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Calcium-Independent Receptor for α-Latrotoxin and Neurexin 1α Facilitate Toxin-Induced Channel Formation: Evidence That Channel Formation Results from Tethering of Toxin to Membrane

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ABSTRACT

α-Latrotoxin binding to the calcium-independent receptor for α-latrotoxin (CIRL-1), a putative G-protein-coupled receptor, stimulates secretion from chromaffin and PC12 cells. Using patch clamp techniques and microspectrofluorimetry, we demonstrate that the interaction of α-latrotoxin with CIRL-1 produces a high conductance channel that permits increases in cytosolic Ca^{2+}. α-Latrotoxin interaction with CIRL-1 transiently expressed in bovine chromaffin cells produced a 400-pS channel, which rarely closed under Ca^{2+}-free conditions. The major effect of overexpressing CIRL-1 was to greatly increase the sensitivity of chromaffin cells to channel formation by α-latrotoxin. α-Latrotoxin interaction with CIRL-1 transiently overexpressed in non-neuronal human embryonic kidney 293 (HEK293) cells produced channels that were nearly identical with those observed in chromaffin cells. Channel currents were reduced by millimolar Ca^{2+}. At α-latrotoxin concentrations below 500 pM, channel formation occurred many seconds after binding of toxin to CIRL-1 indicating distinct steps in channel formation. In all cases there was a rapid, sequential addition of channels once the first channel appeared. An analysis of CIRL-1 mutants indicated that channel formation in HEK293 cells is unlikely to be transduced by a G-protein-dependent mechanism. α-Latrotoxin interaction with a fusion construct composed of the extracellular domain of CIRL-1 anchored to the membrane by the transmembrane domain of vesicular stomatitis virus glycoprotein, and with neurexin 1α, an α-latrotoxin receptor structurally unrelated to CIRL-1, produced channels virtually identical with those observed with wild-type CIRL-1. We propose that α-latrotoxin receptors recruit toxin to facilitate its insertion across the membrane and that α-latrotoxin itself controls the conductance properties of the channels it produces.

Since the demonstration that black widow spider venom and its active component α-latrotoxin (Ltx) produce massive exocytosis at the frog neuromuscular junction (Longenecker et al., 1970; Frontali et al., 1976; Pumplin and Reese, 1977; Fesce et al., 1986), the toxin has been the focus of intense investigation. Ltx also causes release from a variety of other neurons and cells [for reviews see Meldolesi et al. (1986) and Surkova (1994)]. An early hypothesis for the action of Ltx was that the toxin itself produces divalent ion-permeable channels in the plasma membrane. This was based on the observation that Ltx inserts into artificial bilayers to form high conductance, divalent ion-permeable channels (Finkelstein et al., 1976). The flux of Ca^{2+} through such channels could stimulate exocytosis and thus contribute to the actions of Ltx. [At the neuromuscular junction, Mg^{2+} can substitute for Ca^{2+} to support Ltx-induced secretion (Misler and Hurlburt, 1979).] Indeed, Ltx produces channels in PC12 cells (Wanke et al., 1986), neuroblastoma cells (Hurlbut et al., 1994), and rat adrenal chromaffin cells (Barnett et al., 1996).

However, already in the early studies there was evidence that in biological membranes the toxin was not acting alone on the bilayer but interacting with specific receptors. Ltx was found to bind in a saturable manner with nanomolar affinity to synaptosomal membranes (Tzeng and Siekevitz, 1979;

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ABBREVIATIONS: Ltx, α-latrotoxin; CIRL, calcium-independent receptor for α-latrotoxin; HEK293, human embryonic kidney 293; TMR, transmembrane region; p120, CIRL-1 extracellular domain; VSV-G, vesicular stomatitis virus glycoprotein; GFP, green fluorescent protein; HA, hemagglutinin; PCR, polymerase chain reaction; DPBS, Dulbecco's phosphate-buffered saline; PSS, physiological salt solution; CCh, carbachol; IP_3, inositol trisphosphate.

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Rosenthal et al., 1990). The binding was both Ca^{2+}-dependent and Ca^{2+}-independent. These findings raised the possibility that receptors may contribute to the insertion or conductance properties of Ltx or by themselves form channels when the ligand bound. Recently, two families of high affinity Ltx receptors have been cloned. Calcium-independent receptor for α-latrotoxin (CIRL), or latrophilin, binds Ltx in a Ca^{2+}-independent manner (Krasnoperov et al., 1997; Lelianova et al., 1997), and neurexin 1α binds Ltx in a Ca^{2+}-dependent manner (Petrenko et al., 1990). Both are able to support Ltx-induced, Ca^{2+}-dependent secretion from chromaffin or PC12 cells (Krasnoperov et al., 1997; Bittner et al., 1998; Sugita et al., 1999).

We have focused on the function of CIRL in Ltx-induced secretion. The primary sequence of CIRL predicts a G-protein-coupled receptor with significant homology to members of the secretin receptor family. To date, three members of the CIRL family of receptors have been identified (Sugita et al., 1998; Krasnoperov et al., 1999). CIRL-1 and CIRL-3 are expressed primarily in brain, whereas CIRL-2 is ubiquitously expressed. No endogenous ligand or G-protein-activated effector has yet been identified for any of the CIRL receptors. Immunoblotting indicates that bovine chromaffin cells express CIRL-1 or a closely related protein (M. A. Bittner and R.W.H., submitted). Binding of toxin to the endogenous receptor occurs in the absence of Ca^{2+}, and subsequent addition of Ca^{2+}-containing medium results in Ca^{2+} influx and secretion. Overexpression of CIRL-1 by transient transfection increased 10-fold the sensitivity of chromaffin cells to the effects of Ltx. These observations raise the possibility that the Ltx-induced channels previously observed in rat chromaffin cells (Barnett et al., 1996) directly result from the interaction of Ltx with CIRL-1 or a closely related receptor. Alternatively, a recent report has suggested a mechanism whereby the interaction of Ltx with CIRL (Latrophilin) activates endogenous neuronal channels to elicit secretion (Davletov et al., 1998).

