaryov *et al*., 1992; Ullrich *et al*., 1995). This could be explained by the fact that only some splice variants of neurexin Iα interact with the toxin (Davletov *et al*., 1995) and that very little neurexin is expressed in brain membranes as opposed to latrophilin (Figure 1C), whose affinity to LTX is also ~10-fold higher (Figure 1; Davletov *et al*., 1995).

On the other hand, knockout of the neurexin Iα gene in mice causes a decrease in the Ca^{2+}-dependent binding of LTX to synaptosomes (Geppert *et al*., 1998). Surprisingly, the Ca^{2+}-independent binding is also reduced, while the affinities of the remaining Ca^{2+}-dependent and -independent sites are unchanged. Moreover, in the absence of neurexin, Ca^{2+} still enhances both the binding and the excitatory effect of LTX, indicating that latrophilin and its signalling mechanism are also sensitive to Ca^{2+}. Since the level of latrophilin expression in the neurexin-deficient mouse is unknown, this protein could be down-regulated or incorrectly targeted. It is also known that some insulin-secreting pancreatic β-cell lines, expressing more neurexin Iα than neurones, are not responsive to LTX unless transfected with latrophilin (Lang *et al*., 1998). As our *in situ* binding experiments demonstrate, neurexin may potentially mediate LTX binding in some brain regions, including the white matter. Nevertheless, the structure of neurexin precludes its participation in the G protein- and PLC-coupled mechanism and Ca^{2+} signalling described here. Moreover, in the presence of Sr^{2+}, when LTX interacts with latrophilin but not neurexin, all parameters of the toxin-elicited release are very similar to those in Ca^{2+} (Figures 1, 3 and 6). Thus, LTX may exert all of its effects and trigger the same secretory mechanism by interacting only with latrophilin.

Although mediated by latrophilin, the Ca^{2+}-dependent and -independent LTX actions in nerve terminals differ not only by their requirement of the extracellular cation but also by underlying molecular processes. First, as we demonstrate here, the Ca^{2+}-dependent LTX-evoked release is vesicular because it is blocked by botulinum neurotoxins (Figure 6). This mechanism is similar to exocytosis triggered by depolarization, as both stimuli require Ca^{2+}_e. However, LTX increases the sensitivity of the release mechanism to Ca^{2+} or Sr^{2+} by 10-fold. Furthermore, higher concentrations of these cations partially inhibit the toxin effect. The major difference between eliciting release by depolarization and LTX, revealed in our experiments, is that in the absence of Mg^{2+}, LTX does not stimulate Ca^{2+} influx into the cytoplasm but still triggers the Ca^{2+}_e-dependent exocytosis (Figure 4B). Dependence on Mg^{2+} has also been described in frog NMJs where LTX, in the absence of Ca^{2+}_e, increased the frequency of miniature end-plate potentials only in the presence of mM Mg^{2+} (Misler and Hurlbut, 1979). Since Mg^{2+} itself is unable to support depolarization-induced secretion, these data could mean that the toxin acts by sending an intracellular signal that also depends on certain extracellular cations.

Our experiments with intracellular Ca^{2+} chelators (Figure 4A) indicate that the toxin's action requires an increase in the cytoplasmic Ca^{2+} concentration. Since the cation may not need to enter the terminals, this rise of cytoplasmic Ca^{2+} could only be produced from intracellular Ca^{2+} stores. Consistently, drugs depleting Ca^{2+} stores block the Ca^{2+}-dependent toxin effect. These findings suggest that the LTX-stimulated mechanism of exocytosis requires release of Ca^{2+} from internal stores, which can be triggered by the toxin only in the presence of Ca^{2+}_e. This conclusion agrees well with experiments on rat hippocampal neurones and bovine chromaffin cells (Capogna *et al*., 1996; Michelena *et al*., 1997), in which the site of action of Ca^{2+}_e in the toxin effect was hypothetically placed on the external or submembraneous cell surface.

