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THE ROYAL SOCIETY

Norepinephrine exocytosis stimulated by α-latrotoxin requires both external and stored Ca^{2+} and is mediated by latrophilin, G proteins and phospholipase C

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α-latrotoxin (LTX) stimulates massive release of neurotransmitters by binding to a heptahelical transmembrane protein, latrophilin. Our experiments demonstrate that latrophilin is a G-protein-coupled receptor that specifically associates with heterotrimeric G proteins. The latrophilin–G protein complex is very stable in the presence of GDP but dissociates when incubated with GTP, suggesting a functional interaction. As revealed by immunostaining, latrophilin interacts with $G\alpha_{q/11}$ and $G\alpha_o$ but not with $G\alpha_s$, $G\alpha_i$ or $G\alpha_z$, indicating that this receptor may couple to several G proteins but it is not promiscuous. The mechanisms underlying LTX-evoked norepinephrine secretion from rat brain nerve terminals were also studied. In the presence of extracellular Ca^{2+}, LTX triggers vesicular exocytosis because botulinum neurotoxins E, Cl or tetanus toxin inhibit the Ca^{2+}-dependent component of the toxin-evoked release. Based on (i) the known involvement of $G\alpha_q$ in the regulation of inositol-1,4,5-triphosphate generation and (ii) the requirement for Ca^{2+} in LTX action, we tested the effect of inhibitors of Ca^{2+} mobilization on the toxin-evoked norepinephrine release. It was found that aminosteroid U73122, which inhibits the coupling of G proteins to phospholipase C, blocks the Ca^{2+}-dependent toxin's action. Thapsigargin, which depletes intracellular Ca^{2+} stores, also potently decreases the effect of LTX in the presence of extracellular Ca^{2+}. On the other hand, clostridial neurotoxins or drugs interfering with Ca^{2+} metabolism do not inhibit the Ca^{2+}-independent component of LTX-stimulated release. In the absence of Ca^{2+}, the toxin induces in the presynaptic membrane non-selective pores permeable to small fluorescent dyes; these pores may allow efflux of neurotransmitters from the cytoplasm. Our results suggest that LTX stimulates norepinephrine exocytosis only in the presence of external Ca^{2+} provided intracellular Ca^{2+} stores are unperturbed and that latrophilin, G proteins and phospholipase C may mediate the mobilization of stored Ca^{2+}, which then triggers secretion.

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

1. INTRODUCTION

α-latrotoxin (LTX), a protein of 130 kDa, is the main vertebrate-specific neurotoxin isolated from the venom of the black widow spider, *Latrodectus* sp. The toxin causes massive release of different neurotransmitters from nerve terminals of various types of neurons, as indicated by the dramatic increase in the frequency of miniature postsynaptic potentials (reviewed by Rosenthal & Meldolesi 1989). A primary target of LTX is the neuromuscular junction (NMJ) where the toxin apparently triggers exocytosis of only clear synaptic vesicles (Matteoli *et al.* 1988). However, LTX also stimulates secretion of peptides and catecholamines stored in large dense-cored vesicles of sensory neurons, endocrine and neuroendocrine cells (De Potter *et al.* 1997; Grasso *et al.* 1980; Meldolesi *et al.* 1983; Barnett *et al.* 1996). LTX does not normally gain access to the central nervous system *in vivo*, but brain neurons are also very sensitive to this toxin (Grasso *et al.* 1982; Nicholls *et al.* 1982; Meldolesi *et al.* 1984; Capogna *et al.* 1996). These observations suggest that LTX acts on a universal mechanism of secretion that is common for cells possessing regulated exocytosis.

The toxin exerts its action through interaction with a cell-surface receptor, which has been hypothesized to play a major role in the regulation of exocytosis (Rosenthal & Meldolesi 1989)). LTX binds to the receptor independently of Ca^{2+} and can stimulate release in the absence of this cation, provided other divalent cations are present (Misler & Hurlbut 1979; Rosenthal *et al.* 1990). This suggested that the receptor not only provides binding sites for the toxin but may also participate in signal transduction leading to facilitation of spontaneous exocytosis (Vicentini & Meldolesi 1984).

In the quest for the Ca^{2+}-independent LTX receptor, a novel neuronal protein has been found recently and

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termed latrophilin (Davletov *et al*. 1996). Latrophilin is a heptahelical transmembrane protein that belongs to the secretin/calcitonin family of G-protein-coupled receptors (GPCR: Krasnoperov *et al*. 1997; Lelianova *et al*. 1997). Most members of this receptor family bind biologically active peptides and regulate various secretory processes (e.g. Jelinek *et al*. 1993; Lin *et al*. 1992; Chen *et al*. 1993). Purified latrophilin binds LTX in the presence of any cation or in EDTA with affinity similar to that of the receptor found on synaptosomes (Tzeng & Siekevitz 1979; Meldolesi 1981; Davletov *et al*. 1996). Transfection experiments in pancreatic β-cell lines (Lang *et al*. 1998) and *in situ* binding studies to brain sections (Davletov *et al*. 1998) demonstrated that latrophilin is the main receptor and a mediator of LTX activity (see also § 3). Furthermore, COS cells transfected with latrophilin not only acquire the ability to bind LTX but also respond to stimulation with the toxin by increasing the production of cAMP and IP_3 (Lelianova *et al*. 1997). Thus, latrophilin appears to be suited to transducing the signal elicited by the toxin, ultimately leading to stimulation of exocytosis.