There are several key findings in this study that provide insight into the mechanism by which Ltx affects biological membranes. Using patch clamp techniques and microspectrofluorimetry with bovine chromaffin cells and HEK293 cells, we demonstrate that the interaction of Ltx with transiently expressed CIRL-1 results in a high conductance channel that permits a rise in cytosolic Ca^{2+}. Channel formation can occur when CIRL-1 or neurexin 1α is expressed in a nonexcitable cell. Importantly, experiments with CIRL-1 mutants and neurexin 1α indicate that the distinctive channels produced by Ltx interaction with receptor occur with different extracellular binding domains and do not require specific membrane anchoring domains on the receptor. The data suggest that Ltx receptors recruit and tether the toxin to the membrane to facilitate its ability to create channels. The experiments also identify distinct steps in the kinetics of channel formation.

Materials and Methods

Plasmids. The construction of the plasmids encoding CIRL-1 (pCDR7) and the CIRL-1 carboxyl-terminal deletion mutants (pCDR-7TMR and pCDR-1TMR) has been described previously (Krasnoperov et al., 1997; Ichtchenko et al., 1999). To construct the pCDR-p120/vesicular stomatitis virus glycoprotein (VSV-G) plasmid encoding the extracellular region of CIRL-1 (p120; residues 1 through 855) and a single transmembrane domain of VSV-G (Guan and Rose, 1984; Guan et al., 1985), the pCDR7 plasmid was partially digested with *Bsp*lu11I and *Pme*I and the resulting linear fragment encoding p120 was purified. The VSV-G fragment was produced by PCR amplification of the plasmid encoding VSV-G (pSVGL) using a *Bsp*lu11I-tagged forward primer and a *Pme*I-tagged reverse primer. The PCR product was then cut by *Bsp*lu11I and *Pme*I, and the purified fragment was ligated to the p120-encoding fragment described above. A synthetic linker corresponding to amino acids 833 through 855 of CIRL-1 was then inserted into the *Bsp*lu11I site between the p120 and VSV-G inserts to complete the pCDR-p120/VSV-G plasmid. The structures of CIRL-1 and the CIRL-1 mutant receptors are shown schematically in Fig. 1. The plasmid encoding neurexin 1α (pCMVbN1α-1) was a gift from Thomas C. Sudhof (The University of Texas Southwestern Medical Center) and has been described previously (Sugita et al., 1999). The plasmid encoding VSV-G (pSVGL) was a gift from Dr. John K. Rose (The Salk Institute) and has been described previously (Guan and Rose, 1984). The plasmid encoding a mutant green fluorescent protein (GFP; S65T) was a gift from Dr. Ian Macara (University of Virginia) and has been described previously (Helm et al., 1995). The plasmid encoding muscarinic M_3 receptor (pCMVM3) was a gift from Dr. Stephen K. Fisher (University of Michigan).

Cell Culture and Transfection. Bovine adrenal chromaffin cell cultures were prepared and maintained using methods identical with those described in previous studies (Bittner and Holz, 1992). Cells were cultured as monolayers on collagen-coated glass coverslips, which formed the bottoms of 35-mm culture dishes at a density of 600,000 cells per dish and were transfected by calcium phosphate precipitate (Wilson et al., 1996) 14 to 18 h after plating. The cells were cotransfected with precipitates containing equal mass amounts of experimental (pCDR7) or control plasmid (pCMVneo), along with soluble GFP-encoding plasmid (p7sGFP), which served as a marker for transfection.

Human embryonic kidney 293 (HEK293) cells were plated onto poly(L-lysine)-coated 60-mm culture dishes at a density of 500,000 cells per dish and maintained at 37°C and 10% CO_2 in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum. Cells were cotransfected 14 to 18 h later with GFP-encoding plasmid (p7sGFP) and either experimental (pCDR7, pCDR-7TMR, pCDR-1TMR, p120/VSV-G, pCMVbN1α-1, or pCMVM3) or control plasmid (pCMVneo) using the same procedure indicated above for chromaffin cells. Within 24 h after transfection, the culture medium was replaced by medium containing 10 μM cytosine arabinoside to limit mitotic activity. Experiments were performed 36 to 48 h after transfection. On the day experiments were performed, the culture medium was replaced with Ca^{2+}-free extracellular medium (see below) and cells were mechanically dissociated

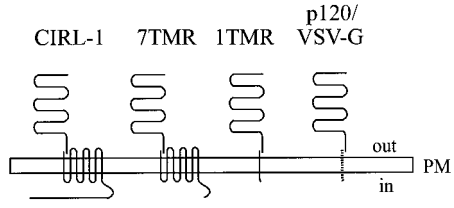

Fig. 1. Structure of CIRL-1 and CIRL-1 mutant receptors. CIRL-1, wild-type receptor; 7TMR, CIRL-1 without its carboxyl-terminal tail; 1TMR, extracellular domain of CIRL-1 (p120) anchored by only one CIRL-1 transmembrane domain; p120/VSV-G, extracellular domain of CIRL (p120) anchored to the membrane by the transmembrane domain of VSV-G. [Modified from Krasnoperov et al. (1999).] Note: the discontinuity between the extracellular domains and the transmembrane domains indicates that each receptor consists of two heterologous subunits resulting from endogenous proteolytic processing of the precursor proteins. Although the p120/VSV-G construct shows similar discontinuity, cleavage of this receptor is unknown.

from the monolayer. They were then centrifuged (5 min at 100*g*), resuspended in culture medium containing 10 μM cytosine arabinoside, and replated onto glass bottom 35-mm culture dishes coated with poly(L-lysine) at a density of 100,000 cells per dish. Cells were used in experiments 2 to 10 h after replating.