Latrophilin is a G protein-coupled receptor and the toxin, therefore, could act via a G protein signalling cascade. In fact, LTX has been shown to induce polyphosphoinositide (PPI) hydrolysis and inositol polyphosphates (IP_3) production in PC12 cells (Vicentini and Meldolesi, 1984; Rosenthal *et al*., 1990) and in COS cells expressing latrophilin (Lelianova *et al*., 1997). Interestingly, these effects were observed only in the presence of Ca^{2+}_e and

were attributed to the activation of a Ca^{2+}-sensitive PLC. Finally, we demonstrate here that activation of PLC by the toxin may account fully for the $Ca^{2+}{}_e$-dependent stimulation of NE release, because it is specifically blocked by U73122. In most systems, this aminosteroid reduces PPI hydrolysis and subsequent Ca^{2+} signalling (Jin *et al*., 1994; Cui and Kanno, 1997) by inhibiting G proteins that control PLC (Thompson *et al*., 1991). PLC activation by a G protein α-subunit has also been implicated in regulation of dopamine and GABA exocytosis from nerve terminals (Zelles *et al*., 1995), although the respective G protein-coupled receptors have not been found. Collectively, these results indicate that neurotransmitter release could be controlled by a mechanism involving PLC activation and release of stored Ca^{2+}; in the presence of $Ca^{2+}{}_e$, LTX could stimulate this machinery via latrophilin and its requisite G protein. Activation of PLC requires the presence of Ca^{2+} as a co-factor (Finch *et al*., 1991). Accordingly, localized downstream of $Ca^{2+}{}_e$ entry (Figure 5), PLC could be one of the Ca^{2+} sensors for exocytosis. Our finding that LTX greatly increases the terminal sensitivity to Ca^{2+} is, therefore, interesting because it suggests that activation of the G protein-coupled receptor may hypersensitize the release machinery to $Ca^{2+}{}_e$. This, by analogy, offers a plausible mechanism of augmentation of exocytosis induced by depolarization.

However, we could detect only a small (5%) positive change in IP_3 formation upon the stimulation of synaptosomes by LTX in Ca^{2+}, while U73122 inhibited the overall PPI hydrolysis (data not shown). The reason why LTX stimulates a large rise in IP_3 production in cell cultures (Vicentini and Meldolesi, 1984; Lelianova *et al*., 1997) but not in nerve endings, could be that in terminals, the toxin induces PPI metabolism only at the active zones where latrophilin is more abundant (Y.A.Ushkaryov, in preparation). Therefore, while serving as a general second messenger that triggers exocytosis of LDCV in endocrine cells (Tse *et al*., 1997), IP_3 may have a local function in neurones, e.g. activation of IP_3-gated calcium channels (Ueda *et al*., 1996). Alternatively, the role of PLC may be to modify the plasma membrane by removing binding sites for vesicular proteins (Schiavo *et al*., 1996) or to directly induce membrane fusion as proposed for other phospholipases (Nishio *et al*., 1996).

As demonstrated here and by others (reviewed by Rosenthal and Meldolesi, 1989), the toxin can stimulate a substantial release of neurotransmitter in the total absence of Ca^{2+}. However, in our system, this Ca^{2+}-independent release was insensitive to botulinum toxins. Therefore, the Ca^{2+}- and SNARE-independent LTX-stimulated NE release is unlikely to be the classical vesicular exocytosis. This release occurs through pores induced by LTX in the cell membrane described previously (e.g. Grasso *et al*., 1980; Hurlbut *et al*., 1994). However, we found that these pores are permeable to not only cations but also small substances, including D-aspartate and FITC (Figure 7). The leakage of NE is slow and begins after a considerable delay, because NE is mostly vesicular and must first leak from vesicles into the cytoplasm and only then could exit through the pore into the extracellular space (Figure 7B). Thus, our data fully support and extend previous observations that LTX action involves pore formation in the presynaptic membrane (McMahon *et al*., 1990). Other

