α -latrotoxin triggers transmitter release via direct insertion into the presynaptic plasma membrane

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α -latrotoxin, a component of black widow spider venom, binds to presynaptic nerve terminals and stimulates massive neurotransmitter release. Previous studies have demonstrated that α -latrotoxin first binds to two high-affinity receptors on nerve terminals, neurexins and CLs (CIRLs and latrophilins), and then executes a critical, second step of unknown nature that stimulates neurotransmitter release. We now demonstrate that incubation of α -latrotoxin with synaptosomes at 0°C results in its peripheral membrane association. Incubation at 37°C, however, converts the toxin into an operationally integral membrane protein, and induces generation of a protease-resistant fragment that consists of the entire N-terminal domain of α -latrotoxin and becomes protease sensitive after lysis of synaptosomes. Our data suggest that α -latrotoxin inserts into the presynaptic plasma membrane after receptor binding, resulting in an intracellular location of the N-terminal sequences. Membrane insertion of the N-terminal domain of α -latrotoxin occurs spontaneously, independently of membrane recycling or transmembrane ion gradients. We postulate that α -latrotoxin acts intracellularly in triggering release, and propose that non-selective cation channels induced by α -latrotoxin may be a by-product of membrane insertion.

Keywords: black widow spider/ α -latrotoxin/membrane insertion/neurotransmitter release/synaptic exocytosis

Introduction

The venom of the black widow spider contains several homologous high molecular weight neurotoxins (Grishin, 1999). Most of these toxins act only on invertebrates, but a single toxin, α -latrotoxin, is specific for vertebrates (Knipper *et al.*, 1986). α -latrotoxin is a 130 kDa protein that binds to high-affinity receptors in nerve terminals, and causes massive stimulation of neurotransmitter release by exocytosis (Frontali *et al.*, 1976; Tzeng *et al.*, 1978). α -latrotoxin is synthesized initially as a precursor of ~160 kDa in the black widow spider, and is processed proteolytically in the venom gland at the N- and C-termini (Kiyatkin *et al.*, 1990; Dulubova *et al.*, 1996). The mature toxin consists of two major domains: an N-terminal domain that contains no striking characteristics apart from two hydrophobic sequences and an abundance of nega-

tively charged amino acids, and a C-terminal domain that is composed of 22 consecutive ankyrin repeats. α -latrotoxin forms dimers in solution (Petrenko *et al.*, 1993) that may assemble further into tetramers or higher order oligomers in the presence of divalent cations (Orlova *et al.*, 2000). Several other black widow spider toxins that only act on invertebrates were found to have very similar protein organization (Kiyatkin *et al.*, 1993; Dulubova *et al.*, 1996; Danilevich *et al.*, 1999).

The mechanism of action of α -latrotoxin has been enigmatic, although most studies suggest that the toxin is active in the absence of extracellular Ca^{2+} , and directly stimulates the secretory apparatus by an unknown mechanism (Gorio *et al.*, 1978; Ceccarelli and Hurlbut, 1980; Capogna *et al.*, 1996). Two classes of receptors for α -latrotoxin have been identified. Neurexins are neuron-specific cell surface proteins that function at least partly as cell adhesion molecules, and bind α -latrotoxin with high affinity in a Ca^{2+} -dependent manner (Ushkaryov *et al.*, 1992; Sugita *et al.*, 1999). CLs (CIRLs and latrophilins) belong to the G-protein-coupled receptor protein family but contain unusually large extra- and intracellular domains (Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997). Unlike neurexins, CLs are not brain-specific but are widely expressed in all tissues; they bind α -latrotoxin independently of Ca^{2+} (Sugita *et al.*, 1998; Ichtchenko *et al.*, 1999). Both classes of receptors are functional and probably contribute to α -latrotoxin action. Binding of the toxin to synaptic cell surface receptors is essential but not sufficient for action. Recent studies revealed that a mutant toxin containing a four residue insertion in the middle of the toxin (LTX^{N4C}) has the same high binding affinity for receptors as wild-type toxin, and even activates hydrolysis of phosphatidylinositides in synaptosomes similarly to wild-type α -latrotoxin, but is nevertheless unable to trigger release (Ichtchenko *et al.*, 1998). Expression of neurexins and CLs in PC12 cells greatly sensitizes them to α -latrotoxin (Bittner *et al.*, 1998; Sugita *et al.*, 1998). The same effect is also observed when deletion mutants of both receptors lacking intracellular sequences are expressed (Sugita *et al.*, 1998, 1999). The mutant receptors obviously are incapable of transducing α -latrotoxin signal from the cell surface, suggesting that activation of neurexins and CLs by α -latrotoxin does not by itself trigger release.

Binding of α -latrotoxin to synaptic membranes induces non-selective cation currents (Wanke *et al.*, 1986; Hurlbut *et al.*, 1994). Furthermore, purified α -latrotoxin at high concentrations spontaneously inserts into artificial lipid bilayers and forms channels, although with low efficiency (Finkelstein *et al.*, 1976). However, the toxin does not simply act in triggering neurotransmitter release as an ionophore for Ca^{2+} or Mg^{2+} , because it is active under conditions when ionophores that conduct divalent cations are unable to trigger release even at a 1000-fold higher

concentration (Khvotchev and Südhof, 1998). At high concentrations, α -latrotoxin possesses a cytolytic activity, and causes leakage of small molecules such as ATP or D-aspartate from the cytoplasm, a characteristic feature of many pore-forming, membrane-active proteins (McMahon *et al.*, 1990; Deri *et al.*, 1993).

α -latrotoxin is a truly fascinating toxin because of its unusual domain structure and action on the synaptic secretory apparatus. It is the only currently known toxin that directly attacks the secretory apparatus by stimulating it exhaustively; its actions are the mirror image of clostridial botulinum and tetanus toxins, which inhibit the same secretory apparatus (Link *et al.*, 1992; Schiavo *et al.*, 1992; Blasi *et al.*, 1993). As such, there is high hope that, similarly to the clostridial neurotoxins, identification of the molecular targets for α -latrotoxin will lead to significant insight into how exocytosis is mediated. In the current study, we explore the hypothesis that α -latrotoxin invades the interior of the synaptic nerve terminal after receptor binding. Using protection from proteolysis as a tool, we show that after binding, α -latrotoxin attached to synaptosomal plasma membranes undergoes a structural change that converts its N-terminal domain into a protease-resistant part. Our data imply that α -latrotoxin inserts into the plasma membrane, partially translocates into the presynaptic cytosol and then triggers neurotransmitter release by an intracellular action.

Results

Partial protection of α -latrotoxin from proteolysis dependent on pre-incubation with synaptosomes at 37°C

Previous studies have shown that binding of α -latrotoxin to its receptors on nerve terminals and activation of these receptors by the toxin are not sufficient to trigger neurotransmitter release (Ichtchenko *et al.*, 1998; Sugita *et al.*, 1998, 1999). This raised the possibility that α -latrotoxin gains access to the cytosol of the nerve terminal, and stimulates exocytosis by an intracellular action. Toxin translocation into the nerve terminal should render it partly or completely inaccessible to externally applied proteases. Therefore, we tested whether all or part of α -latrotoxin becomes protease-resistant when it is incubated with synaptosomes under conditions that promote stimulation of neurotransmitter release.