Immunocytochemistry. Three consecutive sequences encoding the Influenza hemagglutinin (HA) epitope tag, YPYDVPDYA, were introduced using a PCR-based technique into the CIRL-1 coding sequence at the amino terminus. HEK293 cells were plated on poly(L-lysine)-coated glass coverslips that formed the well bottoms of 12-well culture dishes. Cells were maintained and cotransfected with plasmids encoding epitope-tagged CIRL-1 and GFP as described above. Immunocytochemistry was performed 36 to 48 h after transfection. For surface staining, transfected cells were rinsed twice with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 4% (w/v) paraformaldehyde in 0.1 M cacodylic acid (pH 7.0) for 30 min at room temperature. The cells were then rinsed once with DPBS, quenched with 50 mM NH_4Cl in DPBS for 30 min, and rinsed twice with DPBS at room temperature. Nonspecific binding was blocked with 0.1% (w/v) gelatin in DPBS for 20 min, followed by 4% donkey serum in DPBS for 20 min at room temperature. After a single rinse with DPBS at room temperature, the cells were incubated with mouse anti-HA (1:500; 12CA5, Berkeley Antibody Co., Berkeley, CA) in DPBS for 2 h at 4°C. The cells were then rinsed five times with DPBS and incubated with rhodamine-conjugated donkey anti-mouse antibody (1:100) and 5 mg/ml BSA in DPBS for 70 min at 4°C. After this final incubation, the cells were rinsed five times with DPBS at 4°C and mounted in 0.1% paraphenyldiamine, 90% glycerol, and 0.1 M PBS (pH 9.0). Cell surface fluorescence was examined by collecting images with a confocal imaging system (Bio-Rad Laboratories, Hercules, CA) with a 100× objective.

Electrophysiology. Patch electrodes were pulled from 1.5-mm *A* × 1.12-mm i.d. borosilicate glass (Corning 7740) capillaries (A-M Systems, Carlsborg, WA), coated with elastomer (Sylgard; Dow Corning), and fire-polished to 3 to 8 mΩ. The standard extracellular solution was a physiological salt solution (PSS) consisting of (in millimolar): NaCl, 145; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 10; and HEPES, 10 (pH 7.3 at room temperature). Ca^{2+}-free PSS and divalent ion-free PSS were prepared by excluding the appropriate divalent ion-containing salts and including 1 mM EGTA. Electrodes were filled with a solution consisting of (in millimolar): CsOH, 120; CH_4O_3S, 120; CsCl, 20; $MgCl_2$, 1; Mg-ATP, 2; Li-GTP, 0.5; EGTA, 0.25; and HEPES, 20 (pH 7.3). Whole cell or outside-out patch clamp recordings of ionic current were made using an amplifier (Axopatch 200A; Axon Instruments, Burlingame, CA) with a computer interface (ITC-16; Instrutech Corp., Great Neck, NY). Membrane conductance and series resistance were compensated electronically to 75%. Current signals were filtered at 5 kHz (8-pole Bessel) and stored directly onto the computer hard disk for later analysis. Voltage protocols, data acquisition, and analyses were performed using software (Pulse Control; Richard Bookman, University of Miami) developed as an extension of the numerical/graphics program Igor (WaveMetrics, Lake Oswego, OR). Unless otherwise indicated, recordings were made at room temperature from cells or membrane patches voltage clamped at a holding potential of −70 mV.

Calcium Measurements. Measurements of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were made using Fura-2 and dual-wavelength microspectrofluorimetry (Stuenkel, 1994). Loading of cells with Fura-2 was accomplished by incubation at 37°C for 30 min in PSS containing 1 μM acetoxymethyl ester of Fura-2 (Fura-2 AM; Molecular Probes, Eugene, OR) in dimethyl sulfoxide carrier (0.1% final concentration). Emission signals of Fura-2 at alternating excitation wavelengths of 340 and 380 nm were monitored at 500 nm using a photomultiplier-based AR-CM system (SPEX Industries, Edison, NJ). The ratio of emitted light (340/380 excitation) was used as a readout for changes in $[Ca^{2+}]_i$ as described previously (Fajtova et al., 1991). Contamination of the Fura-2 signal by GFP was insignificant.

Reagents and Cell Perfusion. Ltx was purified as previously described (Petrenko et al., 1990). Carbachol (CCh) was purchased from Sigma Chemical Co. (St. Louis, MO). Drugs were dissolved in PSS, Ca^{2+}-free PSS, or divalent ion-free PSS and applied by pressure ejection from fused-silica tubing (300-μm i.d.; Poly Micro Technologies, Phoenix, AZ) positioned immediately adjacent to the cell from which membrane current or Fura-2 fluorescence was being recorded.

Results

Transfection with a Plasmid Encoding CIRL-1 Renders Chromaffin Cells Supersensitive to Channel Formation by Ltx. Bovine adrenal chromaffin cells were transiently cotransfected with plasmids encoding GFP (p7sGFP) and either CIRL-1 (pCDR7) or control plasmid (pCMVneo). Cells in the whole-cell patch clamp configuration were continuously voltage clamped at a holding potential (V_h) of −70 mV and Ltx was applied by local perfusion. In the initial experiments, Ltx was applied under Ca^{2+}-free conditions to ensure that only those receptors that bind Ltx in the absence of Ca^{2+} were being studied. Cells overexpressing CIRL-1 exhibited large changes in membrane current when challenged with 5 pM Ltx (Fig. 2A; $n = 4$). In contrast, control cells without overexpressed CIRL-1 showed no change in membrane current when challenged with the same concentration of toxin (Fig. 2B; $n = 6$). Higher concentrations of Ltx (50 or 100 pM) produced channel formation in control cells due to interaction with endogenous Ltx receptors (see below).

