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Reconstitution of the purified α-latrotoxin receptor in liposomes and planar lipid membranes. Clues to the mechanism of toxin action

H.Scheer, G.Prestipino[1] and J.Meldolesi

Department of Pharmacology, CNR Center of Cytopharmacology and Institute S.Raffaele, University of Milano, 20129 Milano, and [1]CNR Institute of Cybernetics and Biophysics, 16032 Camogli, Italy

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The receptor of α-latrotoxin (the major toxin of the black widow spider venom), purified from bovine synaptosomal membranes, was reconstituted into small unilamellar liposomes. These (but not control) liposomes exhibited high-affinity, specific binding of [^{125}I]α-latrotoxin. In the receptor-bearing liposomes α-latrotoxin induced depolarization and stimulated ^{45}Ca efflux. These responses to α-latrotoxin, that were observed only in the presence of external divalent cations, resembled those previously demonstrated in mammalian brain synaptosomes. The α-latrotoxin-activated ion fluxes are therefore, at least in part, the result of the direct interaction of the toxin with its receptor. When control and receptor-bearing liposomes were pre-incubated with α-latrotoxin and then added to a solution bathing a planar lipid bilayer membrane, single channel cationic conductances were observed. In the presence of the receptor, the conductances induced by α-latrotoxin were markedly different from those observed without the receptor, but not identical to those recently characterized by patch clamping in the cells of a line (PC12) sensitive to α-latrotoxin. These results demonstrate that the reconstituted receptor is functional, and suggest that the cationic channel activated by the toxin–receptor interaction is modulated by additional component(s) in the membrane of synapses and cells.

Key words: black widow spider venom/ion channels/membrane conductances/receptor/reconstitution

Introduction

α-Latrotoxin (α-LTx) is a neurotoxic protein of high mol. wt (130 000) which is purified from the venom of the European black widow spider, *Latrodectus mactans tredecimguttatus*. Nanomolar concentrations of α-LTx cause massive exocytotic release of a wide variety of neurotransmitters at central and peripheral synapses of vertebrates (Hurlbut and Ceccarelli, 1979; Frontali *et al.*, 1976; Tzeng *et al.*, 1978; Meldolesi, 1982; Grasso *et al.*, 1982). Specific, high-affinity binding sites for α-LTx, the α-LTx receptors, have been described in neurons (Tzeng and Siekevitz, 1979) where they are localized in the pre-synaptic membranes (Valtorta *et al.*, 1984) and in PC12 cells, a neurosecretory cell line which is toxin sensitive (Grasso *et al.*, 1982; Meldolesi *et al.*, 1983). Other tissues, be they secretory or excitable, contain no α-LTx receptors and are insensitive to the toxin (Hurlbut and Ceccarelli, 1979; Meldolesi, 1982).

Binding of α-LTx to its receptor in calcium-containing media induces a tetrodotoxin-insensitive depolarization of the plasma membrane and stimulation of a verapamil-insensitive calcium influx (Grasso *et al.*, 1982; Nicholls *et al.*, 1982). In calcium-free but divalent cation-containing media, binding of toxin, plasma membrane depolarization and release of neurotransmitter are still observed (Hurlbut and Ceccarelli, 1979; Misler and Hurlbut, 1979; Tzeng *et al.*, 1978; Meldolesi, 1982; Nicholls *et al.*, 1982; Meldolesi *et al.*, 1983). In divalent cation-free media, binding is no longer able to cause depolarization or release (Misler and Hurlbut, 1979; Nicholls *et al.*, 1982; Meldolesi *et al.*, 1983).

The precise mechanism(s) through which α-LTx induces its effects are still obscure. An interesting property of α-LTx is its spontaneous insertion across artificial lipid bilayers with the appearance of discrete, high conductance, cation-selective channels (Finkelstein *et al.*, 1976; Robello *et al.*, 1984). One previous hypothesis was therefore that the action of the toxin is due to its channel-forming activity (Finkelstein *et al.*, 1976). This model, however, leaves unexplained the strict tissue specificity of α-LTx action, which depends on the distribution of the α-LTx receptor.

We have recently purified the α-LTx receptor from bovine synaptosomal membranes using an α-LTx-containing immunoaffinity matrix. In SDS–polyacrylamide gel electrophoresis the purified protein is of high mol. wt (~200 000) and consists of two subunits (66 000 and 54 000). The purified protein still retains its property of binding α-LTx with an affinity similar to that observed in the native membrane ($K_A = 3 \times 10^{10} \times M^{-1}$) (Scheer and Meldolesi, 1985).