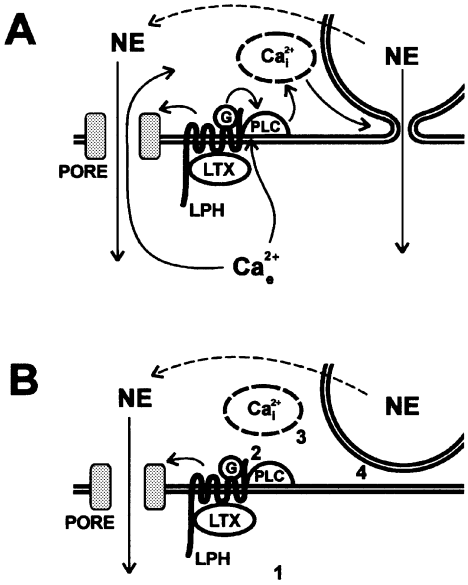

Fig. 9. A model of processes underlying the $Ca^{2+}{}_e$-dependent and -independent modes of LTX action (see text).

novel findings of the current work include the demonstration that LTX induces pore opening only in the presynaptic membrane (Figure 7) and that the toxin is unable to make pores in heterologous cells in spite of the receptor being expressed on their surface (Figure 8). In concert with other published work (Wanke *et al*., 1986; Lang *et al*., 1998), our observations further distinguish between the pores formed by LTX in lipid bilayers and in neuronal membranes and suggest that this pore may be a functional part of the presynaptic release site.

The two proposed mechanisms of LTX action are summarized schematically in Figure 9. Upon binding to latrophilin (LPH), LTX opens a pore in the cell membrane and activates a G protein-coupled PLC. Ca^{2+} can enter the cytoplasm through the toxin-induced pores and stimulate exocytosis directly (at high $[Ca^{2+}]_e$) or by further activating PLC (at low and high $[Ca^{2+}]_e$) (Figure 9A). LTX causes exocytosis only in the presence of $Ca^{2+}{}_e$, although it does not stimulate Ca^{2+} influx into the cytoplasm in the absence of Mg^{2+}. Activation of PLC by latrophilin and Ca^{2+} leads to the production of IP_3, which mobilizes Ca^{2+} from internal stores, providing a synergistic mechanism involving both $Ca^{2+}{}_e$ and $Ca^{2+}{}_i$. The rise of the cytoplasmic $[Ca^{2+}]$ triggers exocytosis. LTX cannot stimulate SV exocytosis when (1) it is applied in a $Ca^{2+}{}_e$-free medium, (2) PLC in terminals is inhibited by U73122, (3) the $Ca^{2+}{}_i$ pools are depleted by thapsigargin, or (4) exocytosis is blocked by the cleavage of SNARE proteins by botulinum toxins (see 1–4 in Figure 9B). High $[Ca^{2+}]_e$ in the presence of Mg^{2+} could reverse the effects of thapsigargin by replenishing the stores but will be insufficient to overcome the block of PLC (Figure 5B), consistent with a central role of PLC in the LTX-elicited neuroexocytosis. However, under a range of conditions (1–4) when active exocytosis is arrested (Figure 9B), LTX still opens the pore, and neurotransmitter gradually leaks from vesicles into the cytoplasm (dashed line) and quickly escapes via the pore.

Our results, indicating that LTX evokes vesicular

secretion only in the presence of Ca^{2+}_e, seem to be at variance with previous observations addressing the toxin's effect in the absence of Ca^{2+}. Those experiments where quantal (vesicular) release was measured in nominally Ca^{2+}-free conditions (Misler and Hurlbut, 1979; Ceccarelli and Hurlbut, 1980) were conducted on tissue preparations, i.e. under conditions when it is difficult to remove all extracellular and stored Ca^{2+}. These results should be reconsidered in view of our finding that LTX greatly increases the sensitivity of exocytosis to minimal $[Ca^{2+}]_e$ that is insufficient for depolarization to trigger release. Another group (Capogna *et al*., 1996) argued that BoNT F blocks quantal secretion triggered by LTX in hippocampal slices in a Ca^{2+}-independent manner. However, 2.8 mM Ca^{2+}_e was present throughout that experiment, and the observed exocytosis was actually Ca^{2+}-dependent. In a similar way, when LTX was pre-bound to rat chromaffin cells in the absence of Ca^{2+}, exocytosis could be quickly stimulated or blocked by the addition or removal of Ca^{2+}_e (Barnett *et al*., 1996). As our model also predicts, LTX may act at different $[Ca^{2+}]_e$ in cells expressing PLC isozymes with distinct Ca^{2+} sensitivities.