The current responses to Ltx under Ca^{2+}-free conditions in both CIRL-1- (Fig. 3A, 5 pM Ltx; $n = 4$) and control-transfected cells (Fig. 3C, 50 pM Ltx; $n = 4$) were characterized by distinct inward current steps and occasional current fluctuations reflecting channel closures or blockade (Hurlbut et al., 1994; Barnett et al., 1996). The average amplitudes of the current steps in CIRL-1- and control-transfected cells were 28.3 ± 1.0 pA ($n = 11$) and 27.2 ± 0.6 pA ($n = 13$), respectively. The reversal potential for the channel currents in both CIRL-1- and control-transfected cells was approximately 0 mV (data not shown). Using this reversal potential, we calculated an average channel conductance of approximately 400 pS. The Ca^{2+} sensitivity of the channels formed by Ltx interaction with endogenous Ltx receptor and with recombinant CIRL-1 in bovine chromaffin cells were qualitatively similar. When 2 mM Ca^{2+} was present in the extracellular bath, current records obtained following Ltx application to CIRL-1- (Fig. 3B, 5 pM Ltx; $n = 6$) and control-transfected

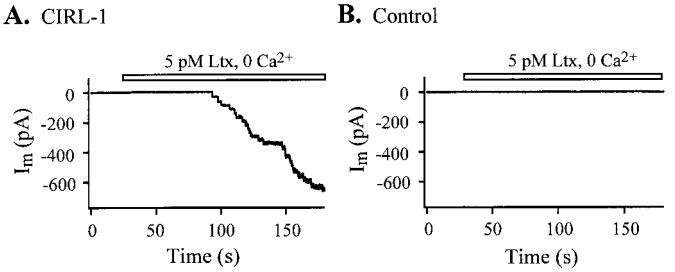

Fig. 2. Overexpression of CIRL-1 in bovine chromaffin cells facilitates Ltx-induced channel formation. CIRL-1- (A) and control-transfected (B) chromaffin cells were voltage clamped at a holding potential of −70 mV and whole-cell membrane current (I_m) was monitored before and during continuous application of 5 pM Ltx under Ca^{2+}-free conditions (indicated by open bars). A large inward current developed in CIRL-1-transfected cells ($n = 4$) but not in control-transfected cells ($n = 6$).

cells (Fig. 3D, 50 pM Ltx; $n = 4$) were characterized by a higher incidence of current fluctuations, which usually obscured the identification of unitary current steps. Hence, Ltx interacts with recombinant CIRL-1 or the endogenous Ltx receptor to produce similar channels. The major effect of transiently expressing CIRL-1 in chromaffin cells was to increase greatly the sensitivity to channel formation by Ltx.

Low Concentrations of Ltx Induce Channels in HEK293 Cells Transiently Transfected with Plasmid Encoding CIRL-1. HEK293 cells were transiently transfected with a plasmid encoding HA-tagged CIRL-1. Immunocytochemical staining with HA antibody revealed plasma membrane expression of HA-tagged CIRL-1 in transfected cells (Fig. 4). Little or no protein was detected in intracellular compartments. Nontransfected cells or cells transfected with pCMVneo were unstained (data not shown).

Cells overexpressing CIRL-1 exhibited large changes in membrane current when challenged with 5 pM Ltx under Ca^{2+}-free conditions (Fig. 5A; $n = 12$). The current responses were similar to those observed in chromaffin cells in that they were characterized by distinct inward steps that had an average amplitude of 27.6 ± 0.8 pA ($n = 44$). Current responses to low concentrations of Ltx (5 or 50 pM) were not observed under Ca^{2+}-free conditions in any of the six cells examined that had been transfected with control plasmid (pCMVneo) (Fig. 5B).

Unexpectedly, a high concentration of Ltx (500 pM) was able to cause channel formation in cells without transfected CIRL-1. The appearance of these channels had a longer latency after the beginning of the perfusion with Ltx than with lower concentrations of Ltx in CIRL-1-transfected cells. These current responses may have resulted from Ltx interaction with endogenous receptors that may be very few in number and/or that bind Ltx with low affinity. [Antibody to CIRL-1 did not detect the protein in blots of nontransfected HEK293 cells. Nevertheless, the effects in nontransfected HEK293 cells of high Ltx concentrations are likely to be mediated by an endogenous receptor. Pretreatment of nontransfected HEK293 cells with conconavalin A, which blocks Ltx interaction with endogenous receptors (Tzeng and Siekevitz, 1979; Meldolesi et al., 1983), prevented Ltx-induced $^{45}Ca^{2+}$ uptake into untransfected HEK293 cells (M. A. Bittner and R.W.H., unpublished observations).] They displayed distinct inward current steps that had an average amplitude of 28.4 ± 1.1 pA ($n = 14$); these were similar to those induced by much lower Ltx concentrations in CIRL-1-transfected cells.