As the purified receptor was still biologically active with respect to its toxin-binding ability, we have undertaken reconstitutive experiments to determine the direct effects of α-LTx interactions with its receptor. Two experimental systems were investigated: small, unilamellar liposomes and planar lipid bilayer membranes. The effects of α-LTx on receptor-containing membranes were compared with those induced by the toxin in control membranes.

Results

Reconstituted liposomes were small, with variable diameter ranging from 40 to 120 nm (Figure 1). On the basis of experimental observations (retention of [^{14}C]sucrose throughout reconstitution) and theoretical considerations (Szoka and Papadjopoulos, 1981), the calculated internal volume was ~0.5 μl/ml of reconstituted liposomes. With the method used for preparation of liposomes no multilamellar structures were observed, there is therefore a clearly defined intra- and extraliposomal space. The total membrane surface area of a 1 ml liposomal preparation was calculated to be ~10^{10} μm^2.

Binding of [^{125}I]α-LTx to liposomal preparations was observed only in membranes reconstituted in the presence of the α-LTx receptor; membranes devoid of receptor exhibited no binding. At a final concentration of 0.2 nM α-LTx, specific binding of α-LTx was 1.41 ± 0.18 fmol/50 μl membranes (mean ± SEM; $n = 4$). Replacing Ca^{2+} with 2 mM Mg^{2+} or leaving divalent cations out of the medium had no evident effect on specific binding. Assuming that the binding affinity of the reconstituted receptor is the same as that of the membrane-bound and solubilized receptor ($K_A = 3 \times 10^{10} \times M^{-1}$), the density of receptors available for binding can be calculated to be ~70/μm^2, i.e. about two receptor molecules for every three liposomes.

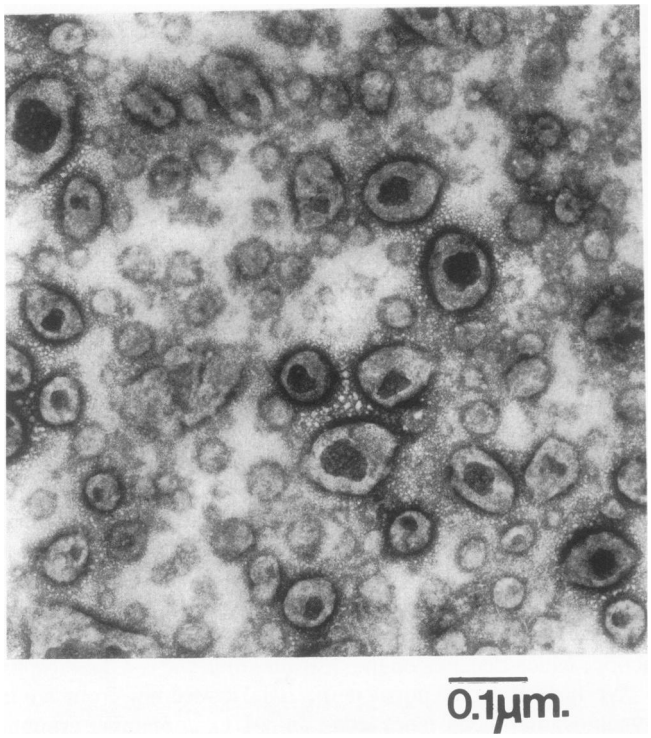

Fig. 1. Negative staining of reconstituted liposomes. 5 μl of membrane suspension was fixed on grid with an excess of 0.5% Mg uranyl acetate in veronal acetate buffer (pH 6) and examined with a Philips EM400 electron microscope. Magnification 100 000 ×.

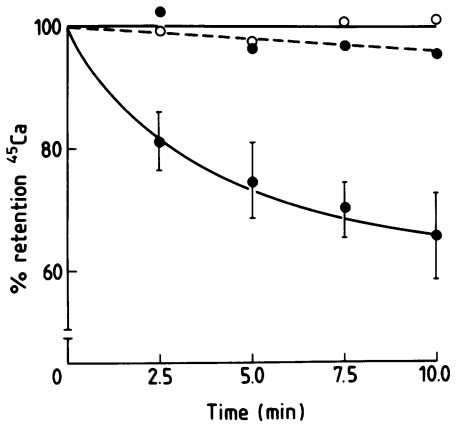

Fig. 2. Time course of ^{45}Ca efflux from liposomes treated with α-LTx. 100% = radioactivity present in untreated liposomes at zero time. ○: control liposomes (no α-LTx receptor), 2 mM $CaCl_2$; ●: liposomes reconstituted with α-LTx receptor, incubated with 2 mM $CaCl_2$ (—) or without divalent cations added, with EGTA, 0.5 mM (- - -). Concentration of α-LTx: 1 nM.