We iodinated recombinant α -latrotoxin and incubated it at 0°C for 30 min with Krebs-bicarbonate buffer either in the absence of synaptosomes (control; top panel in Figure 1), or in the presence of Percoll-purified synaptosomes (test; bottom panel in Figure 1). This incubation was performed to bind the toxin to the synaptosomes. Half of the samples were then transferred to 37°C for 30 min (37°C pre-incubation), while the other half was left at 0°C (0°C pre-incubation). These pre-incubations were performed because uptake of α -latrotoxin into the cytosol, be it by endocytosis or by membrane translocation, may require physiological temperatures and could be blocked at 0°C. After the 0 and 37°C pre-incubations, all samples except for controls (Figure 1, lanes 1 and 2) were digested with trypsin for 10 min at 37°C to proteolyse surface-exposed α -latrotoxin. The concentration of trypsin used was optimized in preliminary experiments with synapto-

Fig. 1. α -latrotoxin fragments protected from proteolysis as a function of pre-incubation with synaptosomes. 125 I-labeled α -latrotoxin (0.5 nM) was added on ice to Krebs-bicarbonate buffer without synaptosomes (top panel) or with synaptosomes (bottom panel). Buffers and synaptosome solutions contained either physiological concentrations of Ca^{2+} and Mg^{2+} (lanes 1–6), or were Ca^{2+} - and Mg^{2+} -free (lanes 7–10), and included 1 μM concanavalin A in lanes 5, 6, 9 and 10. Solutions were pre-incubated for 30 min at 0°C (lanes 1, 3, 5, 7 and 9) or at 37°C (lanes 2, 4, 6, 8 and 10), and then exposed to trypsin (0.2 mg/ml) for 10 min at 37°C (lanes 3–10). Total protein was TCA precipitated, and [125 I] α -latrotoxin in the precipitates was analyzed by SDS–PAGE and autoradiography. The positions of molecular weight markers are shown on the left, and the migration of intact α -latrotoxin (α -Ltx) and of its major proteolytic fragments (labeled A–E) is identified on the right. The degradation product that contaminates α -latrotoxin preparations and is degraded immediately by trypsin is marked by a white asterisk.

somes to completely degrade extracellular proteins but leave intracellular proteins intact (data not shown). After trypsin digestions, proteins were precipitated with trichloroacetic acid (TCA) and analyzed by SDS–PAGE and autoradiography (Figure 1).

The results show that without addition of trypsin, [125 I] α -latrotoxin was stable during the incubations (Figure 1, lanes 1 and 2). In the absence of synaptosomes, addition of trypsin quantitatively digested α -latrotoxin into small soluble fragments (Figure 1, bands E) under all incubation conditions. In the presence of synaptosomes, however, larger fragments of [125 I] α -latrotoxin emerged that were protected from proteolysis and remained bound to the synaptosomes. The pattern of protected proteolytic fragments varied dramatically with the pre-incubation temperature: in the 0°C pre-incubated sample, 130 kDa full-length α -latrotoxin (α -Ltx) was digested quantitatively, first to a 120 kDa fragment (band A), and then to 75 (band B) and 40–43 kDa fragments (band D) (Figure 1,

Fig. 2. Interaction of α -latrotoxin with synaptosomal membranes. Synaptosomes were reacted with ^{125}I -labeled α -latrotoxin (0.5 nM) at 0°C (lane 1), pre-incubated for 30 min at 0 or 37°C, and then lysed hypo-osmotically and washed with Krebs-bicarbonate buffer (lane 2) or with 0.1 M sodium carbonate (lanes 3 and 4). Alternatively, synaptosomes were first lysed and washed with Krebs-bicarbonate buffer to prepare synaptosomal membranes, and then incubated with ^{125}I -labeled α -latrotoxin at 0 or 37°C, followed by a second wash with Krebs-bicarbonate or sodium carbonate buffer (lanes 5–7). Equivalent amounts of the various samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Samples were analyzed by autoradiography for α -latrotoxin, and by immunoblotting for peripheral (rabphilin and munc18-1) and integral membrane proteins (Syt1 = synaptotagmin I and syntaxin).

lane 3). In the 37°C pre-incubated sample, a small amount of full-length α -latrotoxin was protected, and band A was missing (Figure 1, lane 4). The 75 kDa B-fragment was observed in the 37°C sample similarly to the 0°C pre-incubated samples. A striking difference between the 37 and 0°C pre-incubated samples, however, was that a new 55 kDa fragment (band C) emerged in the 37°C pre-incubated sample; this 'C fragment' was the most abundant protected fragment, and was completely absent in the 0°C pre-incubated samples (Figure 1, lane 4). Note that the [^{125}I] α -latrotoxin preparation used in the experiment shown in Figure 1 was contaminated with an endogenous proteolytic fragment of α -latrotoxin that is derived from the C-terminus of the toxin and presumably produced during purification of the recombinant protein (Figure 1, asterisks in lanes 1 and 4). The proteolytic fragment was present only in some of the recombinant α -latrotoxin preparations (e.g. see Figure 3 below), and was digested quantitatively during the trypsin incubations under all conditions.

Specificity of the partial protection of α -latrotoxin from proteolysis by pre-incubation with synaptosomes

The most plausible explanation for the partial protection of α -latrotoxin, especially of the C fragment, is that part of the toxin translocates across the synaptosomal plasma membrane. However, several potential artifacts could also have caused partial protection. For example, fragments of α -latrotoxin could be protected from proteolysis by the receptors to which the toxin is bound, or the massive increase in presynaptic membrane cycling induced by α -latrotoxin may trap parts of the toxin molecule inside the nerve terminal. To test these possibilities, we investigated proteolysis of the toxin under conditions that abolish the

Fig. 3. Fragment C is protected from proteolysis independently of protease cleavage specificity: effect of the 37°C pre-incubation time. Synaptosomes were reacted with ^{125}I -labeled α -latrotoxin (0.5 nM) on ice, pre-incubated at 37°C for the times indicated and exposed to trypsin (0.2 mg/ml), subtilisin (1 mg/ml) or pronase (0.2 mg/ml) for 10 min at 37°C. Digested synaptosomes were pelleted to remove unbound fragments of α -latrotoxin, resuspended in boiling SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography or immunoblotting as shown. Note that all proteases generate similar patterns of proteolytic fragments of α -latrotoxin which are identified by the letters A–D on the right; α -Ltx marks the position of full-length α -latrotoxin. The positions of molecular weight markers are indicated on the left.

stimulation of exocytosis by α -latrotoxin without affecting binding to its receptors.

Concanavalin A is a lectin that binds to carbohydrates on the α -latrotoxin receptors, neurexins and CLs, close to the membrane. Concanavalin A has little effect on the binding of α -latrotoxin to its receptors but inhibits neurotransmitter release stimulated by α -latrotoxin (Filippov *et al.*, 1990; Boehm and Huck, 1998; M.Khvotchev and T.C.Südhof, unpublished observation), possibly because it forms a physical barrier between the membrane and the toxin-binding sites on the receptors. When we pre-treated the synaptosomes with concanavalin A in regular Krebs-bicarbonate buffer, we found that concanavalin A completely blocked the generation of proteolysis-resistant fragments of [^{125}I] α -latrotoxin in the 0 and 37°C pre-incubated samples (Figure 1, lanes 5 and 6), suggesting that after receptor binding, α -latrotoxin undergoes interactions with the synaptosomes that are blocked by concanavalin A.

The concanavalin A result rules out the possibility that receptor binding by itself protects fragments of

α -latrotoxin from proteolysis, but does not address the hypothesis that synaptic vesicle exo- and endocytosis triggered by α -latrotoxin leads to an artifactual protection of part of the toxin from proteolysis. To address this hypothesis, we studied the effects of divalent cations. Similarly to concanavalin A, α -latrotoxin still binds to synaptosomal receptors in the absence of divalent cations (Mg^{2+} or Ca^{2+}), but is unable to release neurotransmitters (Misler and Hurlbut, 1979; Misler and Falke, 1987; Ichtchenko *et al.*, 1998; M.Khvotchev and T.C.Sudhof, unpublished observation). This was not due to structural damage to the synaptosomes in the absence of Mg^{2+} or Ca^{2+} because re-introduction of Ca^{2+} into the buffer after application of α -latrotoxin fully restored the ability of the toxin to stimulate norepinephrine release. Unlike concanavalin A, the absence of Ca^{2+} and Mg^{2+} had no effect on the pattern of proteolysis of α -latrotoxin in synaptosomes (Figure 1, lanes 7 and 8). Fragment C was protected from proteolysis even though no synaptic vesicle exocytosis was induced, indicating that partial protection from proteolysis is not due simply to the secondary effects of α -latrotoxin on membrane traffic. Furthermore, in the absence of Ca^{2+} and Mg^{2+} , concanavalin A was unable to block the protection of α -latrotoxin from proteolysis (Figure 1, lanes 9 and 10), presumably because concanavalin A requires divalent cations for binding to carbohydrates, and thus is inactive in the absence of Ca^{2+} and Mg^{2+} . In addition to demonstrating that stimulation of synaptic vesicle traffic is not required for the partial protection of α -latrotoxin upon interaction with synaptosomes, these results suggest that the block of the action of α -latrotoxin on neurotransmitter release in the absence of Ca^{2+} and Mg^{2+} occurs downstream of the interaction of the toxin with the synaptosomal plasma membrane.