Despite these new findings, the precise mechanism of the process mediated by latrophilin remains unknown, and so we have attempted to decipher some of its pathways. Experiments were performed to demonstrate the association of latrophilin with G proteins and to determine the specific Gα subunit(s) involved in this interaction. The mechanisms underlying the LTX activity in brain nerve terminals were investigated using a pharmacological approach. Our data indicate that (i) $G\alpha_q$ and $G\alpha_o$ interact with latrophilin in a specific and functional manner; (ii) the toxin only causes exocytosis in the presence of extracellular Ca^{2+}; and (iii) to exert its Ca^{2+}-dependent activity, LTX also requires intact intracellular Ca^{2+} stores. These results suggest that receptor/G-protein-regulated intracellular Ca^{2+} stores may be involved in the control of exocytosis.

2. RESULTS

(a) *Latrophilin is coupled to G proteins*

Affinity chromatography of solubilized rat brain membranes on immobilized LTX affords a 25 000-fold purification of latrophilin. This procedure was used to study the association of latrophilin with G proteins. After extensive washes of the column with 0.2 M and 0.6 M NaCl, latrophilin is routinely eluted with 1 M NaCl. If chromatography is conducted in the presence of 20 μM Mg^{2+} and 2 mM GDP, a 40 kDa protein can be detected by Coomassie staining of the eluate fraction separated in a sodium dodecylsulphate (SDS) gel. This protein was shown to be a G protein α-subunit by Western blotting using an antibody that recognizes all α-subunits of heterotrimeric G proteins (figure 1). The band could also be stained with specific anti-$G\alpha_o$ and anti-$G\alpha_{q/11}$ antibodies but not with the antibodies directed at $G\alpha_i$ and $G\alpha_z$ (figure 1) or $G\alpha_s$ (not shown). To verify the specificity of the co-elution of latrophilin and the G protein(s) from the affinity column, a control column containing immobilized bovine serum albumin (BSA) was synthesized. When the BSA column was used in chromatography of solubilized brain membranes, no $G\alpha_{q/11}$ or $G\alpha_o$ could be detected in the eluate (not shown). Thus, these Gα-subunits seem to specifically interact with latrophilin bound to immobilized LTX.

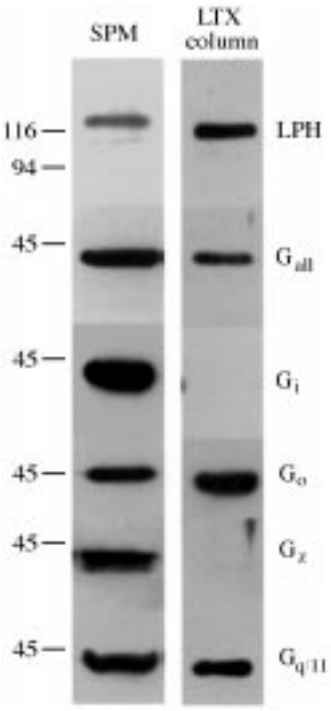

Figure 1. Heterotrimeric G proteins $G\alpha_{q/11}$ and $G\alpha_o$ co-purify with latrophilin using LTX affinity chromatography. Synaptosomal plasma membranes (SPM) were solubilized and loaded onto the LTX affinity column, which was then extensively washed and eluted with 1 M NaCl (see Davletov *et al*. 1996). Equivalent aliquots of SPM and the LTX column eluate were separated in a 10% SDS gel, transferred onto Immobilon membrane and immunostained with antibodies to latrophilin (LPH) or G protein α-subunits (as shown on the right). Numbers on the left denote the position of molecular weight standards.

To demonstrate further the specificity of this interaction, a second round of affinity chromatography was introduced. The eluate from the toxin column was loaded onto a wheat germ agglutinin (WGA) column and eluted with N-acetylglucosamine. Since G proteins are not glycosylated, they do not bind to the lectin column (not shown); however, if a G protein interacts with the glycosylated toxin receptor, it should be eluted from the WGA column in parallel with latrophilin. Indeed, as figure 2 (upper panel) demonstrates, elution of the G protein from the lectin column perfectly matched that of latrophilin. Furthermore, if during chromatography of brain proteins the LTX column was washed with GTP instead of GDP and then eluted with 1 M NaCl, much less G protein remained in the latrophilin fraction (figure 2, lower panel). Thus, excess of GTP is able to reverse the interaction of latrophilin with its requisite G protein(s), suggesting that this association is not only physical but also functional.