Flux experiments

α-LTx induced a ^{45}Ca efflux from liposomes reconstituted with the α-LTx receptor (Figure 2). The observed efflux was slow (~30% of ^{45}Ca released in 10 min) and dependent on the presence of divalent cations in the medium. Co^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+} (2 mM, added as chlorides) effectively substituted for Ca^{2+} in the external medium. In divalent cation-free medium α-LTx caused no ^{45}Ca release. Control (i.e. receptor-free) liposomes, either in the presence or absence of divalent cations, did not release ^{45}Ca in response to α-LTx (Figure 2). Experiments

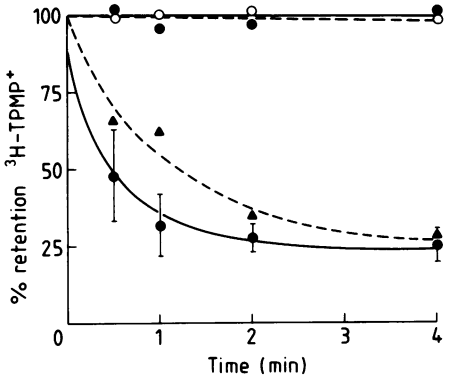

Fig. 3. Time course of [^{3}H]TPMP$^+$ efflux from liposomes treated with α-LTx. The distribution of [^{3}H]TPMP$^+$ monitors the membrane potential generated by the addition of valinomycin (1 μM) to liposomes ± α-LTx receptor, containing 0.1 M KCl + 0.5 mM $MgCl_2$ + 10 mM Hepes–NaOH buffer, pH 7.4, and suspended in 0.1 M NaCl with or without divalent cation, in the same buffer. 100% = radioactivity present in untreated liposomes at zero time. ○: control liposomes (no α-LTx receptor), 2 mM $CaCl_2$; solid symbols: liposomes reconstituted with α-LTx receptor, 2 mM $CaCl_2$ (—●—), 2 mM $MgCl_2$ (---▲---); no divalent cations added, 0.5 mM EGTA (---●---). Concentration of α-LTx: 1 nM.

designed to induce influx of ^{45}Ca were inconclusive due to the small intraliposomal space available.

Valinomycin, an ionophore highly selective for K^+, was used to generate potential across liposomal membranes. As measured by the accumulation of the lipophilic cation [^{3}H]methyltriphenylphosphonium (TPMP$^+$) and calculated by the Nernst equation (Bakeeva *et al.*, 1970), the membrane potential was slightly over 200 mV (217 ± 14 mV; n = 9). In receptor-reconstituted liposomes, α-LTx induced a rapid and extensive efflux of [^{3}H]-TPMP$^+$, i.e. a depolarization (Figure 3). This efflux was dependent on the presence of external Ca^{2+}. 2 mM Mg^{2+} could substitute for Ca^{2+}, although the rate and extent of efflux appeared reduced. α-LTx-induced depolarization was always incomplete, in contrast to the essentially complete efflux of [^{3}H]TPMP$^+$ with the external presence of 30 mM KCl (either with control or receptor-containing liposomes). The quantitative difference of the depolarization effects of α-LTx and high external K^+ suggests the existence of a subpopulation of liposomes insensitive to the toxin, consistent with binding data (see above). Control liposomes did not respond to α-LTx in any detectable manner (Figure 3).