Our data and previous findings lead us to the following conclusions. (i) Both the Ca^{2+}_e-dependent and -independent LTX activities are mediated by the G protein-coupled receptor, latrophilin. (ii) LTX triggers vesicular exocytosis only in the presence of Ca^{2+}_e. (iii) The toxin increases the sensitivity of exocytosis to external Ca^{2+}. (iv) In the absence of Mg^{2+}, the Ca^{2+}_e-dependent LTX-stimulated release occurs without Ca^{2+} influx but depends on the mobilization of Ca^{2+} from intracellular stores. (v) The Ca^{2+}_e-dependent release mechanism stimulated by LTX involves PLC. (vi) The Ca^{2+}-independent LTX action is based on the formation of a presynaptic membrane pore permeable to small substances. Future experiments will have to address the site at which Ca^{2+} acts to mediate the toxin-evoked exocytosis, and the nature of the non-selective pore induced by the toxin.

Materials and methods

Latrotoxin binding to synaptosomes and COS cells

LTX was purchased from Neurogen or purified from the venom of *Latrodectus lugubris tredecimguttatus* and iodinated according to the published method (Ushkarev and Grishin, 1986). Different concentrations of $[^{125}I]$LTX were mixed with purified rat brain synaptosomes, supplemented with 2 mM Mg^{2+} alone or with 2 mM Sr^{2+} or Ca^{2+} in buffer A (50 mM Tris, pH 8.0, 0.15 M NaCl). Controls included a 100-fold excess of unlabelled LTX. After an 8-min incubation, terminals were quickly separated from the unbound toxin by filtration through glass-fibre filters (GF/F, Whatman) and the radioactivity of filters was measured. Neurexin Iα (splice variant B/B/A/B) (Ulrich *et al*., 1995) was re-cloned into pcDNA3.1 (Invitrogen) and transfected (in parallel with latrophilin) into exponentially growing COS-7 cells using SuperFect reagent (Qiagen) as vehicle. The binding of iodinated LTX to harvested cells was measured under controlled conditions as described for synaptosomes. Mg^{2+} was present throughout all binding procedures to enable the comparison with release experiments.

Latrophilin isolation

Latrophilin was isolated from solubilized plasma membranes of rat brain synaptosomes by affinity chromatography on immobilized LTX as described (Davletov *et al*., 1996), except that the adsorption of proteins to the LTX column was performed in 2 mM Mg^{2+} with or without 2 mM Sr^{2+} or Ca^{2+}. Equivalent aliquots of eluted fractions were separated in 8% SDS–polyacrylamide gels, proteins transferred onto PVDF membrane and visualized using the respective antibodies and the Chemiluminescence Substrate System (Pierce).

LTX binding to rat brain sections.

Animals were sacrificed, the brains removed and rapidly frozen in isopentane cooled on dry ice. Cryostat sections (12 μm thick) were prepared from the unfixed tissue and thaw-mounted onto gelatin-coated slides. The sections were air-dried (10 min) and pre-incubated for 15 min in buffer A containing 2.5 mM $MgCl_2$ and either 2 mM Ca^{2+} or 10 mM EGTA. All solutions used subsequently contained either 2 mM Ca^{2+} or 10 mM EGTA. The sections were incubated with 1 nM $[^{125}I]$LTX for 40 min at 23°C and washed six times for 10 min at 4°C. Following a brief wash in cold water, the sections were fixed for 15 min at 23°C in 4% glutaraldehyde in buffer A, dipped in cold water and dried overnight at 4°C. The slides were exposed to Hyperfilm βmax (Amersham) for 24–56 h to obtain a linear film response to both high and low signals. For quantitative analysis, autoradiographs were digitized using a PowerLook II scanner (U-Max) and Photoshop software (Adobe). The Sigma software (Jandel) was used to determine the optical density in 30 areas on each autoradiograph corresponding to brain regions (Figure 2) and to perform statistical evaluation. A low, uniform non-specific labelling of control sections, incubated in the presence of 100 nM non-iodinated LTX, was subtracted from mean optical densities of experimental sections. To produce differential pictures, images obtained in the absence of Ca^{2+} were aligned with and subtracted from those in the presence of the ion, then combined with brain section outlines.