As in chromaffin cells, extracellular Ca^{2+} changed the char-

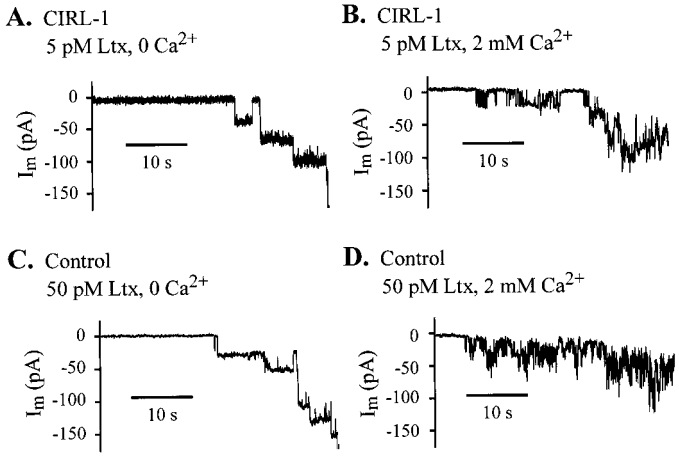

Fig. 3. Channels resulting from interaction of Ltx with transfected CIRL-1 and with the endogenous Ltx receptor in chromaffin cells exhibit similar characteristics. CIRL-1- (A, B) and control-transfected (C, D) chromaffin cells were voltage clamped at a holding potential of −70 mV and whole-cell membrane current (I_m) was monitored before and during continuous application of 5 pM Ltx (CIRL-1-transfected cells) or 50 pM Ltx (control cells) in the absence (left) or presence (right) of extracellular Ca^{2+}. Current records represent expanded time segments surrounding the initial current responses and do not indicate the onset of each toxin application. In Ca^{2+}-free solution, large (20 to 40 pA) unitary current steps were seen in both CIRL-1- ($n = 4$) and control-transfected ($n = 6$) cells. Increased current fluctuation was associated with the current steps when Ca^{2+} was present ($n = 6$ and $n = 4$, respectively).

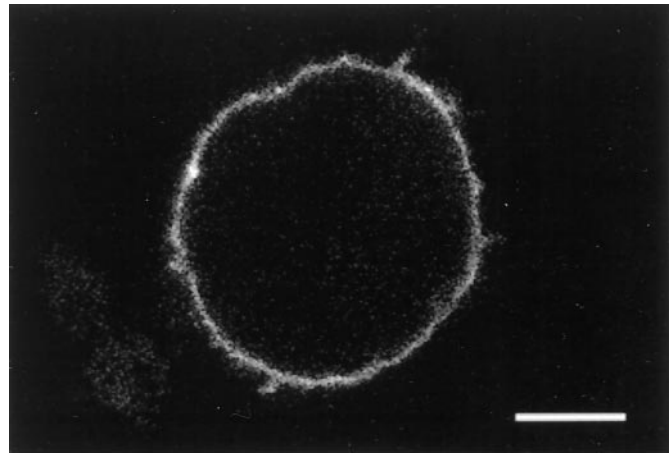

Fig. 4. CIRL-1 is expressed as a membrane protein in HEK293 cells. Confocal fluorescence image of HA epitope-tagged CIRL-1 in transfected HEK293 cells. Note the increased fluorescence intensity along the cell surface. Bar, 7 μm.

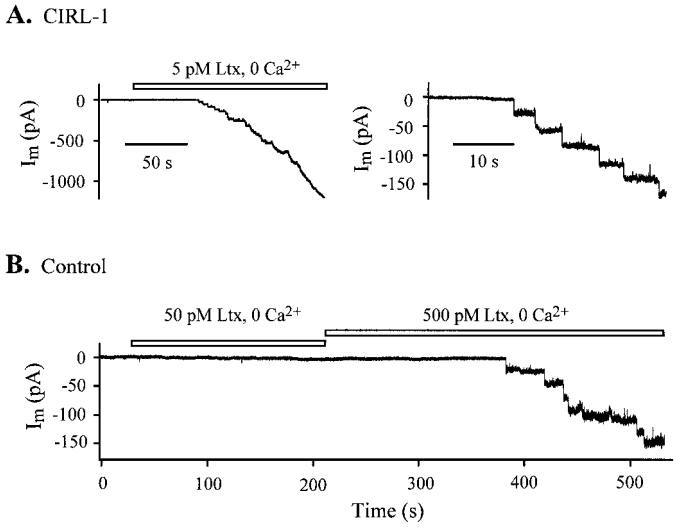

Fig. 5. Expression of CIRL-1 in HEK293 cells facilitates Ltx-induced channel formation. CIRL-1- (A) and control-transfected (B) HEK293 cells were voltage clamped at a holding potential of −70 mV and whole-cell membrane current (I_m) was monitored before and during continuous application of 5, 50, or 500 pM Ltx in the absence of extracellular Ca^{2+} (open bars indicate periods of specified treatments under Ca^{2+}-free conditions). The current record in A, right, represents an expanded time segment surrounding the initial current response depicted in A, left. As in chromaffin cells, low concentrations (5 pM) of Ltx produced a large inward current in CIRL-1-transfected HEK293 cells ($n = 12$), whereas in control cells ($n = 6$), a higher concentration (500 pM) of Ltx was required to elicit a current response.

acteristics of the Ltx-induced conductance in HEK293 cells transfected with CIRL-1. The Ltx-induced currents fluctuated rapidly in the presence of extracellular Ca^{2+} (Fig. 6A; $n = 7$). Figure 6B demonstrates the effect of Ca^{2+}on Ltx-induced channels that initially appeared in the absence of Ca^{2+}. A cell was perfused for 84 s with 5 pM Ltx in Ca^{2+}-free medium. After 31 s, typical channels appeared and the inward current increased over the next 53 s. When the perfusion solution was switched to a Ltx-free, Ca^{2+}-containing solution the current immediately decreased. The current increased when Ca^{2+} was subsequently removed and decreased again when Ca^{2+} was reintroduced. In contrast to the effects of Ca^{2+}, extracellular Mg^{2+} had no effect on the Ltx-induced current (data not shown). When HEK293 cells transfected with CIRL were perfused with Ltx in a divalent ion-free extracellular solution, discrete current steps developed that exhibited an average amplitude (29.6 ± 1.2 pA; $n = 17$) similar to that observed when 2 mM Mg^{2+} was present. These data indicate that Ca^{2+} but not Mg^{2+} reduces the conductance of Ltx-induced channels.