Planar lipid membranes

Liposomes with or without α-LTx receptor were pre-incubated for 1 min with various concentrations of α-LTx, and then applied to the solution bathing the *cis* surface of planar lipid bilayer membranes. After the addition of the liposomes, fluctuations of current flowing across the membrane appeared. The rate of appearance of these fluctuations was decisively different with the two types of liposomes used. With receptor-containing liposomes, individual channel openings were recorded within 1 min after the addition of liposomes. Thereafter, the current flowing through the membrane increased rapidly, even when low concentrations of α-LTx were used, reaching high values within a few minutes (e.g. 2.5 nA at 90 mV, with an α-LTx concentration of 6.5×10^{-11} M in the *cis* chamber; Figure 4A). In contrast, long latencies (30–40 min) were observed after the addition of liposomes free of receptor, even though the concentration of α-LTx applied was greater (3.5×10^{-10} M). Compared with the results obtained with receptor-containing liposomes, the total current passing

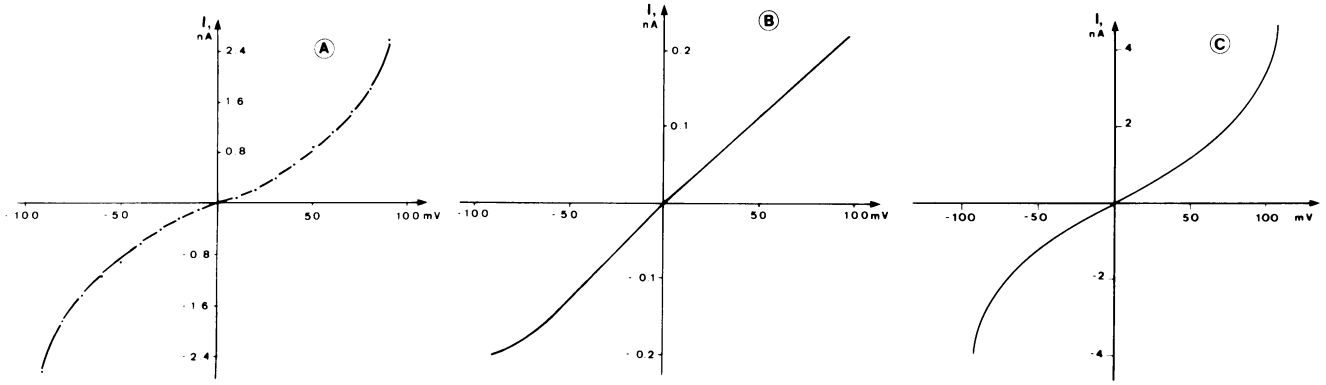

Fig. 4. I/V plot of currents induced in planar lipid membranes by α-LTx applied: **(A)** together with receptor-bearing liposomes (20 μl); **(B)** with control liposomes (20 μl); **(C)** alone, after application of receptor-bearing liposomes (20 μl) and washing. Triangular wave, 0.005 Hz. Concentration of α-LTx in the *cis* chamber: 0.065 **(A)**, 0.2 **(B)** and 0.1 **(C)** nM.

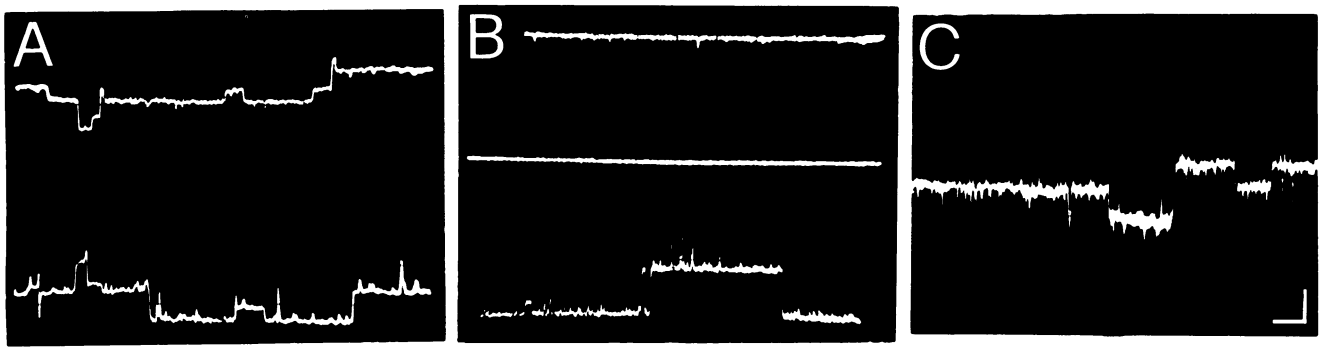

Fig. 5. Current fluctuations induced in planar lipid membranes by α-LTx applied as in Figure 4A–C. Voltage clamped at (from top to bottom) **A**: + and −90 mV; **B**: +90, 0 and −90 mV; **C**: +80 mV. Filter set: **A** = 50; **B** and **C** = 70 Hz. Horizontal bar = 1 sec; vertical bar = 20 pA. Concentrations of α-LTx in the *cis* chamber: 0.065 **(A)**, 0.2 **(B)** and 0.1 **(C)** nM.