Synaptosomal release and uptake experiments

Synaptosomes were prepared from rat brains on a Ficoll (Pharmacia) gradient and resuspended in 5 ml of buffer B (132 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 2.5 mM $MgSO_4$, 10 mM D-glucose, 0.1 mM Na_2EDTA, 1.2 mM NaH_2PO_4 and 20 mM HEPES, pH 7.2). 1 ml of this suspension, containing 50 μM pargyline (Sigma) and 0.5 mM ascorbic acid, was then incubated with 2.5 μCi (6 nmol) of $[^3H]$NE (New England Nuclear Products) for 30 min at 37°C. After three washes with buffer B, 150 μl of these pre-loaded synaptosomes were mixed with 50 μl of stimulants, KCl, LTX or A23187 (Calbiochem), incubated for 0–20 min at 37°C and rapidly sedimented in a microcentrifuge. Aliquots of the supernatant were combined with scintillation liquid (Sigma) and counted in a scintillation counter. Before the addition of a stimulating agent, the terminals were treated for 5 min with U73122 or U73343 (0–50 μM), for 20 min with reserpine (50 μM) (Calbiochem), or for 2 h with BoNT C or D (100 nM, Research Biochemicals International). For experiments with intracellular Ca^{2+} inhibitors, synaptosomes pre-loaded with $[^3H]$NE were resuspended in buffer B containing 1 mM EGTA and 10 μM thapsigargin, 50 μM ryanodine or 100–400 μM BAPTA-AM, incubated for 30 min at 37°C, washed in buffer B with thapsigargin or ryanodine and exposed to 1–10 nM LTX. D-[2,3-^3H]Aspartate (2.5 μCi, 6 mmol, Amersham) was preloaded into synaptosomes for 5 min (McMahon and Nicholls, 1990) and its secretion measured as that of NE. Lysis of synaptosomes was assessed by monitoring release of lactate dehydrogenase using a cytotoxicity kit (Boehringer Mannheim) or by Western blotting of synaptophysin in the supernatant. Insignificant disintegration of synaptosomes (2–4%) did not progress after the first 2 min of LTX application. The influx of $^{45}Ca^{2+}$ into synaptosomes was measured using rapid filtration as described above for LTX binding studies. The incubation solutions contained 0.1–1.2 mM Ca^{2+} and 1–10 μM $^{45}Ca^{2+}$. The radioactivity of filters was determined by liquid scintillation spectrometry.

Diffusion of dye in frog neuromuscular junctions

Experiments were performed at 20–22°C on *cutaneus pectoris* muscles excised from male frogs (*Rana esculenta*). Isolated nerve muscle preparations were pinned in a Plexiglas chamber and equilibrated for 90 min in a Ca^{2+}-free Ringer solution (110 mM NaCl, 2.1 mM KCl, 5 mM HEPES pH 7.25) containing 1.8 mM MgCl and 2 mM EGTA. Nicotinic acetylcholine receptors were labelled with 3.75 μg/ml rhodamine-conjugated α-bungarotoxin (Molecular Probes) for 30 min in the same medium. The nerve terminals were then exposed for 10 min to 5 μM FITC or 5 μM FITC-conjugated dextran (M_r 4400) (Sigma), extensively washed and imaged to measure the background. The procedure was then repeated on the same terminals but in the presence of 1 nM LTX. If the toxin was not included during the second incubation with the dye, no labelling of terminals occurred. Experiments were conducted on nine NMJs from three nerve muscle preparations. FITC fluorescence was quantified in the same characteristic areas of a terminal, 10–90 min after washing the terminals exposed to the toxin. Preparations were viewed with a Zeiss Axioskop fixed-stage microscope equipped with epifluorescence (546±18 nm excitation and 590 nm emission for rhodamine; 450–490 nm excitation and 515–565 nm emission for FITC). Images were captured at a minimal intensity of excitation light using a

high-sensitivity CCD video camera (Jai 758; Datacell) and analysed with Image-Pro software (Media Cybernetics). Digital processing involved the subtraction of the background image.

Statistical analysis

All data are means (±SEM) of duplicate or triplicate measurements obtained in multiple independent experiments. Student's *t*-test was applied to verify the significance of observed changes.

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