Increases in Cytosolic Calcium Induced by Ltx Interaction with CIRL-1 Require Extracellular Calcium. It has been demonstrated in chromaffin cells that Ltx interaction with CIRL-1 or with endogenous receptors causes an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) (Bittner et al., 1998). Because CIRL-1 has seven transmembrane domains and is thought to be a G-protein-coupled receptor, increases in $[Ca^{2+}]_i$ could result from Ca^{2+} influx through the channel and/or from a phospholipase C-mediated increase in IP_3 and release of Ca^{2+} from intracellular stores. Measurement of Fura-2 fluorescence was used to detect Ltx-induced changes of $[Ca^{2+}]_i$ in HEK293 cells transiently transfected with CIRL-1-encoding plasmid (pCDR7). When the extracellular solution contained 2 mM Ca^{2+}, local application of 50 pM Ltx to CIRL-1-transfected cells elicited robust increases in $[Ca^{2+}]_i$ (Fig. 7A; $n = 12$) that were characterized by a rapid rise followed by a sustained plateau. Under Ca^{2+}-free conditions, local application of 50 pM Ltx to CIRL-1-transfected cells did not elicit changes in $[Ca^{2+}]_i$ in any of the 14 cells investigated (Fig. 7B). Addition of Ca^{2+} to the extracellular solution produced immediate increases in $[Ca^{2+}]_i$ (Fig. 7B, $n = 3$) despite extended wash periods (up to 100 s) during which the Ltx stimulus was absent. The latter response was likely due to Ca^{2+} influx through channels previously formed by the Ltx-CIRL-1 interaction. The lack of a rise in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} was not due to the inability of the cells to respond to phospholipase C activation. Application of the muscarinic agonist carbachol in the absence of extracellular Ca^{2+} increased $[Ca^{2+}]_i$ in HEK293 cells transiently transfected with a muscarinic receptor (M_3 receptor, Fig. 7C). Intracellular Ca^{2+} rose rapidly and then smoothly declined toward resting level in 7 of 10 transfected cells. Thus, although HEK293 cells permit functional coupling between transiently transfected G-protein-coupled receptors and phospholipase C, Ltx acting through CIRL-1 does not increase $[Ca^{2+}]_i$ by such a mechanism. More likely the increase in $[Ca^{2+}]_i$ occurs via influx through the channel.

Effects on Conductance of Varying Ltx Concentration Applied to CIRL-1-Transfected HEK293 Cells. Following Ltx application in the absence of Ca^{2+}, there was a latency before the onset of current response that varied inversely with the Ltx concentration (Fig. 8A). Ltx-induced current was detected almost immediately (by 3.4 ± 1.0 s; $n = 5$) following the application of 500 pM Ltx. In contrast, latencies of 16.4 ± 3.4 s ($n = 8$) and 37.4 ± 4.2 s ($n = 9$), respectively, were observed before channel formation following the application of 50 and 5 pM Ltx. The latencies were highly reproducible with an approximately linear relationship between the reciprocal of the lag time and toxin concentration (Fig. 8B). Despite the effect of toxin concentration on the latency, the average sizes of single channel current steps were virtually independent of toxin concentration (Fig. 8, A and C).

The latency could reflect the kinetics of Ltx interaction with CIRL-1 or steps subsequent to toxin binding. To exam-

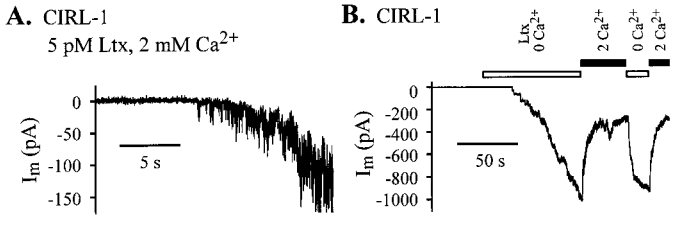

Fig. 6. Extracellular calcium changes the conductance effects of Ltx in CIRL-1-transfected HEK293 cells. A, Ltx (50 pM) was applied 10 s before the beginning of the record in the presence of 2 mM Ca^{2+}. B, Ltx (5 pM) was first applied in the absence of Ca^{2+} (open and solid bars indicate periods of specified treatments in the absence and presence of extracellular Ca^{2+}, respectively). Discrete current increases were observed (difficult to see with the expanded I_m scale). Where indicated, the perfusion solution was changed to 2 mM Ca^{2+} (without Ltx) and back to 0 Ca^{2+} (without Ltx). Note the difference in the time scales.