through the membrane was always lower (~1/50 at −90 mV) (Figure 4B). The voltage dependencies of the currents induced by α-LTx in the presence or absence of receptor are plotted in Figure 4A and B. With toxin alone a rectification was observed at negative potentials > −70 mV. This phenomenon had already been observed by Robello *et al.* (1984) in cholesterol-containing membranes exposed to α-LTx. In contrast with receptor no such rectification was observed, but the current increase induced by voltage was greater than ohmic.

Figure 5A and B illustrates single current fluctuations induced in planar membranes by α-LTx + liposomes with or without receptor. Without receptor (Figure 5B) the fluctuations were infrequent, especially at positive voltages, indicating the persistent opening of the channels. At negative voltages the currents tended to decrease. The fluctuations induced by α-LTx in the presence of receptor were more frequent than those just described (Figure 5A). Channel openings and closings were seen not only at negative but also at positive potentials. Also the size of individual fluctuations induced in planar membranes by α-LTx with and without receptor was distinctly different. Without receptor, two major classes of negative conductance steps were observed, centered (at −90 mV) around 60–80 and 240 pS (Figure 6C). Under these conditions the average conductance ± SE of the steps was 159.6 ± 11.5 pS. With the receptor, on the other hand, large fluctuations were very rare, and almost symmetrical fluctuations were recorded, most of which centered around 65 pS (averages ± SE: 89.6 ± 8.6 and 75.6 ± 5.4 pS at − and +90 mV, respectively).

In the experiments described so far, the aqueous solution bathing the planar membrane contained both monovalent (NaCl, 0.1 M) and divalent ($CaCl_2$, 50 μM) cations. Attempts to withdraw Ca^{2+} from the solutions by the addition of an excess of EGTA were unsuccessful because they invariably led to the rupture of the membrane. Thus, the divalent cation dependency of α-LTx effects revealed by the experiments with liposomes reconstituted with the α-LTx receptor (Figures 2 and 3) could not be further demonstrated by parallel results in planar lipid membranes.

To test whether the marked differences between the total current elicited by α-LTx in receptor-bearing versus control liposomes was because receptor-containing liposomes were more fusible with the planar membrane, experiments were carried out in which receptor-bearing liposomes were added to the *cis* chamber for 20 min and subsequently washed out with 2.5 volumes of buffer prior to the addition of α-LTx. The results of these experiments were somewhat variable, also because in many cases the washing procedure led to the damage and rupture of the membrane. In some experiments (Figures 4C and 5C), however, the conductances induced by α-LTx application to the membranes first treated with receptor-bearing liposomes, and then washed, were indistinguishable in size, kinetics and voltage dependence from those observed in the membranes exposed to receptor-bearing liposomes pre-incubated with α-LTx.

In a separate series of experiments the effects of control and receptor-containing liposomes, but without added toxin, were investigated. With either type of liposome no changes in the conductances across the planar membranes could be observed. This result was obtained both when experiments were carried out in