In addition to the controls described above, we found that protection of the toxin was not changed when synaptosomes were incubated in the presence of high concentrations of ionophores, such as ionomycin, a divalent cation-selective ionophore, or gramicidin A, valinomycin and monensin that drastically increase membrane permeability to monovalent cations (data not shown). Substitutions of sodium with choline, or chloride with gluconate in the media were also without effect (data not shown). Together, these data suggest that after binding of α -latrotoxin to its synaptosomal receptors, the toxin undergoes a change that occurs equally at 0 and at 37°C and can be blocked by concanavalin A, followed by a further transformation that is dependent on the 37°C pre-incubation. The question then arises, what is the nature of the interaction of α -latrotoxin with synaptosomes that results in these temperature-dependent changes?

Pre-incubation at 37°C with synaptosomes transforms α -latrotoxin into an operationally integral membrane protein

In its receptor-bound state, α -latrotoxin presumably constitutes a peripheral membrane protein, which can be dissociated from the membranes by washing with alkaline buffers or chaotropic agents. If α -latrotoxin penetrated the plasma membrane after pre-incubation at 37°C, it should be transformed into an integral membrane protein that cannot be readily stripped from the membrane. To test this, we pre-incubated [^{125}I] α -latrotoxin with synaptosomes at

0 and 37°C, lysed the synaptosomes hypotonically and washed the synaptosomal membranes extensively with Krebs-bicarbonate buffer or 0.1 M Na_2CO_3 pH 11. The resulting samples were analyzed by SDS-PAGE and autoradiography for α -latrotoxin, and by immunoblotting for control proteins (Figure 2). As controls, we used peripheral membrane proteins, rabphilin and munc18-1, that should be washed off under denaturing conditions, and intrinsic membrane proteins, synaptotagmin I and syntaxin, that should not be washed off.

α -latrotoxin pre-incubated with synaptosomes at 0°C was not removed by washes with Krebs-bicarbonate buffer but was almost completely extracted by washing with carbonate buffer (Figure 2, lanes 2 and 3). A similar behavior was observed for the peripheral membrane proteins, rabphilin and munc18-1, while intrinsic membrane proteins were unaffected. When α -latrotoxin was pre-incubated at 37°C, however, it could not be washed off with carbonate buffer, although the treatment had no effect on the properties of rabphilin and munc18-1 (Figure 2, lane 4). Synaptotagmin I and syntaxin also remained unchanged, suggesting that the 37°C pre-incubation transforms α -latrotoxin into an operationally intrinsic membrane protein.

The proteolysis data in Figure 1 indicated that the conversion of α -latrotoxin into a partly protease-resistant form does not involve endocytosis since little full-length α -latrotoxin became protected. We therefore tested whether the conversion of α -latrotoxin into an operationally integral membrane protein requires intact synaptosomes, or can also be achieved with synaptosomal membranes. We lysed synaptosomes and resuspended the synaptosomal membranes in Krebs-bicarbonate buffer. The membranes were then pre-incubated with [^{125}I] α -latrotoxin at 0 or 37°C, washed with carbonate buffer at pH 11 and analyzed for [^{125}I] α -latrotoxin by SDS-PAGE and autoradiography (Figure 2, lanes 5–7). Again, we observed that the 37°C pre-incubation converted α -latrotoxin into an operationally integral membrane form that cannot be stripped off with alkaline buffer under conditions where control peripheral membrane proteins such as rabphilin and munc18-1 were washed off. Similar results were obtained when we used chaotropic 1 M sodium perchlorate as a stripping buffer (data not shown). Thus, incubation of α -latrotoxin at 37°C with membranes containing the appropriate receptors spontaneously converts the toxin into a form resembling an integral membrane protein. This conversion appears to be mediated only by the toxin once it is bound to the appropriate receptors since it can be observed in synaptosomal membranes, and a similar conversion was also obtained with COS cell membranes expressing recombinant α -latrotoxin receptors (data not shown).

Time and protease dependence of proteolytic protection of α -latrotoxin

The partial protection of α -latrotoxin from proteolysis (Figure 1) and its conversion into an operationally integral membrane protein (Figure 2) upon incubation with synaptosomes at 37°C suggest that part of the toxin may translocate into the synaptosomal plasma membrane. To explore this hypothesis, we tested if the proteolysis-resistant fragments, especially fragment C (Figure 1), are

dependent on the protease used, and what time of exposure to 37°C is required to produce fragment C (Figure 3). We employed three proteases (trypsin, subtilisin and pronase) that have very different cleavage specificity, using a relatively short time of protease digestion (10 min). However, in contrast to the first set of proteolysis experiments (Figure 1), we omitted in this and all following experiments the control without synaptosomes because no protected fragments were observed, and we pelleted the synaptosomes at the end of the experiment to separate bound protected fragments from released small peptides of [¹²⁵I]α-latrotoxin.

The results demonstrate that the overall pattern of digestion is very similar for the three proteases, resulting in the same three fragments (bands A, B and D) in the 0°C pre-incubated sample, and the emergence of fragment C only in the 37°C pre-incubated sample with a similar time dependence of pre-incubation (Figure 3). Virtually no fragment C was observed after 1 min of 37°C pre-incubation, but protection of fragment C was robust after 3 min, and almost complete for all three proteases after 10 min of pre-incubation. Fragment B was constant independently of the time of 37°C pre-incubation, while fragment D decreased at longer 37°C pre-incubation times for all three proteases. As evidenced by the control immunoblots for APP (a cell surface protein) and rab3A (an intracellular synaptic vesicle protein), the protease treatments efficiently destroy extracellular markers but leave intracellular proteins largely unaffected (Figure 3). These data demonstrate that the pattern of partial protection from proteolysis of [¹²⁵I]α-latrotoxin is independent of the substrate specificity of the protease used, with fragment C invariably generated only after a 37°C pre-incubation.

Fragment C is the only α-latrotoxin fragment that is inaccessible to external proteases

The generation of fragment C as a function of the 37°C pre-incubation correlates very well with the requirement for the 37°C pre-incubation for converting α-latrotoxin into an operationally integral membrane protein, suggesting that fragment C may correspond to a part of α-latrotoxin that becomes a component of the presynaptic active zone and thus permanently protected from external proteases. To address this question, we subjected α-latrotoxin bound to synaptosomes to extended trypsin proteolysis as a function of pre-incubation at 0 or 37°C in order to cleave exhaustively all surface-accessible proteins (Figure 4A).

Very short times of trypsin digestion (1–3 min) resulted in a ladder of multiple proteolytic fragments that was similar for the 0 and 37°C samples (Figure 4A, lanes 2–5). However, after only 3 min digestion, fragment C became abundant in the 37°C sample but not in the 0°C sample (lanes 4 and 5). The difference between the 0 and 37°C samples was more pronounced after longer incubations with trypsin, until finally, after digestions for 40 or 70 min, no protected fragments remained in the 0°C sample, while fragment C was the only major fragment retained in the 37°C sample (lanes 10 and 11). The apparent decrease in the amount of fragment C after extended proteolysis was probably due to partial damage to synaptosomes. These observations show, in agreement with the membrane-

Fig. 4. Fragment C is the only proteolytic product of α-latrotoxin in synaptosomes remaining after extensive trypsin digestion, but is degraded upon lysis of synaptosomes. (A) Synaptosomes were pre-incubated with [¹²⁵I]-labeled α-latrotoxin (0.5 nM) for 30 min at 0 or 37°C, and then digested with trypsin (0.2 mg/ml) at 37°C for 1, 3, 15, 40 or 70 min as indicated. After proteolysis, synaptosomes were pelleted by centrifugation, and only fragments associated with the synaptosomes were analyzed by SDS-PAGE and autoradiography. The typical proteolytic fragments of α-latrotoxin are identified on the right (letters A–D). The asterisk in lane 1 marks a degradation product that is present in the batch of α-latrotoxin used in these experiments but is completely digested by trypsin in all proteolysis samples. (B) Protected fragments of α-latrotoxin are completely degraded upon lysis of synaptosomes. Synaptosomes were pre-incubated with [¹²⁵I]-labeled α-latrotoxin (0.5 nM) for 30 min at 0 or 37°C, digested with trypsin (0.2 mg/ml) at 37°C for 10 min, and then recovered with the proteolytic α-latrotoxin fragments bound to the synaptosomal membrane (lanes 1 and 2). These synaptosomes were then lysed hypoosmotically in a solution either lacking (lanes 3 and 4) or containing trypsin (lanes 5 and 6), and incubated for an additional 10 min at 37°C prior to analysis by SDS-PAGE and autoradiography or immunoblotting.

binding properties (Figure 2), that the protected fragments of α-latrotoxin bound to synaptosomes at 0°C are only shielded transiently from proteolysis and can be accessed by trypsin, whereas fragment C generated after pre-incubation at 37°C is completely inaccessible to trypsin.