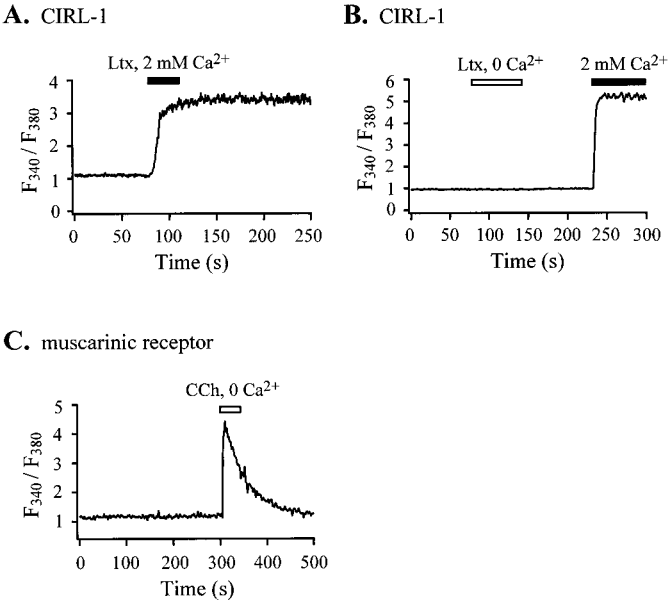

Fig. 7. Ltx causes increases in cytosolic Ca^{2+}only in the presence of extracellular calcium in CIRL-1-transfected HEK293 cells. Intracellular Ca^{2+} was monitored by microspectrofluorimetry of Fura-2. A, Ltx (50 pM) was applied to a CIRL-1-transfected cell in the presence of 2 mM Ca^{2+}. Similar responses were obtained in 12 cells. B, Ltx (50 pM) was applied to a CIRL-1-transfected cell in the absence of Ca^{2+}. There was no change in the Fura-2 fluorescence until solution containing 2 mM Ca^{2+} was perfused. Similar results were obtained in 14 cells. C, An HEK293 cell transfected with a plasmid encoding a muscarinic receptor (M_3) was perfused with a Ca^{2+}-free solution. At the indicated time, CCh (100 μM) was perfused in the continuing absence of Ca^{2+}. Application of CCh produced elevations in $[Ca^{2+}]_i$ in 7 of 10 cells. Open and solid bars indicate periods of specified treatments in the absence and presence of extracellular Ca^{2+}, respectively.

ine these possibilities, Ltx application was limited to a brief pulse followed by a rapid washout and ensuing responses were observed. In Fig. 8D, the current response to a 5-s pulse of 50 pM Ltx was examined in the absence of Ca^{2+}. If the lag period was the result of kinetic constraints related to binding of toxin to CIRL-1, removal of Ltx before the onset of current changes would be predicted to prevent the response. Instead, channel formation occurred after removal of Ltx. Thus, rate-limiting steps occur in channel formation subsequent to Ltx binding to CIRL-1.

Ltx Causes Channel Formation in Outside-Out Patches from CIRL-1-Transfected HEK293 Cells. Because the primary sequence of CIRL-1 predicts that it is a G-protein-linked receptor, Ltx binding to the extracellular domain may activate an intracellular signal transduction pathway that regulates channel formation. To test this possibility, the effects of Ltx on outside-out membrane patches from CIRL-1-transfected HEK293 cells were investigated. In five of eight patches, local perfusion of Ca^{2+}-free, Ltx (50 pM)-containing solution resulted in the appearance of distinct, unitary current steps (Fig. 9A). Patches that did not respond to 50 pM Ltx were also unresponsive to subsequent application of 500 pM Ltx, suggesting that the patches lacked CIRL-1. The average amplitude (27.8 ± 1.3 pA; $n = 20$) of the Ltx-induced current steps recorded from patches was nearly identical with the average amplitude of steps recorded in the whole-cell configuration. These results suggest that formation of the conductance pathway depends on Ltx-CIRL-1 interaction but not on a consequent activation of a cytosolic signal transduction cascade. In each patch exhibiting Ltx-induced channel activity, a cascade of many current steps as observed in whole-cell recordings always followed the first current step. A plot of the time course of this phenomenon is shown in Fig. 9B.

Mutants of CIRL-1 with Carboxyl-Terminal Deletions Support Ltx-Dependent Conductance Increases. The roles of the long cytosolic tail and the transmembrane domains of CIRL-1 in mediating Ltx-induced channel formation were investigated with two carboxyl-terminal CIRL-1 deletion mutants (see Fig. 1). One mutant, 7TMR, consisted of the extracellular domain (p120) along with the seven transmembrane domains and connecting loops but without the long cytosolic, carboxyl tail. The other mutant, 1TMR, consisted of the extracellular domain (p120) and only the first transmembrane domain and the first intracellular segment. Large increases in membrane conductance were observed following application of 50 pM Ltx in cells expressing either mutant (Fig. 10). However, there were differences in the resulting conductances. In HEK293 cells expressing the CIRL-1 deletion mutant without the carboxyl-terminal tail