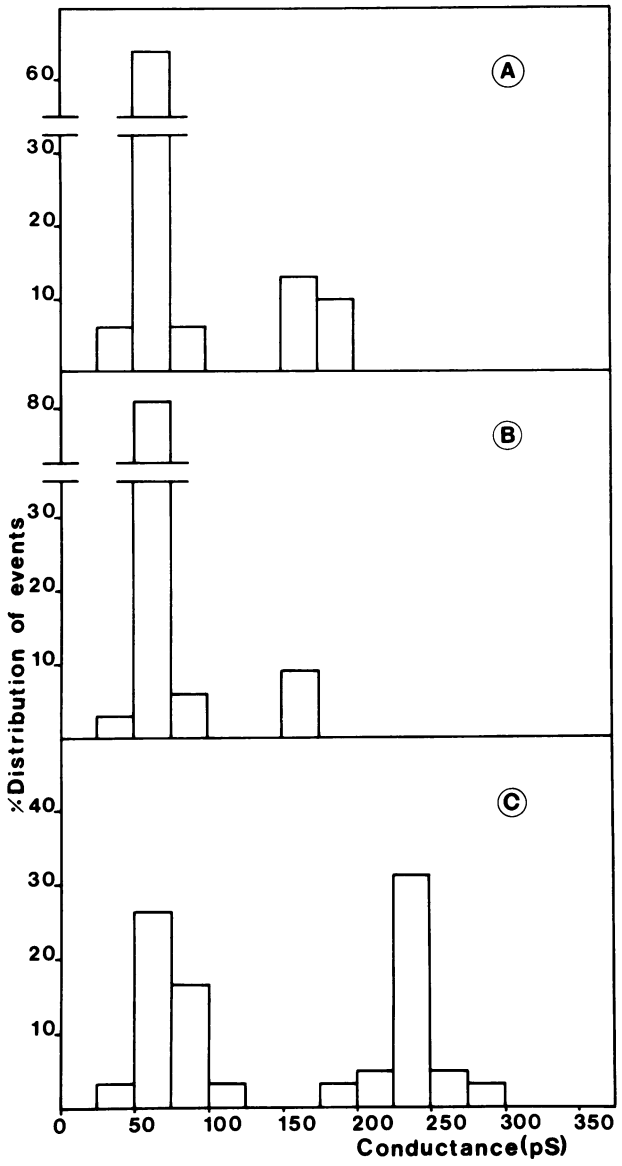

Fig. 6. Amplitude histogram of conductance fluctuations in voltage-clamped planar lipid membranes treated with α-LTx. **(A)** and **(B)** 0.065 nM α-LTx applied together with 20 μl of receptor-bearing liposomes, voltage clamped at − and +90 mV, respectively; **(C)** 0.2 nM α-LTx applied together with 20 μl of control liposomes, voltage clamped at −90 mV. Number of analyzed events: **A** = 30; **B** = 32; **C** = 60.

regular medium (see Materials and methods) or in the same buffer supplemented with either 10 mM $CaCl_2$ or 200 mM sucrose in the *cis* chamber, manipulations known to increase the extent of liposomal fusion with planar bilayer membranes (Cohen *et al.*, 1984).

Discussion

In a variety of cellular preparations (synapses of the central and peripheral nervous system; PC12 cells) α-LTx induces depolarization not inhibitable by tetrodotoxin and influx of Ca^{2+} not blocked by the organic Ca^{2+} channel blockers (Grasso *et al.*, 1982; Nicholls *et al.*, 1982; Meldolesi *et al.*, 1983). These effects of α-LTx appear to be due to the opening of a channel permeable to both mono- and divalent cations. In order to obtain information on the process(es) underlying the ion transport effects of α-LTx, we have reconstituted the purified α-LTx receptor into artificial membranes. Our previous studies had demonstrated that the purified receptor maintains the ability to bind α-LTx specifically and with high affinity. Here we show that the binding ability is also maintained after reconstitution, and that the reconstituted receptor is functional, i.e. it responds to the application of α-LTx by initiating ion fluxes similar in some respects to those elicited by α-LTx in intact cells and synapses. In small unilamellar liposomes containing the α-LTx receptor, two effects of the toxin could be mimicked: (i) collapse of the membrane potential imposed on the liposomes by valinomycin-induced K^+ efflux and monitored by following the distribution of [^{3}H]TPMP$^+$; (ii) stimulation of Ca^{2+} efflux. The difference in the rate of these two effects, as is evident by direct comparisons of Figures 2 and 3, is probably dependent on the different physicochemical features of the tracers employed, the different pathways they travel (diffusion through the lipid bilayer versus flux through a hydrophilic channel) and the influence of the membrane potential. The results reported here with reconstituted liposomes bearing the α-LTx receptor are similar to those previously reported in guinea-pig brain synaptosomal preparations (Nicholls *et al.*, 1982). In particular, depolarization was found to be absolutely dependent on the presence of divalent cations in the external medium, with Ca^{2+} more effective than Mg^{2+}. The similarity between the results in synaptosomes and in the reconstituted system strongly indicates that these toxin-induced ion fluxes are, at least in part, a direct and not a secondary effect of α-LTx interaction with its receptor.