(b) *Exocytotic nature of the Ca^{2+}-dependent LTX-evoked secretion*

LTX has been shown to act both in the presence and absence of Ca^{2+} (Misler & Hurlbut 1979, Lang *et al*.

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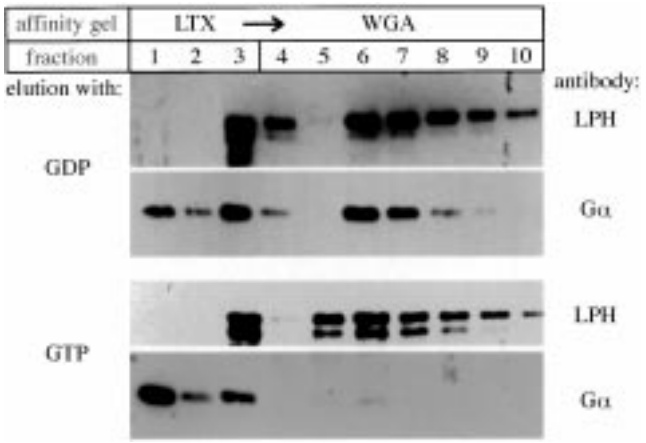

Figure 2. GDP stabilizes the G protein–latrophilin complex, whereas GTP reverses this interaction. The LTX affinity column was loaded with synaptosomal plasma membranes, washed and first eluted with 2 mM GDP or GTP (lanes 1 and 2, upper or lower panels, respectively) and then with 1 M NaCl (lane 3). This eluate was loaded on a second affinity column containing immobilized WGA, which was then washed and eluted with 100 mM N-acetylglucosamine (lanes: 4, flow through; 5, buffer wash; 6–10, consecutively eluted fractions). After electrophoresis and Western blotting, the protein bands were stained with antibodies against latrophilin (LPH) or Gα. Note that when the LTX column is eluted with GTP, much less G protein remains in the complex with latrophilin.

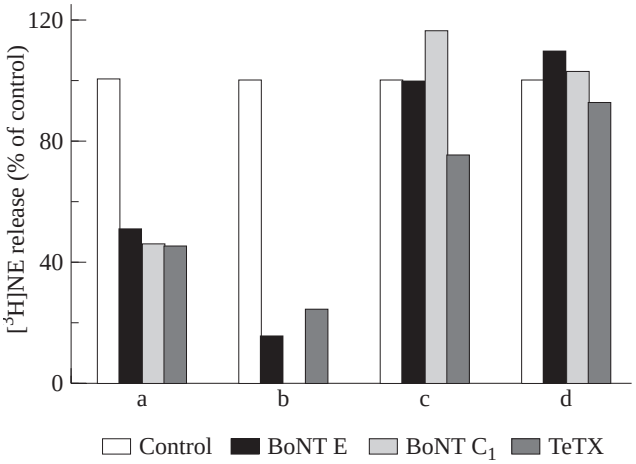

Figure 3. Clostridial neurotoxins block the Ca^{2+}-dependent LTX-evoked exocytosis of norepinephrine (NE) from rat brain synaptosomes but do not inhibit the Ca^{2+}-independent component of this release. a, total release of [^{3}H]NE after 5 min stimulation with 5 nM LTX in the presence of 100 mM Ca^{2+}; b, the Ca^{2+}-dependent component of the same release; c, the Ca^{2+}-independent component of the same release; d, the Ca^{2+}-independent LTX-stimulated release in the presence of 15 μM U73122. The amount of release in each case is expressed as a percentage of control. Empty bars, control experiments where no clostridial neurotoxins were used; filled bars, release from synaptosomes poisoned for 2 h at 37 °C with respective neurotoxins as shown below the graph.

1998). However, the role of this cation in the toxin's action remained unclear. Therefore, the nature of norepinephrine (NE) release triggered by LTX with or without Ca^{2+} was investigated using clostridial neurotoxins. These neurotoxins inhibit exocytosis by proteolysing different SNARE proteins that participate in vesicular docking and/or fusion (Sollner *et al*. 1993). Botulinum neurotoxins (BoNT) E and Cl and tetanus toxin (TeTX) were used to cleave SNAP-25, syntaxin (and SNAP-25) and synaptobrevin, respectively (Schiavo *et al*. 1993; Blasi *et al*. 1993; Foran *et al*. 1996; Schiavo *et al*. 1992). Each of these toxins greatly reduced the overall LTX-stimulated secretion of NE from synaptosomes in the presence of Ca^{2+} (figure 3, a). However, when the Ca^{2+}-dependent and Ca^{2+}-independent components of the LTX effect were analysed separately, only the Ca^{2+}-dependent component was found to be sensitive to the cleavage of the SNARE proteins (figure 3, b, c and d). Upon the treatment of synaptosomes with BoNT E or TeTX, LTX was able to evoke only *ca*. 15% and 25% of NE release it normally causes in a Ca^{2+}-sensitive manner, whereas BoNT Cl totally blocked any Ca^{2+}-specific LTX effect. In contrast, botulinum neurotoxins did not inhibit, and TeTX only partially reduced the effect of LTX observed in the absence of Ca^{2+} (figure 3, c). Furthermore, when the Ca^{2+}-dependent toxin-stimulated secretion was blocked by a specific inhibitor of phospholipase C (PLC, see below), the Ca^{2+}-independent LTX effect was still insensitive to clostridial toxins (figure 3, d). This result suggests that the Ca^{2+}-independent LTX-induced NE release may be non-vesicular because it does not depend on the intactness of the SNARE proteins.