The question arises of whether fragment C is protected from proteolysis because it is translocated into the synaptosomal cytosol, and thus inaccessible from the outside, or because it becomes shielded from proteolysis by an artifactual process, for example by forming insoluble aggregates. To test this, we investigated whether the protected proteolytic fragments of α-latrotoxin can be made accessible to trypsin by lysing synaptosomes (Figure 4B). We first treated synaptosomes with [¹²⁵I]α-latrotoxin followed by 0 and 37°C pre-incubations and a short trypsin digestion essentially as described above, and then osmotically lysed the synaptosomes in the absence and presence of trypsin. Trypsin treatment of the synaptosomes resulted in the typical protected proteolytic fragments of α-latrotoxin (Figure 4B, lanes 1 and 2) that were not degraded any further by lysis in the absence of

trypsin (lanes 3 and 4). Lysis in the presence of trypsin, however, led to a complete degradation of all proteolytic α -latrotoxin fragments (lanes 5 and 6). This result thus demonstrates that the protected fragments are shielded by the integrity of the synaptosomes and not, for example, by an aggregation process. Since not only the C fragment but also the other fragments were degraded upon lysis of the synaptosomes, this result suggests that possibly the other, more labile fragments are also associated with a more transient translocation of the parts of the toxin to which they correspond.

Effect of trypsin digestion on synaptosome function

The protease protection experiments were designed to digest the synaptosomal surface selectively without significantly damaging the integrity and interior of the synaptosomes. This experimental design predicts that the synaptosomes should still function in release after proteolysis since their interior is intact. However, proteolysed synaptosomes may be unable to respond to α -latrotoxin after protease digestion since the surface receptors are digested. We therefore tested the effect of trypsin treatment on the ability of synaptosomes to release glutamate as a function of stimulation (Figure 5). Synaptosomes were loaded with [3 H]glutamate, treated with trypsin in a superfusion chamber and washed extensively with superfusion buffer. Afterwards, glutamate release was measured as a function of KCl depolarization, hypertonic sucrose or α -latrotoxin stimulation. The baseline glutamate efflux was increased slightly after trypsin treatment of the synaptosomes (asterisks in Figure 5A and B), presumably because trypsin caused limited damage of the plasma membrane. However, the secretory response of the synaptosomes to KCl depolarization or hypertonic sucrose was unimpaired; thus the synaptosomes were still functional after the trypsin digestion. The response to α -latrotoxin, in contrast, was abolished by the trypsin treatment, consistent with the notion that toxin binding to cell surface receptors was required for action (Figure 5A and B).

We next explored the effect of trypsin digestion on neurotransmitter release triggered by α -latrotoxin if trypsin is added after application of the toxin (Figure 5C). When a short pulse of α -latrotoxin is applied to nerve terminals, a 2–4 min lag period follows before neurotransmitter release reaches maximal levels. This lag period resembles the time of 37°C pre-incubation required for generation of fragment C (Figure 3). After the lag period, release continued for >10 min, even though no additional α -latrotoxin is applied. To test whether trypsin cleavage changes the stimulated release after toxin application, we superfused synaptosomes with a 1 min pulse of α -latrotoxin, followed by continuous addition of trypsin starting 10 s or 2 min after the toxin application (Figure 5C). Interestingly, trypsin had no major effect on continuing release of glutamate stimulated by α -latrotoxin, suggesting that once fragments of α -latrotoxin become protease resistant, they continue to trigger exocytosis even if the other parts of α -latrotoxin and its receptors are digested by trypsin. Note that the application of trypsin without a preceding pulse of α -latrotoxin induced a small and slowly developing efflux

Fig. 5. Glutamate release from synaptosomes treated with trypsin. (A) Effect of trypsin treatment on glutamate release stimulated by KCl depolarization or hypertonic sucrose. Synaptosomes loaded with [3 H]glutamate were superfused with Krebs-bicarbonate buffer containing trypsin (0.2 mg/ml for 5 min at $\sim 30^\circ\text{C}$), followed by a 5 min wash with normal Krebs-bicarbonate buffer. Glutamate release was then monitored from trypsin-treated and control synaptosomes as a function of stimulation with 30 s pulses of 25 mM KCl or hypertonic sucrose (0.5 M). There is a small elevation of basal glutamate release in trypsinized synaptosomes (indicated by the asterisk), but no difference in stimulated release. (B) Trypsin treatment abolishes exocytosis triggered by α -latrotoxin. Trypsinized and control synaptosomes prepared as described above were stimulated with α -latrotoxin (5 nM for 1 min) as indicated. Note that α -latrotoxin acts with an ~ 1 min delay, and its effect reaches maximum after 3–4 min, similar to the appearance of proteolytic fragment C in Figure 3. (C) Application of trypsin after α -latrotoxin is bound to synaptosomes does not affect the stimulation of glutamate release. Trypsin (0.2 mg/ml) was introduced into the superfusion buffer 10 s or 2 min after α -latrotoxin treatment (5 nM for 1 min). As a control, trypsin was added to synaptosomes that had not been exposed to α -latrotoxin (open squares), demonstrating that trypsin by itself induces a small leakage of glutamate (asterisk) which presumably accounts for the increase in the initial baseline release observed in trypsinized synaptosomes (asterisks in A and B).

of glutamate, presumably due to decreased stability of synaptosomal membranes after the digestion (asterisk in

Figure 5C). Similar results were obtained with more aggressive proteases, pronase and proteinase K, although non-specific leakage of glutamate was significantly larger with these enzymes (data not shown).

Impaired translocation of a mutant α -latrotoxin that does not trigger release

Previously, we described mutant α -latrotoxin generated by the insertion of four amino acids between the N-terminal domain and the 22 C-terminal ankyrin repeats of the toxin (Ichtchenko *et al.*, 1998). The mutant toxin, designated Ltx^{N4C}, was unable to trigger neurotransmitter release in synaptosomes but faithfully reproduced all other tested properties of wild-type α -latrotoxin, such as high-affinity binding to neuexins and CLs and stimulation of hydrolysis of phosphatidylinositides in synaptosomes. A plausible hypothesis would be that the mutant α -latrotoxin is inactive because its ability to interact with the membrane is impaired. To test this, we labeled wild-type and mutant α -latrotoxins with ¹²⁵I, pre-incubated them with synaptosomes at 37°C for increasing amounts of time, and digested them with trypsin as described above. To ensure identical specific activities of the toxins, we performed all labeling and purification steps in parallel using equal amounts of the proteins and the ¹²⁵I label. Incubation of both toxins with synaptosomes at 37°C without trypsin treatment produced no changes in the full-length toxin band (Figure 6A, lane 2 versus lane 1). This indicates that neither toxin is processed or covalently modified during the incubation at physiological temperatures, and that both toxins bind to synaptosomes equally well at 0 and 37°C. The pattern of trypsin-resistant fragments produced by the wild-type and mutant toxins in synaptosomes and the kinetics of their accumulation were identical (Figure 6A). Typical bands A, B, C and D described above were also present for Ltx^{N4C}. Accumulation of fragment C was prominent after 3 min and almost complete after 10 min of incubation at 37°C. However, the amount of protected fragments generated by trypsin was significantly less for the mutant compared with the wild-type toxin. This is evident from a quantitation of fragment C relative to the total amount of α -latrotoxin bound to synaptosomes, demonstrating that under identical conditions, protection of the mutant is only ~25% of that for the wild-type toxin (Figure 6B). When the synaptosomes were disrupted by sonication, however, both α -latrotoxins were largely degraded by trypsin, showing that the protection of fragments requires intact synaptosomes (Figure 6A, lane 8). Control blots for neuexins confirmed that the trypsin treatment effectively digested the surface of the synaptosomes. In contrast, rab3A/3C, an internal synaptic vesicle protein, was only digested after sonication (Figure 6A). These data are consistent with the notion that an activity of α -latrotoxin downstream of receptor binding cannot be executed as efficiently by the mutant toxin as by wild-type toxin, providing a rationale for the inactivity of the mutant toxin in neurotransmitter release.