The experiments with planar lipid membranes were complicated by the fact that α-LTx alone, when added to the solution bathing the membrane surface, becomes spontaneously inserted across the lipid bilayer and induces the appearance of large conductances (Finkelstein *et al.*, 1976; Robello *et al.*, 1984). In addition, our attempts at direct receptor reconstitution into planar membranes were unsuccessful. The receptors were thus first reconstituted into liposomes, these were pre-incubated with α-LTx and then applied to the planar membranes. Under these conditions clear differences were revealed by the parallel study of the membrane conductances induced by α-LTx applied with and without receptor. These differences can be classified into two groups. The first includes: (i) the latency of the appearance of current fluctuations, 10−60 s with receptor, 30−40 min without; (ii) the dependence of the fluctuations on the toxin concentration: without receptor, higher concentrations were needed; (iii) the total current amplitude which, at −90 mV, was ~50-fold greater in the presence of the receptor. These results are all explained by the high affinity of the α-LTx-receptor binding, that causes large numbers of toxin molecules to interact rapidly with the membrane. The second group of differences concerns the features of the α-LTx-induced current fluctuations. In particular, when the receptor was present: (i) fluctuations appeared not only at negative but also at positive voltages; (ii) they were more frequent and (iii) they were mostly of smaller amplitude.

In order to interpret these results it would be important to have precise knowledge of the nature of the interaction between liposomes and membranes. The results of our washing experiments demonstrated that such an interaction is stable. We can thus assume that, in the conditions of our experiments, some receptor-bearing liposomes fused with the planar membranes, and that this fusion accounted for the appearance of single-channel events. Consistent with this interpretation are the statistical results reported in Figure 6. Due to the rapid current increase occurring in the receptor-containing preparations, the sensitivity of single-channel recording remained adequate only for very short time (a few seconds). Our analyses were therefore carried out on a

small number of events. Nevertheless, the results obtained clearly showed that the large negative current fluctuations, typical of α-LTx alone, were very rare in the presence of the receptor, while the smaller fluctuations recorded were uniform (conductance centered around 65 pS) and apparently symmetrical.

At cells and synapses, the crux of the problem of α-LTx-induced cation fluxes concerns the nature of the channel involved, i.e. whether this channel is composed by a domain of the toxin molecule that gets inserted across the lipid bilayer after the binding of another domain to the receptor, or by component(s) of the membrane, most likely the receptor molecule itself. Up to now, no function other than toxin binding has been recognized for the α-LTx receptor. Addition to the solution bathing the planar membrane of liposomes bearing reconstituted receptor in the absence of added toxin was unable to cause the appearance of any channel activity in the membrane. While this finding is certainly not a final proof for the non-ionophoric nature of the receptor, it does not support this possibility. On the other hand, recent patch clamp studies in PC12 cells have revealed that the channel (permeable to Na^+, K^+ and Ca^{2+}) activated by α-LTx in this cellular target is small, ~15 pS in conductance (Wanke *et al.*, 1986). This value is much smaller than not only the large conductances activated by α-LTx alone, but also the 65 pS fluctuations that predominate in the presence of the receptor. Whatever the nature of the channel, it appears that its features in the plasma membrane of the cellular targets of α-LTx are different from all conductances investigated in the reconstituted system, and might therefore result from more complex interactions between the toxin, the receptor and its immediate environment, possibly involving other components of the plasma membrane. The work that we have initiated needs to be further developed in order to yield a closer reconstruction of the ionic events triggered in the plasma membrane of sensitive cells and synapses by the interaction with α-LTx.

Materials and methods

α-LTx was purified from homogenates of spider venom glands as previously described (Frontali *et al.*, 1976). The α-LTx receptor was purified from Triton X-100-solubilized bovine synaptosomal membranes as described elsewhere (Scheer and Meldolesi, 1985), except that the dialysis medium used to remove urea was 0.1M KCl, 0.5 mM $MgCl_2$ and 10 mM Hepes−KOH (pH 7.4). [^{3}H]$TPMP^+$; (specific activity 37 Ci/mmol), ^{45}Ca (specific activity 10 mCi/mg) and [^{3}H]1,2-dipalmitoyl-L-3-phosphatidylcholine (specific activity 55 Ci/mmol) were purchased from Amersham International, UK. Soybean L-α-phosphatidylcholine (Type II-S; Sigma) was purified according to Kagawa and Racker (1971) prior to use. Cholesterol and valinomycin were obtained from Sigma. All other materials were reagent grade. Unless otherwise stated, all operations were carried out at 0°C.