(c) *Role of intracellular Ca^{2+} stores*

These findings indicated that Ca^{2+} plays a very important role in the LTX-triggered NE exocytosis. Since latrophilin interacts with $G\alpha_q$, a G protein known to participate in regulation of inositol-1,4,5-triphosphate (IP_3) production and thus in the mobilization of Ca^{2+} from intracellular stores (Selbie & Hill 1998), the involvement of such stores in LTX action was tested using several drugs that either inhibit phospholipases or deplete Ca^{2+} stores. In these experiments 100 μM extracellular Ca^{2+} were used because we found (Davletov *et al*. 1998) that this cation concentration is sufficient to cause a maximal response to LTX. Out of several drugs tested (figure 4*a*), only the specific inhibitor of phospholipase C, aminosteroid U73122 (1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino} hexyl)-1*H*-pyrrole-2,5-dione) specifically and completely blocked the Ca^{2+}-dependent release stimulated by LTX. A close homologue of this drug, U73343 (1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}-hexyl)-2,5-pyrrolydinedione) had no effect, nor did the other inhibitors of phospholipases A and C tested: ET-18-OCH3 (1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphorylcholine; Wu *et al*. 1998), AACOCF3 (arachydonyl-trifluoromethyl ketone; Bartoli *et al*. 1994) and manoalide (Bennett *et al*. 1987) (figure 4*a*). The inhibitory action of U73122 is partially reversed by higher [Ca^{2+}] (up to 1.2 mM). Importantly, U73122 affects only the Ca^{2+}-dependent LTX-evoked NE secretion (figure 4*b*). The drug did not inhibit the Ca^{2+}-independent effect of the toxin; this lack of inhibitory activity persisted even when the SNARE proteins were cleaved (figures 4*b*, 3*d*). In fact, release stimulated by LTX in the absence of Ca^{2+} was only slightly attenuated by manoalide, whereas none of the other drugs inhibited this release.

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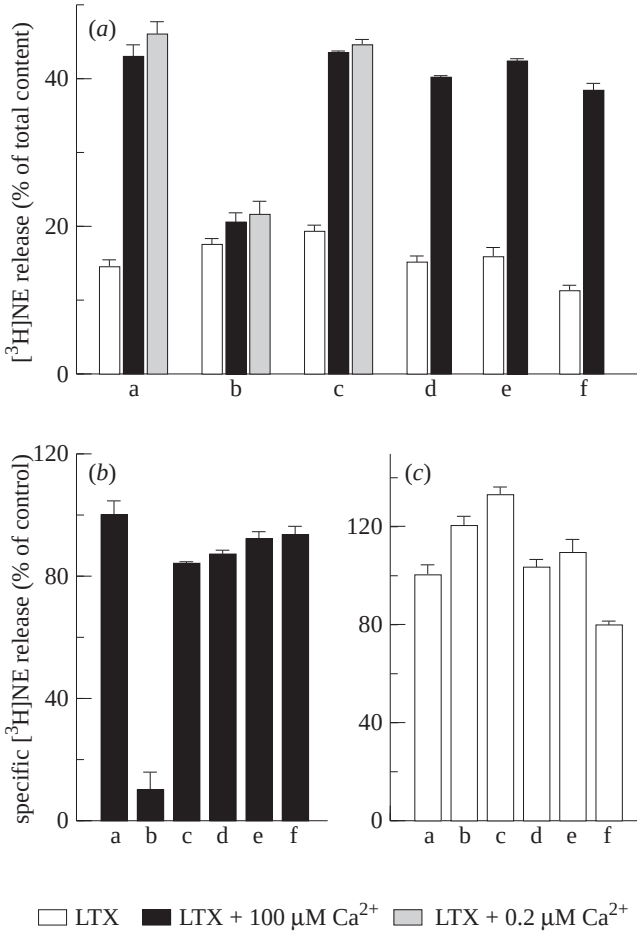

Figure 4. Only specific PLC inhibitor, U73122, blocks the Ca^{2+}-dependent NE release stimulated by LTX in synaptosomes. The following conditions were used: a, no inhibitors; b, 20 μM U73122; c, 20 μM U73343; d, 30 μM ET-18-OCH_3; e, 40 μM $AACOCF_3$; f, 10 μM manoalide. (*a*) [^{3}H]NE secretion (plotted as a percentage of its total synaptosomal content) was measured 5 min after stimulation with 5 nM LTX in the presence of 100 μM EGTA (open bars) or 100 mM (black bars) and 1.2 mM (hatched bars) free Ca^{2+}. (*b*) The Ca^{2+}-dependent and (*c*) Ca^{2+}-independent (right) components of LTX-elicited [^{3}H]NE secretion; data extracted from that in panel (*a*) and expressed as a percentage of release in control (no inhibitor present). Note that U73122 does not affect the Ca^{2+}-independent component of LTX-evoked NE release.