Fragment C of α -latrotoxin corresponds to the N-terminal domain

The proteolysis of [¹²⁵I] α -latrotoxin as a function of 37°C pre-incubation identified a single fragment (band C) that was protected consistently, independently of the protease

Fig. 6. Relative resistance to proteolysis of wild-type (Ltx^{WT}) and mutant α -latrotoxin (Ltx^{N4C}) bound to synaptosomes. **(A)** Accumulation of trypsin-resistant fragments of wild-type and mutant α -latrotoxins as a function of pre-incubation at 37°C. Identical amounts of both α -latrotoxins were ¹²⁵I-labeled to the same specific activity, and bound to synaptosomes at 0°C. Synaptosomes were then pre-incubated at 37°C for the indicated times (0–30 min), and treated with trypsin (0.2 mg/ml) for 10 min at 37°C. The sample in lane 8 was sonicated before trypsin digestion. Samples were prepared and analyzed as described in Figures 1A and 5. **(B)** Time course of the accumulation of fragment C from wild-type and mutant α -latrotoxins in synaptosomes. Experiments described in (A) were analyzed on a PhosphorImager. The amount of fragment C was determined by integration of a rectangular area around the corresponding band and normalized for the total amount of ¹²⁵I-labeled α -latrotoxin bound to the synaptosomes (lane 1). Data are from a single representative experiment performed in quadruplicate.

used. To elucidate the part of α -latrotoxin from which this fragment is derived, we generated site-specific antibodies to different regions of α -latrotoxin. Three parts of the toxin were selected: the extreme N-terminus, the C-terminal half of the N-terminal domain and the C-terminal seven ankyrin repeats (Figure 7). We then pre-incubated synaptosomes with unlabeled α -latrotoxin at 0 and 37°C as described above, digested the samples with trypsin for 1 h and analyzed the resulting fragments by immunoblotting with the three site-specific α -latrotoxin antibodies that were affinity purified (Figure 7A). As a further control, we performed the trypsin digestions at 0 and at 37°C in order to exclude the possibility that the 37°C incubation for trypsin digestion in itself may convert the 0°C pre-incubated α -latrotoxin sample into a partially 37°C pre-incubated sample (Figure 7A).

Immunoblotting of the synaptosomes that contained no α -latrotoxin revealed that in spite of the affinity purification, the N-terminal antibody (T2946) cross-reacted with

Fig. 7. Mapping of the protected fragment C of α -latrotoxin to the N-terminus. **(A)** Immunoblot analysis of synaptosomes incubated without (lane 1) or with α -latrotoxin (lanes 2–6). Synaptosomes were pre-incubated with the toxin (8 nM) for 30 min at 0 or 37 $^{\circ}$ C, followed by a 1 h treatment with trypsin (0.2 mg/ml) at 0 or 37 $^{\circ}$ C as indicated. Samples were analyzed by SDS-PAGE and immunoblotting with the affinity-purified site-specific polyclonal antibodies whose epitopes are described in **(B)**. The names of antibodies used are boxed and shown on the right. Antibody T2946 recognizes endogenous 40 and 80 kDa brain proteins (labeled by asterisks) in addition to α -latrotoxin. The contaminating proteolytic fragment of α -latrotoxin that is present in the preparation used for the current experiment is recognized by antibody T2947, and is labeled by a white asterisk in the bottom panel. The positions of molecular weight markers are indicated on the left, and the typical proteolytic fragments of α -latrotoxin are identified on the right. **(B)** Schematic diagram of the structure of α -latrotoxin, the regions of the toxin used for the production of site-specific antibodies and its proteolysis-resistant fragments recognized by the antibodies. The residue numbers corresponding to the parts of α -latrotoxin used for the production of antibodies T2946, U987 and T2947 are depicted above the bar diagram. Antibody names are boxed and shown above horizontal bars indicating the positions of the corresponding antigens. Two hydrophobic segments found in the N-terminal domain of α -latrotoxin are marked HS1 and HS2. The first and last ankyrin repeats are marked R1 and R22. Proteolysis-resistant fragments of α -latrotoxin typically found in protease protection experiments are shown below the bar diagram with their names on the left and the approximate molecular weights on the right.

at least two major brain proteins of ~80 and 40 kDa. These proteins were largely protease resistant and presumably intracellular (Figure 7A, lane 1). In undigested samples, all three antibodies recognized α -latrotoxin (lane 2). The endogenous proteolytic product that contaminated this recombinant α -latrotoxin preparation reacted only with

the C-terminal antibody (asterisk in lane 2, Figure 7A). Fragment B was recognized by the C-terminal antibody only. When trypsin treatment was performed at 37 $^{\circ}$ C instead of less aggressive treatment at 0 $^{\circ}$ C, fragment B was completely digested in the 0 $^{\circ}$ C sample and greatly reduced in the 37 $^{\circ}$ C sample (Figure 7A, middle panel, lanes 5 and 6 versus lanes 3 and 4), in agreement with previous findings. Fragment D was recognized exclusively by antibody U987 generated against the C-terminal part of the N-terminal domain of α -latrotoxin (lane 3 and data not shown), and present only in the 0 $^{\circ}$ C sample digested with trypsin at 0 $^{\circ}$ C. Both antibodies from the N-terminal domain, however, reacted selectively with fragment C that was generated only in the 37 $^{\circ}$ C pre-incubated sample, independently of whether the trypsin digestions were performed at 0 or 37 $^{\circ}$ C (Figure 7A). These data demonstrate that the N-terminal domain of α -latrotoxin but not the ankyrin repeats becomes selectively protected from proteolysis after 37 $^{\circ}$ C pre-incubation with synaptosomes.

Based on this and previous experiments, we can now assign the observed proteolysis-resistant fragments to the defined structural domains of α -latrotoxin (Figure 7B). When synaptosomes were pre-incubated with the toxin at 37 $^{\circ}$ C, subsequent trypsin digestion resulted in two major products, fragments C and B. Their combined mol. wt, 55 and 75 kDa, adds up to that of the full-length toxin, suggesting that they are produced by cleavage of α -latrotoxin into two fragments rather than by independent proteolytic events. Since fragment C was recognized by the very N-terminal antibody T2946, it consists of the whole N-terminal domain of α -latrotoxin and may include the beginning of the ankyrin repeats domain (Figure 7B). Consequently, fragment B should represent the remaining 20 or so ankyrin repeats. The doublet D of 40–43 kDa is present in the 0 $^{\circ}$ C sample and almost completely absent after the pre-incubation at 37 $^{\circ}$ C. Fragment D is only recognized by antibody U987, generated against the C-terminal part of the N-terminal domain of α -latrotoxin, but not by the N- or C-terminal antibodies. It is likely that the same proteolytic site on the toxin molecule is used to generate resistant fragments B, C and D in the 0 and 37 $^{\circ}$ C samples, because fragment B is always present. Therefore, fragment D probably constitutes a truncated fragment C lacking ~100 N-terminal amino acid residues (Figure 7B). Band A, observed in the 0 $^{\circ}$ C sample, is a product of partial proteolysis of α -latrotoxin only at the N-terminus without digestion of the cleavage site on the boundary between the N- and C-terminal domains. Similarly, full-length α -latrotoxin, observed only in the 37 $^{\circ}$ C sample, presumably corresponds to partially translocated toxin with an undigested site on the boundary between the N- and C-terminal domains.