Reconstitution

700 μl of the purified receptor (0.5 pmol) was added to a 300 μl solution containing (final concentrations) soybean L-α-phosphatidylcholine (5 mg/ml), cholesterol (6.5 μM), d-octyl-β-D-glucopyranoside (10 mM), Triton X-100 (2%), KCl (0.1 M), $MgCl_2$ (0.5 mM) and Hepes−KOH (10 mM; pH 7.4). The Triton X-100 was removed by addition of 0.3 ml Biobeads SM-2 (Biorad) and an overnight incubation on a rotating apparatus. Following removal of beads, a second incubation with Biobeads SM-2 was carried out for another 6 h. The concentration of Triton X-100 remaining was non-detectable (<0.05%) as judged from spectrophotometric measurements (280 nm). Subsequently, preparations were extensively dialyzed: first against 0.1 M KCl, 0.5 mM $MgCl_2$ and 10 mM Hepes−NaOH (pH 7.4) followed by 0.1 M NaCl and 10 mM Hepes−NaOH (pH 7.4). Control liposomes were prepared in parallel, except that the initially added 700 μl solution contained no receptor.

Binding of α-LTx

α-LTx was iodinated by the Bolton−Hunter method as previously described (Meldolesi *et al.*, 1982) to a specific activity of 2 Ci/μmol. Binding of [^{125}I]α-LTx was determined by the addition of 50 μl of liposomes (^{3}H-labeled by inclusion of [^{3}H]phosphatidylcholine in the reconstitution mixture) to 50 μl of 0.1 M NaCl, 10 mM Hepes−NaOH (pH 7.4), 2 mM $CaCl_2$, 1 mg/ml bovine serum albumin and 20 fmol [^{125}I]α-LTx. Following a 10-min incubation at 20°C, the preparation was transferred onto ice. Free [^{125}I]α-LTx was separated from bound [^{125}I]α-LTX by elution through a 1-ml column of Sepharose 2B resin prepared in 1-ml syringes. Non-specific binding was measured by the addition of an excess of non-radioactive α-LTx (6 nM). By comparison of the recovery of [^{3}H]phosphatidylcholine incorporated into liposomes with that of [^{125}I]α-LTx, an estimate of toxin binding was obtained.

Flux experiments

In order to measure ^{45}Ca efflux liposomes were loaded by including ^{45}Ca and [^{3}H]phosphatidylcholine throughout the reconstitution, followed by extensive dialysis. Efflux of ^{45}Ca was tested by addition of 50 μl of reconstituted liposomal preparation into 500 μl of 0.1 M NaCl and 10 mM Hepes−NaOH (pH 7.4). Following addition of either divalent cations and/or α-LTx and the appropriate incubation time, aliquots were filtered through Whatman GF/C filters. Radioactivity remaining on filters following one wash with 500 μl of buffer was determined in a Beckman scintillation counter using NEN Atomlight. Retention of ^{45}Ca and [^{3}H]phosphatidylcholine was not affected by prior incubation with 0.5 mM EGTA, but was reduced to <1% of input by prior incubation with 1% Triton X-100. To measure membrane potential, non-radioactive liposomes (containing 0.1 M KCl and suspended in 0.1 M NaCl, see above) were loaded with [^{3}H]$TPMP^+$ by adding to 40 μl of the liposomal preparation 2 μl each of [^{3}H]-$TPMP^+$, tetraphenylborate and valinomycin (final concentrations: 1.5, 3 and 1 μM, respectively). Following a 15-min incubation, the preparation was diluted to 1 ml with buffer and tested for release of [^{3}H]$TPMP^+$ as described above for ^{45}Ca. Values were corrected for small non-specific binding of [^{3}H]$TPMP^+$ to filters (typically, 0.5−0.8% of input).

Planar lipid membranes

Membranes were formed from a solution containing 18 mg L-α-phosphatidylcholine and 2 mg cholesterol in 1 ml decane as described by Mueller and Rudin (1969). The aqueous medium was 0.1 M NaCl, 50 μM $CaCl_2$ and 5 mM Tris−HCl (pH 7.4). The diameter of the hole separating the two 5-ml compartments was 1 mm. The current from an applied voltage was measured using Ag/AgCl electrodes using a voltage clamp as input circuit and a current−voltage converter as output circuit (Analog Devices AD 515L), with a 10^8-10^9 Ω feedback resistor. Test substances were added to the front (*cis*) compartment. The rear (*trans*) compartment was defined to be at zero potential. Positive current is the flow of positive ions into the *trans* compartment. All measurements were carried out at ambient temperature. Continuous data were recorded by a stereo−video cassette recorder (SONY SL-HF 100 EC) and a modified digital−audio processor (SONY PCM-701 ES).

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