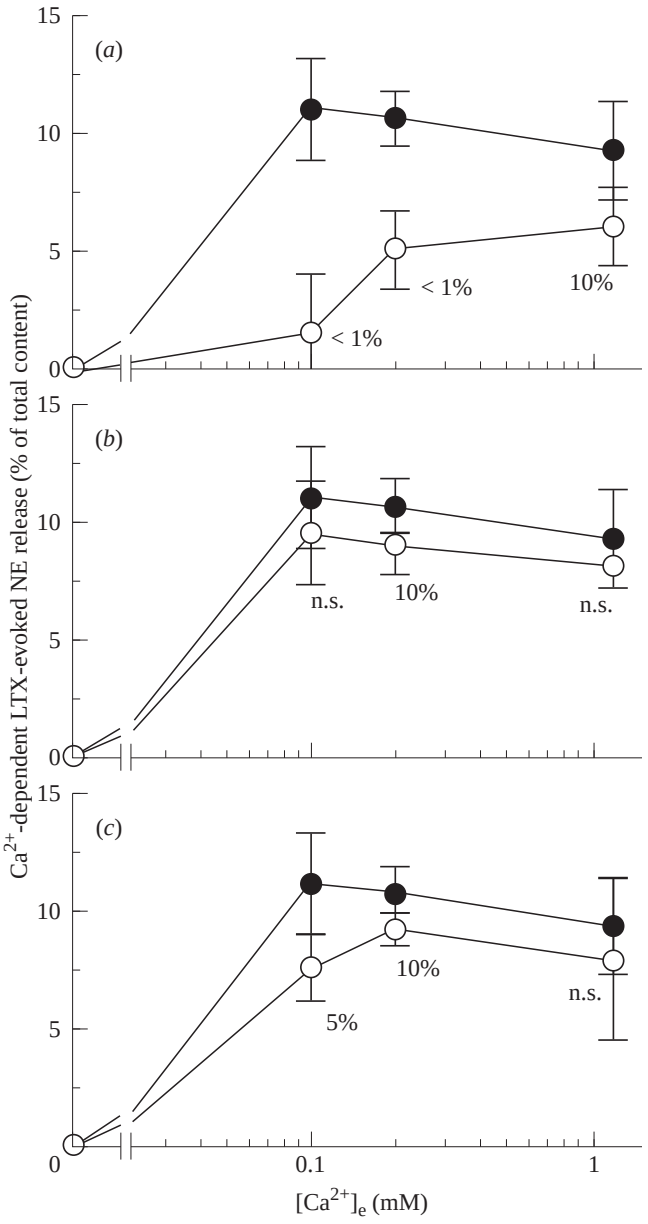

Figure 5. Effect of inhibitors of intracellular Ca2+ stores on the LTX-evoked [^{3}H]NE release and its dependence on $[Ca^{2+}]_e$. Filled symbols pertain to stimulated release in the absence of an inhibitor; open symbols show evoked secretion in the presence of 10 μM (*a*) thapsigargin, (*b*) 1.5 mM neomycinor, (*c*) 10 mM caffeine. The probability that the observed differences are insignificant (Student's criterion) is given near each value (n.s. no significant difference). Note that thapsigargin and, to some extent, caffeine inhibit the toxin's action at 100 μM Ca^{2+} and that higher $[Ca^{2+}]_e$ reverses these inhibitory effects.

Aminosteroid U73122 has been proposed to block intracellular Ca^{2+} release by inhibiting PLC (Smith *et al.* 1990; Jin *et al.* 1994) or by irreversibly inactivating G protein(s) involved in Ca^{2+} signalling (Thompson *et al.* 1991; Wu *et al.* 1998). Therefore, several other pharmacological agents were tested that are known to cause dissipation of Ca^{2+} from intracellular stores or inhibition of PLC: thapsigargin (Thastrup *et al.* 1990), neomycin (Lin *et al.* 1997) and caffeine (McPherson *et al.* 1991). Of these, only thapsigargin (figure 5*a*) potently inhibited the LTX-stimulated NE secretion in the presence of 100 mM Ca^{2+}. Increasing extracellular $[Ca^{2+}]$ tends to reverse the inhibitory effect of thapsigargin. Again, the Ca^{2+}-independent neurotransmitter release caused by LTX was unaffected (data not shown).