The N-terminal domain of α -latrotoxin becomes detergent insoluble after pre-incubation at 37 $^{\circ}$ C

What happens to the N-terminal domain of α -latrotoxin after it inserts into the presynaptic plasma membrane of the synaptosomes? The mode of membrane association as a function of pre-incubation temperature showed that the toxin is converted into a tightly membrane-associated form after the 37 $^{\circ}$ C pre-incubation (Figure 2), while the effect of proteolysis on α -latrotoxin-stimulated glutamate release suggests that the proteolysis-resistant parts of

Fig. 8. Interaction of α -latrotoxin with synaptosomal membranes and the insoluble cytoskeletal cytomatrix. Synaptosomes were pre-incubated with ^{125}I -labeled α -latrotoxin at 0 or 37°C, digested with trypsin and recovered by gentle centrifugation as described above. Trypsinized synaptosomes were sonicated vigorously and washed extensively with Krebs-bicarbonate buffer, 0.1 M sodium carbonate pH 11, 5 M urea, or 2% NP-40. Solubilized samples were centrifuged at 200 000 g, and pellets were analyzed by SDS-PAGE followed by autoradiography for ^{125}I - α -latrotoxin and by immunoblotting for the control proteins munc18-1, synaptotagmin I (SytI) and syntaxin.

the toxin may mediate the stimulation of release by α -latrotoxin (Figure 5). To explore the nature of the membrane association of the proteolysis-resistant fragments, we performed standard protease protection experiments with α -latrotoxin pre-incubated at 0 or 37°C. Digested synaptosomes were then separated from trypsin, sonicated vigorously to disrupt the plasma membrane and extracted extensively with Krebs-bicarbonate buffer, 0.1 M sodium carbonate pH 11, 5 M urea or 2% NP-40. Unsolubilized material was collected by high-speed centrifugation and analyzed for α -latrotoxin and control proteins (Figure 8). After sonication, all observed fragments remained bound to the insoluble compartment. Extraction with alkaline buffer or 5 M urea aimed at disrupting hydrophilic and ionic interactions resulted in significant dissociation of the fragments in the 0°C pre-incubated sample, in agreement with previously described results for unproteolyzed toxin (Figure 2). However, protease-resistant fragments in the 37°C sample were only partially extracted. This suggests that the conformational change observed in α -latrotoxin incubated with synaptosomes at 37°C involves not only fragment C but also fragment B. Extraction of sonicated synaptosomes with non-ionic detergents, such as NP-40, did not solubilize any of the resistant fragments significantly, indicating that these fragments are not simply bound to phospholipid membranes but are subject to additional interactions, possibly protein-protein interactions. Immunoblots for control proteins confirmed that the various extraction buffers efficiently solubilized abundant peripheral and intrinsic membrane proteins as expected (Figure 8). Similar results were obtained when synaptosomes were disrupted by hypotonic lysis (data not shown).

Discussion

α -latrotoxin induces massive and exhaustive exocytosis of secretory vesicles in neural and endocrine cells (Frontali *et al.*, 1976; Tzeng *et al.*, 1978; Grasso *et al.*, 1982). In

order to act, α -latrotoxin has to bind to cell surface receptors, but receptor binding in itself is not sufficient to trigger exocytosis (Ichtchenko *et al.*, 1998; Sugita *et al.*, 1998, 1999). α -latrotoxin induces the formation of non-selective cation channels in neurons (Grasso *et al.*, 1980; Meldolesi *et al.*, 1984; Wanke *et al.*, 1986), suggesting that it either directly forms a channel, or induces the opening of a pre-existing channel, although it is unclear which mechanism applies. The induction of cation channels by α -latrotoxin indicated that it may trigger neurotransmitter release as an ionophore, allowing Ca^{2+} to flow into synaptic terminals (Hurlbut *et al.*, 1994). However, the ability of the toxin to trigger neurotransmitter release cannot be explained by its ionophore action alone because α -latrotoxin stimulates exocytosis in the absence of external Ca^{2+} , provided that Mg^{2+} is present (Misler and Hurlbut, 1979; Misler and Falke, 1987; Capogna *et al.*, 1996). Under these conditions, α -latrotoxin probably causes Mg^{2+} influx, but this again cannot account for how α -latrotoxin triggers release because ionophores such as ionomycin that conduct Ca^{2+} and Mg^{2+} (Liu and Hermann, 1978) do not stimulate neurotransmitter release in the presence of Mg^{2+} when Ca^{2+} is absent (Khvotchev and Südhof, 1998). Together, the various observations suggest that Mg^{2+} influx alone does not trigger neurotransmitter release, and that α -latrotoxin probably performs a post-receptor activity that is distinct from channel formation and capable of directly activating the secretory apparatus.

In the present study, we have explored a previously ignored possibility, namely that α -latrotoxin gains direct access to the synaptic cytosol, either by endocytosis or by membrane translocation, in order to trigger exocytosis. To address this possibility, we have studied the binding and protease accessibility of α -latrotoxin incubated with synaptosomes under different conditions, and used site-specific antibodies to map the observed protease-resistant fragments of the toxin. Our results show that after receptor binding, α -latrotoxin inserts into the presynaptic plasma membrane, and translocates its N-terminal domain into the synaptic nerve terminal. This observation provides a mechanism of action for α -latrotoxin, and suggests an explanation for the channel activity of the toxin as a by-product of its translocation.

When α -latrotoxin is pre-incubated with synaptosomes at 0°C, it binds to the membranes as a peripheral membrane protein that is washed off by alkaline buffers or chaotropic agents (Figure 2). Subsequent trypsin digestion of the 0°C bound α -latrotoxin degrades most of the toxin (~70–80%) into small soluble fragments (bands E in Figure 1). However, a minority of the toxin (~20–30%) is partially protected from proteolysis and cleaved into three proteolysis-resistant fragments that remain bound to synaptosomes (bands A, B and D, Figure 1). These fragments are independent of the protease used, and are only observed when synaptosomes are present, suggesting that their protection depends on an interaction of the toxin with the synaptosomes (Figure 3). The protected proteolytic fragments are generated by sequential proteolytic steps in α -latrotoxin: initial clipping of the ~100 N-terminal amino acids to generate fragment A, and subsequent cleavage of the boundary between N- and C-terminal parts of α -latrotoxin to

generate fragments B and D (Figure 7). Extensive trypsin digestion degrades all of these fragments, indicating that they are all still surface accessible as would be expected from the peripheral nature of α -latrotoxin binding to synaptosomes at 0°C (Figures 2 and 4A). However, treatment of synaptosomes with concanavalin A prior to addition of α -latrotoxin does not significantly inhibit α -latrotoxin binding to its receptors but blocks protection of fragments A, B and D from proteolysis (Figure 1). Furthermore, lysis of synaptosomes abolishes the protection of fragments A, B and D from proteolysis (Figure 4B). Together, these results indicate that after binding to synaptosomes at 0°C, α -latrotoxin undergoes a conformational change that leads to protection of fragments A, B and D. This early conformational change requires synaptosomes, occurs at 0°C and is blocked by concanavalin A, but its precise nature is unclear. By analogy with other studied toxins, it is likely that α -latrotoxin oligomerizes on the membrane surface and interacts directly with the membrane, resulting in the appearance of proteolysis-resistant fragments (Bhakdi *et al.*, 1986), a view that is supported further by the ability of the toxin to form oligomers in solution (Petrenko *et al.*, 1993; Orlova *et al.*, 2000). The fact that the A, B and D fragments are only protected in intact synaptosomes but completely degraded upon lysis of the synaptosomes suggests that they may involve a transient and reversible insertion of parts of the toxin into the plasma membrane.