Thus, LTX stimulates mobilization of intracellular stored Ca^{2+} and requires external Ca^{2+} to exert this action. It is, however, possible that during the toxin application, extracellular Ca^{2+} enters the cytoplasm through the pores induced by LTX in the plasma membrane (Hurlbut *et al.* 1994). The entry of Ca^{2+} would activate PLC and produce a rise in the cytoplasmic Ca^{2+} concentration that would further stimulate exocytosis. If this is the case, then PLC inhibitors should be able to block the effect of Ca^{2+}. Indeed, U73122 strongly inhibited not only the LTX-evoked neurotransmitter secretion but also NE release triggered by high K^+ (figure 6; see also Carrasco *et al.* 1997).

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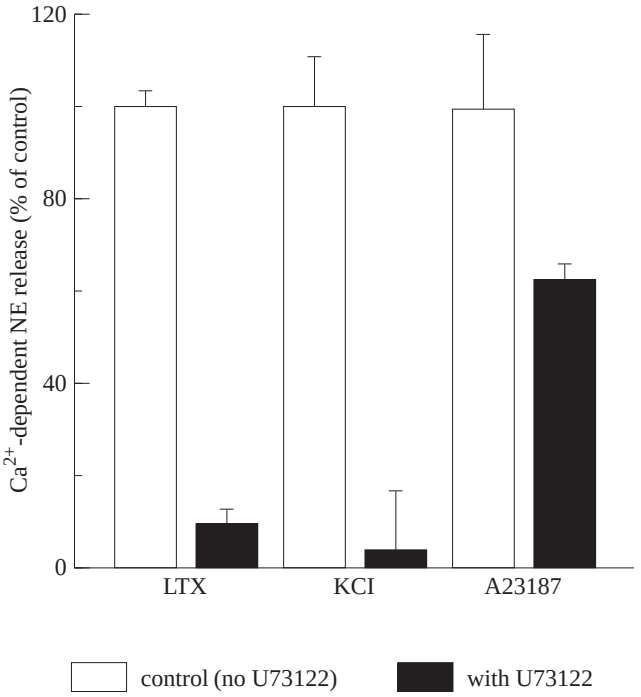

Figure 6. Comparison of the inhibitory effect of U73122 on NE release evoked in synaptosomes by different stimulants of secretion. Synaptosomes preloaded with [^{3}H]NE were stimulated in the presence of 1.2 mM Ca^{2+} by 5 nM LTX, 30 mM KCl or 0.5 µM Ca^{2+} ionophore, A23187. Data plotted represent only the Ca^{2+}-dependent component of secretion and are expressed as a percentage of control. Note the incomplete inhibition by U73122 of the ionophore-stimulated release.

However, when synaptosomes were stimulated using a Ca^{2+} ionophore, A23187, the aminosteroid was not as effective, suggesting that both LTX and K^+ act in a more specialized manner, possibly by stimulating the exocytotic machinery more locally.

(d) *Release in the absence of extracellular Ca^{2+}*

The experiments reported so far characterized the Ca^{2+}-dependent LTX-evoked NE release. However, these experiments did not reveal the nature of the Ca^{2+}-independent effect of the toxin. This component of the toxin's activity, which remained insensitive to any treatment (additional data not shown), was suggested to represent cytoplasmic leakage through pores induced by LTX. To directly demonstrate the existence of these LTX-operated pores, uptake of fluorescent dyes by nerve endings at the frog NMJ was monitored. As previously shown (Davletov *et al*. 1998), fluorescein isothiocyanate (FITC), but not its conjugate with dextran, can enter specifically nerve terminals only after stimulation with LTX. This effect occurs in the total absence of extracellular Ca^{2+}, i.e. when endocytosis is fully blocked (Ceccarelli & Hurlbut 1980; Henkel & Betz 1995). Here, the cytoplasmic, non-vesicular nature of this staining was confirmed by following the changes of fluorescence of the terminals loaded with FITC. Upon the removal of FITC from the bathing solution, the fluorescence of terminals quickly decreased (figure 7) in accordance with the kinetics of a simple diffusion process dependent only on the size of the membrane pores. About 70% of the initial fluorescence