Warming α -latrotoxin bound to synaptosomes from 0 to 37°C results in a dramatic change in the nature of the association of the toxin with the synaptosomal membranes. At 37°C, α -latrotoxin is converted from a peripheral into an operationally integral membrane protein that is not dissociated by alkaline or chaotropic solutions (Figure 2). Trypsin proteolysis of the 37°C bound toxin uncovers a major new protected fragment, the ~55 kDa band C (Figures 1 and 3). As determined by epitope mapping, this fragment is comprised of the entire N-terminal domain of α -latrotoxin, and extends into the first ankyrin repeats (Figure 7). Fragment C includes the cleavage site after the first ~100 N-terminal residues that is the first site of proteolysis in the 0°C sample to generate fragment A; thus the shielding of this particular hypersensitive cleavage site is the major change upon warming α -latrotoxin bound to synaptosomes to 37°C. Strikingly, only fragment C withstands prolonged digestion by trypsin, whereas all other proteolytic fragments of α -latrotoxin produced in synaptosomes after pre-incubations at 0 or 37°C are proteolysed (Figure 4A). Furthermore, protection of fragment C is significantly decreased in a mutant α -latrotoxin (Ltx^{N4C}) that is unable to trigger neurotransmitter release (Figure 6). Together, these observations strongly imply that fragment C corresponds to a part of α -latrotoxin that inserts into, and translocates across, the plasma membrane at physiological temperatures to become inaccessible to proteases added from the outside. The fact that fragment C is completely degraded by trypsin after lysis of synaptosomes demonstrates that this fragment is not intrinsically resistant to proteolysis. Fragment C requires several minutes of pre-incubation at 37°C for its generation, a time course that is similar to the lag period required for α -latrotoxin to trigger maximal neurotransmitter release after binding to synapto-

somes. Once α -latrotoxin is bound to synaptosomes at 37°C, digestion of the surface-exposed parts of the toxin and its receptors does not interfere with stimulated release (Figure 5), suggesting that translocated fragment C mediates the release reaction since release is not stopped when the other parts of α -latrotoxin are digested by proteases.

Many bacterial protein toxins bind to the cell surface but perform their functions in the cytoplasmic milieu. Most of these toxins use endocytosis as a vehicle for intracellular delivery (Montecucco *et al.*, 1994), although some toxins translocate directly across the plasma membrane (e.g. CyaA toxin; reviewed in Ladant *et al.*, 1999). The following evidence argues against an endocytotic mechanism of α -latrotoxin translocation and suggests that it translocates directly across the plasma membrane: (i) pre-incubation of the toxin with synaptosomes at 37°C for 30 min, certainly sufficient time for endocytosis to occur, does not result in significant protection of full-length toxin from externally applied proteases; (ii) after the 37°C pre-incubation, α -latrotoxin assumes the properties characteristic of an integral membrane protein; (iii) incubations at 37°C with synaptosomal membranes alone confer on α -latrotoxin the properties of an operationally integral membrane protein, suggesting that the toxin needs only a membrane and a receptor for translocation; (iv) the same toxin domain (fragment C) becomes inaccessible to externally applied proteases independently of which protease is used, but can be degraded by proteases upon lysis of synaptosomes; and (v) as described above, α -latrotoxin induces non-specific ion channels or pores in membranes which may arise as a by-product when the toxin inserts into the phospholipid membrane at the cell surface. With these properties, α -latrotoxin to our knowledge is the first animal toxin that translocates directly across the plasma membrane into the cytosol. Significant conformational transitions were observed previously in bacterial toxins during membrane binding and pore formation (Shepard *et al.*, 1998). However, a conformational transition alone, such as assembly of protease-resistant structures on the membrane surface of synaptosomes, cannot underlie the observed partial protection of α -latrotoxin from proteolysis because such structures would not become protease-sensitive by simple lysis of the synaptosomes (Figure 4B). Furthermore, the similarity of the patterns of proteolytic fragments obtained with proteases with different cleavage specificity and the resistance of fragment C to extended proteolysis are also inconsistent with a conformational change alone.

Based on the observations described above, we would like to propose a model for α -latrotoxin action (Figure 9). The toxin first binds to presynaptic receptors on nerve terminals, leading to the targeting of α -latrotoxin to the presynaptic active zone. After binding, α -latrotoxin spontaneously undergoes a major conformational change, presumably via oligomerization and/or a direct interaction with the membrane. This occurs at 0°C, is blocked by concanavalin A and is attenuated in the mutant toxin Ltx^{N4C}. Upon elevation of the incubation temperature to 37°C, bound α -latrotoxin spontaneously inserts into the presynaptic plasma membrane and partially translocates across it. The N-terminal domain of α -latrotoxin contains two hydrophobic sequences that are conserved in all

Fig. 9. Model of α -latrotoxin action. The model proposes that α -latrotoxin is recruited to presynaptic membranes by binding two receptor families, neurexins and CLs. Binding is followed by an unidentified step, probably a conformational change, interaction with the membrane or multimerization which renders α -latrotoxin partly resistant to proteases. This step is blocked by concanavalin A and attenuated in the mutant toxin, Ltx^{N4C}. Both binding and conformational change occur efficiently at 0°C. The next step requires physiological temperatures and results in the complete translocation of the N-terminal domain of the toxin. Finally, the translocated domain exerts an unknown intracellular effect that causes massive and exhaustive exocytosis of synaptic vesicles. Both receptors are shown only for the initial step. CLs are omitted from the drawing in later steps for brevity, but are thought to function similarly to neurexins.

known latrotoxins at similar positions (Dulubova *et al.*, 1996). These sequences could interact with the membrane and function in the insertion and translocation of the toxin. It is striking that the N-terminal ~100 residues of α -latrotoxin, including one of the two hydrophobic sequences, are the first to be cleaved off in the 0°C pre-incubated samples but are completely protected in the 37°C samples, suggesting that the N-terminal residues are exposed in the 0°C pre-incubated samples and insert into the membrane upon warming to 37°C. We do not know whether insertion of the toxin only involves the N-terminal domain or other parts of the toxin as well, with a final state which protects only the N-terminal part from extensive proteolysis. Independently of which is correct, the end point of the insertion is that the entire N-terminal domain of α -latrotoxin, probably with at least two of the ankyrin repeats, becomes protected from proteolysis in a process that is inhibited at low temperatures. Finally, the internalized N-terminal part of the toxin exerts a direct action on the secretory machinery, resulting in the continuous fusion of secretory vesicles.

The overall mechanism of action proposed here for α -latrotoxin is not unique but resembles the behavior of other toxins that first bind to extracellular receptors and then act intracellularly (reviewed in Montecucco, 1998). However, our findings show that α -latrotoxin exhibits some intriguing and unexpected features that raise questions for future studies. The membrane insertion and partial translocation of an animal toxin is novel. The fact that the ankyrin repeats that constitute a major part of α -latrotoxin remain largely extracellular is surprising since these repeats are usually found only in intracellular proteins. If any part of α -latrotoxin had been predicted to act intracellularly, the ankyrin repeats would have been the logical choice. The targeting of the synaptic secretory apparatus is also an unusual feature. Based on our results, we therefore hypothesize that the translocated part of

α -latrotoxin acts intracellularly to remove a normally existing inhibition of exocytosis, allowing fusion to occur in the absence of Ca^{2+} . This action may be mediated by a direct effect of α -latrotoxin on the insoluble cytoskeletal cytomatrix of the active zone, as suggested by the fact that its N-terminal domain becomes insoluble to detergent or urea extraction after it is incubated with synaptosomes at 37°C (Figure 8). An alternative hypothesis would be that the N-terminal toxin domain induces fusion directly, possibly by exerting a direct fusogenic effect on phospholipid membranes (Lishko *et al.*, 1990). Independently of which hypothesis will be correct, identification of the part of α -latrotoxin that invades into nerve terminals now allows these questions to be addressed.

Materials and methods

Reagents

Production and purification of recombinant wild-type (Ltx^{WT}) and mutant α -latrotoxin (Ltx^{N4C}) were described previously (Ichtchenko *et al.*, 1998). Trypsin (type IX from porcine pancreas, 15.9 kU/mg), pronase (type XIV from *Streptomyces griseus*, 5.1 U/mg) and concanavalin A (type IV) were obtained from Sigma, subtilisin from Boehringer Mannheim, and radiochemicals from NEN DuPont or Amersham.

Preparation of synaptosomes and synaptic membranes

Synaptosomes from mouse or rat forebrain were prepared as described (Khvotchev and Südhof, 1998). All manipulations were performed at 0°C. The tissue was dissected and homogenized in a glass-Teflon homogenizer at 900 g in buffer A (0.32 M sucrose, 5 mM HEPES-NaOH pH 7.4, 0.1 mM EDTA). Homogenates were cleared by low-speed centrifugation (1000 g for 10 min) and centrifuged at 14 500 g for 20 min to obtain the crude synaptosome fraction (P₂). For neurotransmitter release measurements, the P₂ pellet from one mouse forebrain was resuspended in 3 ml of ice-cold gassed (95% O₂/5% CO₂) Krebs-bicarbonate buffer (composition in mM: 118 NaCl, 3.5 KCl, 1.25 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 5 HEPES-NaOH pH 7.4, 11.5 glucose) and used within 2–3 h. For protease protection assays, synaptosomes were purified further. The P₂ pellet was resuspended in 8.5% Percoll in buffer containing 0.25 M sucrose, 5 mM HEPES-NaOH pH 7.4, 0.1 mM EDTA, and layered on the top of a 12%/20% Percoll step gradient in the same buffer. After centrifugation at 18 000 g for 30 min, synaptosomes were recovered from the 12/20% Percoll interface. Percoll was removed by addition of 30 vols of buffer A and centrifugation at 18 000 g for 20 min. Synaptosomes (P₄) were resuspended in ice-cold Krebs-bicarbonate buffer or modified buffer (4 ml per brain). To prepare synaptosomal membranes, pelleted P₄ synaptosomes from one rat forebrain were lysed hypotonically by addition of 5 ml of 5 mM Tris-HCl pH 7.4 containing the protease inhibitor cocktail 'Complete' (Boehringer Mannheim), incubated at 0°C for 30 min and homogenized in a glass-Teflon homogenizer followed by centrifugation at 100 000 g for 40 min. The membrane pellet was washed again with the same buffer, and resuspended in Krebs-bicarbonate or modified buffer. In some experiments, synaptosomes were lysed by sonication (three pulses 20 s each).

Measurements of neurotransmitter release from synaptosomes

These were carried out as described previously (Khvotchev and Südhof, 1998). Synaptosomes were loaded with 140 nM [³H]glutamate (sp. act. 15 Ci/mmol), 66 nM [³H]γ-aminobutyric acid (GABA; 90 Ci/mmol) or 86 nM [³H]norepinephrine (70 Ci/mmol) for 5 min. Buffers for measurements of norepinephrine release additionally contained 0.4 mM ascorbic acid, 30 μM EDTA and 10 μM pargyline. Labeled synaptosomes (0.1 ml) were trapped on glass fiber filters (GF/B, Whatman), overlaid with 50 μl of a 50% Sephadex G-25 slurry, and superfused at 0.8 ml/min with Krebs-bicarbonate buffer at 35°C under continuous aeration with a 95% O₂/5% CO₂ gas mixture. In some experiments, Ca²⁺ and Mg²⁺ in Krebs-bicarbonate buffer were substituted with 0.1 mM EGTA and EDTA, respectively. After 10 min of washing, three 1 min fractions were collected to determine baseline release, and neurotransmitter release was stimulated by application of 25 mM KCl and 0.5 M sucrose for 30 s, or

5 nM of α -latrotoxin for 1 min. The amounts of neurotransmitters secreted into the superfusate and retained in the synaptosomes at the end of the experiment were quantified by liquid scintillation counting. The fractional release rate of neurotransmitters was calculated as the fraction of radioactivity released divided by the amount remaining on the filter at that particular time point.

Iodination of α -latrotoxin

α -latrotoxin was iodinated as described previously (Ushkaryov and Grishin, 1986). Briefly, 100 μ l of 400 nM wild-type or mutant α -latrotoxin were combined with 15 μ l of 1 M Tris-HCl pH 7.4, 10 μ l of Na¹²⁵I solution (1 mCi) and 10 μ l of freshly prepared chloramine T solution (20 mg/ml). The reaction was carried out for exactly 45 s at room temperature, and terminated by addition of 15 μ l of sodium thiosulfate (20 mg/ml) and saturated KI. Bovine serum albumin (BSA) was added to 0.1%, and unincorporated iodine was removed by rapid gel filtration on a 1 ml Biogel P6 spin microcolumn. Iodinated toxin was kept as a 100 nM stock solution in 100 mM Tris-HCl pH 7.4, 0.1% BSA at 0°C and used within 2–3 weeks.

Protease protection assays

Percoll-purified synaptosomes resuspended in normal Krebs-bicarbonate or modified buffer were incubated with unlabeled (8 nM) or iodinated (0.5 nM) wild-type or mutant α -latrotoxin at 0°C. Synaptosomes were then pre-incubated at either 0 or 37°C for the specified times. After pre-incubations at 0 or 37°C, proteases were added to the samples, which were then incubated further with the proteases at 37 or 0°C for the indicated times. In a typical experiment, 0.5 ml aliquots of synaptosomes in Krebs-bicarbonate buffer were incubated with α -latrotoxin at 0 or 37°C, trypsin was then added from a 50 $\times$ stock solution in Krebs-bicarbonate buffer (10 g/l), and digestions were carried out at 37°C for 10 min with occasional mixing of the samples. In some experiments, trypsin treatment was performed at 0°C for 1 h, or at 37°C for various amounts of time, or other proteases were used. After completion of all incubations, samples were placed on ice, phenylmethylsulfonyl fluoride (PMSF) was added to 1 mM, and synaptosomes were sedimented gently by centrifugation at 3000 g for 10 min at 0°C. Pellets were resuspended in 100 μ l of boiling SDS-PAGE loading buffer and separated by 10% PAGE followed by electrotransfer onto a nitrocellulose membrane. Experiments were analyzed by immunoblotting or autoradiography and phosphorimaging using conventional techniques. In experiments depicted on Figure 1, total sample protein was obtained by precipitation with 10% TCA, and analyzed as described.

Membrane experiments

Membranes were prepared from untreated synaptosomes or synaptosomes pre-incubated with iodinated α -latrotoxin at 0 or 37°C, and were resuspended in Krebs-bicarbonate buffer. Membranes from untreated synaptosomes were incubated with 0.5 nM iodinated α -latrotoxin at 0 or 37°C for 30 min. Aliquots of 0.5 ml of membrane suspension were removed to separate tubes and washed twice with Krebs-bicarbonate buffer; 0.1 M Na₂CO₃, 1 mM EDTA; 5 M urea, 25 mM Tris-HCl pH 8.0, 1 mM EDTA; or 2% NP-40, 25 mM Tris-HCl, 1 mM EDTA. Each washing step consisted of vigorous homogenization of pelleted membranes in the extraction buffer (1 ml total), incubation at 0°C for 30 min with occasional mixing and centrifugation at 100 000 g for 40 min at 0°C. Washed membranes were resuspended in 100 μ l of SDS-PAGE sample buffer, and analyzed by immunoblotting and autoradiography.

Production of antibodies

To raise site-specific antibodies against α -latrotoxin, three fragments of the toxin were expressed as a recombinant GST fusion protein. GST fusion protein expression vectors were constructed in the *Xba*I and *Sac*I sites of pGexKG vector by PCR with *Pfu* polymerase using the full-length α -latrotoxin cDNA (pRLtx4) as a template with the following oligonucleotides: pGexLtx236 (encoding residues E¹–D³³ of the mature toxin), oligonucleotides #1923 (GCCTCTAGACGAAGGAGAAG-ATTTAACTTTAGAGG) and #986 (GCGGAGCTCTTAATCGCCA-ATAATATTGGAAGC); pGexLtx2524 (encoding residues E²⁴⁸–L⁴³⁸), oligonucleotides #1925 (GCCTCTAGACGAACAATACTCTTTCCTG-GCTAACC) and #1924 (GCGGAGCTCTTATAGGTATGGTGTGA-TTTCGCCAAC); pGexLtxCys (encoding residues E⁹⁵¹–F¹¹⁷⁷), oligonucleotides #9924 (GCCTCTAGACGAAGAATGCGCAATCTCTCAT-TTTGC) and #1927 (GCGGAGCTCTTAAATTTTCCGCTTTT-TGTTTC). All constructs were verified by sequencing from the ends. GST fusion proteins were purified from BL21 cells using conventional methods and used to raise antibodies in rabbits (numbers T2946, U987

and T2947, respectively). Antibodies were affinity purified from sera essentially as described (Harlow and Lane, 1988).

Supplementary material

Supplementary material for this paper is available at *The EMBO Journal* Online.

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