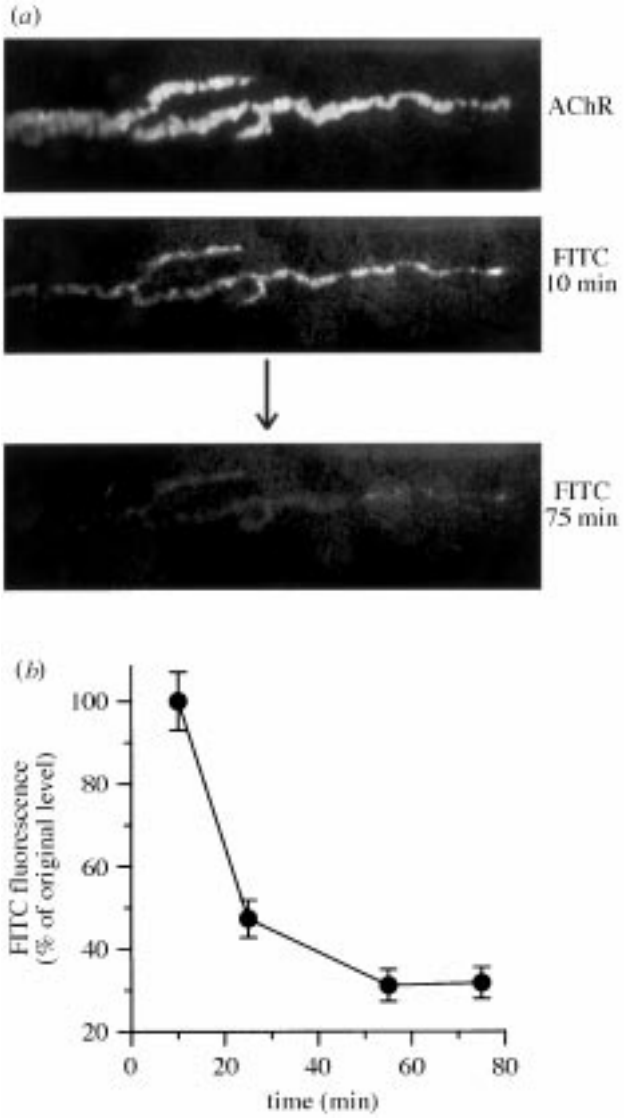

Figure 7. LTX opens large Ca^{2+}-independent pores in the presynaptic membrane of the NMJ. (*a*) Frog nerve–muscle preparations were incubated for 90 min in a buffer containing 2 mM EGTA and NMJs localized by staining with rhodamine-conjugated α-bungarotoxin (upper panel). The addition of 5 mM FITC and 1 nM LTX was followed by extensive washes for 10 min. FITC fluorescence was detected at 515–565 nm with a minimal-intensity excitation light (450–490 nm). Images were acquired immediately after the wash and 15, 45 and 65 min later. If LTX was excluded from the incubation buffer, no fluorescence of terminals was detectable. The fluorescence of terminals was quantified at several characteristic positions and plotted as a percentage of original fluorescence remaining as a function of time (*b*). Note the fast exponential decay of terminal fluorescence due to FITC diffusion out of the terminals.

dissipated within 50 min. This diffusion greatly resembled the efflux of cytoplasmic D-aspartate from synaptosomes upon stimulation with LTX previously reported in the absence of Ca^{2+} (Davletov *et al*. 1998) and was not due to the physical quenching of the dye, which in this case did not exceed 2–3% per 15 min. These results are consistent with the idea that LTX induces in the presynaptic plasma membrane non-selective pores that thereafter remain almost constantly open.

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3. DISCUSSION

LTX causes a massive increase in the frequency of spontaneous fusion events in the presynapse. This effect has been studied for more than 20 years, but its mechanism remains elusive. It is generally understood that a receptor on the presynaptic plasma membrane plays an important role in the toxin's action (Rosenthal & Meldolesi 1989). However, two different hypotheses have been proposed for the function of the receptor in the toxin-evoked release. According to the first hypothesis, LTX needs the receptor just to target the membrane at the right location. The toxin then makes pores in the plasmalemma, as it does in artificial lipid bilayers (Finkelstein *et al*. 1976), and causes influx of Ca^{2+} into the cytoplasm (Grasso *et al*. 1980; Nicholls *et al*. 1982), a process known to lead to exocytosis. The second hypothesis argues that the toxin causes substantial neurotransmitter release even in the absence of Ca^{2+}, provided other divalent cations are present (Rosenthal *et al*. 1990). Notably Mg^{2+}, which is unable to elicit exocytosis when injected through a pipette, can apparently replace Ca^{2+} in supporting the toxin's action (Misler & Hurlbut 1979). This indicates that ion fluxes through the LTX-induced channels may not be the main stimulus in the toxin-evoked release. Indeed, LTX can stimulate insulin secretion from pancreatic β-cells in the absence of any ion fluxes (Lang *et al*. 1998). Moreover, even when Ca^{2+} is present in the medium, it does not need to enter the LTX-sensitive cells to stimulate secretion (Capogna *et al*. 1996; Michelena *et al*. 1997; Davletov *et al*. 1998). These findings suggest that the toxin possesses a signalling function instead of, or in addition to, its channel-forming capability. Therefore, the receptor, apart from providing specifically localized binding sites, is likely to actively participate in the transduction into the cell of the signal elicited by LTX. The presynaptic localization of the LTX receptor and its apparent association with the mechanisms modulating exocytosis warrants great interest in the nature of this protein.

Attempts to isolate the toxin receptor resulted in the discovery of two different neuronal proteins: neurexin and latrophilin (Ushkaryov *et al*. 1994; Davletov *et al*. 1996; Krasnoperov *et al*. 1997; Lelianova *et al*. 1997). Both proteins bind the toxin in Ca^{2+}-containing solutions. However, neurexin will only bind LTX when Ca^{2+} is present, hereas, latrophilin will also interact with LTX in the absence of Ca^{2+} (Davletov *et al*. 1996). Neurexin Iα represents a family of heterogeneous cell-surface receptors homologous to laminin (Ushkaryov *et al*. 1994); its participation in signal transduction is unclear (Missler & Südhof 1998). Latrophilin, on the other hand, is a heptahelical membrane protein. Receptors, which possess this type of structure, have been shown to participate in the generation of various transmembrane signals. Latrophilin may be a GPCR because it physically binds the $G\alpha_o$ subunit of heterotrimeric G proteins and is involved in LTX-evoked upregulation of IP_3 and cAMP in transfected COS cells (Lelianova *et al*. 1997). Apart from that fact, little was known about the processes coupled to this receptor and, therefore, about the mode of LTX action.

In this study, we found that $G\alpha_q$, in addition to $G\alpha_o$, is linked to latrophilin (figure 1). Furthermore, the association of G proteins with latrophilin, although strong in the presence of GDP, is reversed by adding an excess of GTP. This indicates a functional interaction because GPCRs are known to bind G proteins in the GDP-bound (but not GTP-bound) state. We cannot exclude that latrophilin binds G proteins indirectly, via another protein that has not been detected on our gels. However, the ability of LTX to stimulate IP_3 production in latrophilin-expressing cells strongly suggests the existence of a functional link between the receptor and PLC. This link is usually provided by the direct coupling of GPCRs to the G proteins that regulate PLC (Selbie & Hill 1998).

The above hypothesis was confirmed in our experiments where U73122, an inhibitor of the membrane form of phosphatidylinositol-specific PLC, was found to block the LTX-stimulated NE release in synaptosomes (figure 4). This blockade was, however, limited to the secretion caused by the toxin in the presence of sub-millimolar extracellular Ca^{2+}, suggesting the involvement of PLC only in the Ca^{2+}-dependent effect of the toxin. This observation is consistent with the requirement of Ca^{2+} for the stimulation of PLC (Kozawa *et al*. 1987; Finch *et al*. 1991). The cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) by PLC produces IP_3, a second messenger that regulates Ca^{2+} release from intracellular stores. Such stores, which have been implicated in control of exocytosis in gonadotrophs (Tse *et al*. 1997), also play an important role in the toxin-evoked NE release because thapsigargin, by depleting the internal stores, strongly inhibits this secretion (figure 5).

LTX action seems to increase the sensitivity of the presynaptic machinery to Ca^{2+} (Davletov *et al*. 1998). This may be explained by receptor-mediated specific activation of a high-affinity Ca^{2+} sensor. The brain isoform of PLC is known to hydrolyse all phosphoinositides but its activity towards PIP_2 is maximal at micromolar Ca^{2+} (Kozawa *et al*. 1987). Therefore, this enzyme may constitute the high-affinity Ca^{2+} sensor for exocytosis. Interestingly, U73122 also blocks NE release stimulated by depolarization and partially inhibited secretion caused by a Ca^{2+} ionophore, A23187 (figure 6). Thus, PLC-mediated mobilization of Ca^{2+} from internal stores might underlie, at least in part, the mechanism of exocytosis evoked by different stimuli.

Another important conclusion from our experiments is that LTX stimulates SNARE-dependent exocytosis only in the presence of extracellular Ca^{2+}, whereas in the absence of Ca^{2+} a different secretion occurs that does not seem to require intact SNARE proteins (figure 3). One possible explanation of this phenomenon may be the ability of the toxin to open large pores in the plasma membrane (McMahon *et al*. 1990). These pores are specifically induced by LTX only in neuronal cells and are permeable to small dyes (figure 7), neurotransmitters and cations (Davletov *et al*. 1998; Hurlbut *et al*. 1994). Small cytoplasmic substances can quickly escape through these pores. Neurotransmitters from synaptic vesicles may first leak into the cytoplasm (by the reversal of the vesicular uptake system) and then exit through the pores. Alternatively, it is possible that the pores provide binding sites for synaptic vesicles, which then might not require the presence of intact SNARE proteins. It is known that the cleavage of SNARE proteins does not totally prevent the docking and spontaneous fusion of synaptic vesicles (Broadie *et al*. 1995). Total depletion by LTX of synaptic

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vesicles from the frog NMJ bathed in the absence of external Ca^{2+} (Matteoli *et al*. 1988) also speaks in favour of this hypothesis. Therefore, the mechanism of the Ca^{2+}-independent LTX-elicited secretion remains unresolved and will require further characterization.

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