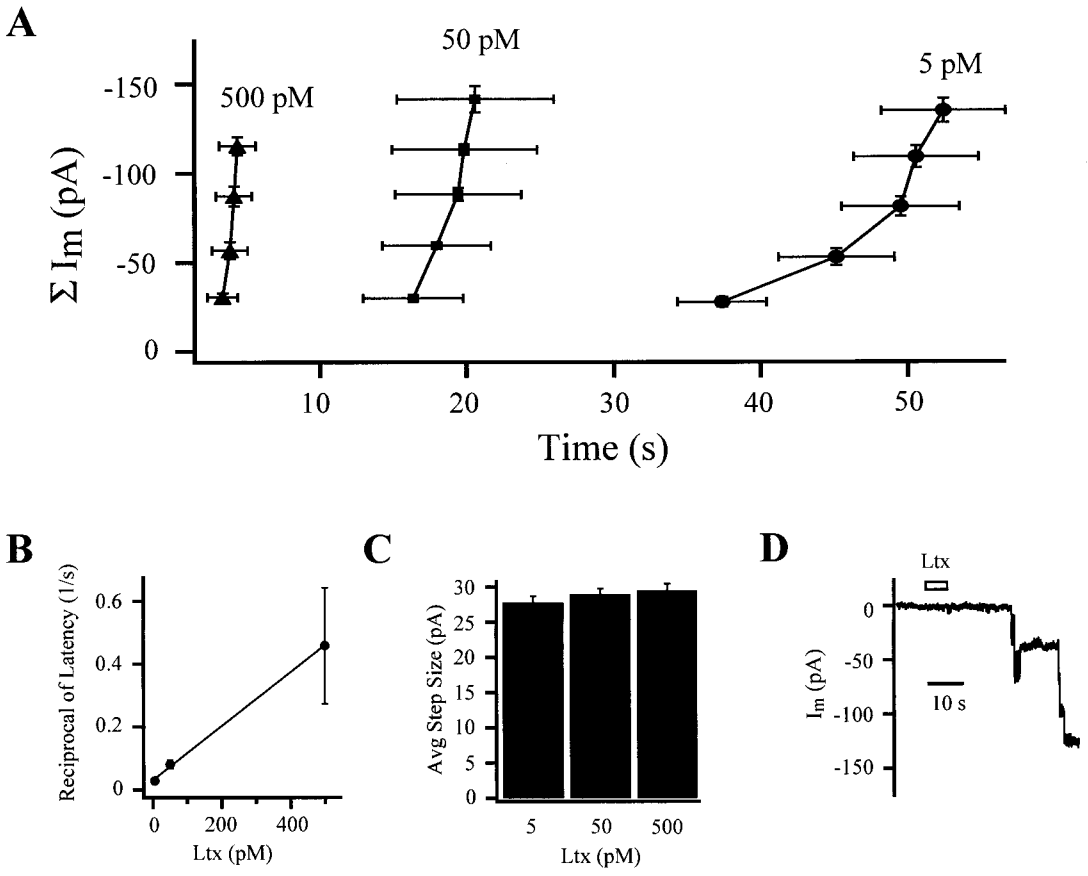

Fig. 8. The effects of various concentrations of Ltx on the time course of appearance and the size of the current steps in the absence of Ca^{2+} in HEK293 cells. A, CIRL-1-transfected HEK293 cells were voltage clamped at −70 mV and whole-cell current (I_m) responses were monitored during continuous perfusion (starting at 0 s) of 5 ($n = 8$), 50 ($n = 7$), or 500 pM ($n = 5$) Ltx in the absence of Ca^{2+}. The means ± S.E. of the time of occurrence and the cumulative size of the first four to five discrete current jumps are plotted. B, the reciprocal of the latency of the first current step from A is plotted versus Ltx concentration. C, the average current steps at the different Ltx concentrations are compared. D, the effects of application of a 5-s pulse of Ltx (50 pM) in the absence of extracellular Ca^{2+} (indicated by the open bar) to a CIRL-1-transfected cell. Note that, after a delay, channel formation occurs after removal of the Ltx. This was observed in three of three cells.

(7TMR), Ltx produced stepwise increases in current when extracellular Ca^{2+} was absent (Fig. 10A; $n = 4$) and responses characterized by rapid current fluctuations when extracellular Ca^{2+} was present (Fig. 10B; $n = 3$). The average amplitude of the individual steps was 29.2 ± 0.5 pA ($n = 14$). These results were similar to those obtained following Ltx interaction with wild-type CIRL-1. In contrast, the currents induced by Ltx in cells expressing the deletion mutant with only the single transmembrane domain (1TMR) exhibited rapid current fluctuations even when extracellular Ca^{2+} was absent (Fig. 10C; $n = 7$). Sustained individual current steps were not observed. Nevertheless, the current responses were reversibly suppressed by extracellular calcium (Fig. 10D; $n = 3$), a result similar with that produced following Ltx interaction with wild-type CIRL-1.

Extracellular Domain of CIRL-1 Fused to Transmembrane Domain of Vesicular Stomatitus Virus Glycoprotein Supports Ltx-Induced Conductance Increases. To determine whether channels resulting from the interaction of Ltx with CIRL-1 require a specific transmembrane domain, a chimeric protein was constructed consisting of the extracellular domain of CIRL-1, p120, fused to the carboxyl-terminal 49 amino acids of VSV-G. This segment of VSV-G consists of a 20-amino acid membrane-spanning domain and a 29-amino acid cytoplasmic domain (Guan and Rose, 1984; Guan et al., 1985). In the absence of extracellular Ca^{2+}, application of 50 pM Ltx to HEK293 cells transfected with p120/VSV-G resulted in stepwise increases in inward current (Fig. 10E, $n = 5$). The average amplitude of the current steps was 29.3 ± 0.9 pA ($n = 11$). In the presence of extracellular Ca^{2+}, currents fluctuated rapidly and individual channels were difficult to resolve (Fig. 10F, $n = 3$). These Ltx-induced conductance effects in the absence and presence of extracellular Ca^{2+} were nearly identical with those produced by Ltx in HEK293 cells transiently expressing wild-type CIRL-1 or 7TMR.

Neurexin 1α Interaction with Ltx Facilitates Channel Formation. Neurexin 1α is a neuronal Ltx receptor that binds the toxin in a Ca^{2+}-dependent manner (Petrenko et al., 1990; Ushkaryov et al., 1992). It is an integral membrane glycoprotein that is structurally unrelated to CIRL-1. The electrophysiological effects of the interaction of Ltx with neurexin 1α have not been described. For the sake of comparison with CIRL-1, the effects on conductance of the interaction of Ltx with neurexin 1α were investigated. In the presence of extracellular Ca^{2+}, application of 50 pM Ltx to HEK293 cells transfected with a plasmid encoding neurexin 1α produced a large inward current characterized by rapid fluctuations (Fig. 11A, $n = 4$). The same concentration of Ltx produced no change in membrane current when extracellular Ca^{2+} was absent (Fig. 11B, upper trace, $n = 5$). To observe single channel current steps, local perfusion of HEK293 cells ($n = 5$) for 5 s with 50 pM Ltx in the presence of extracellular Ca^{2+} (to permit toxin binding) was followed immediately by perfusion with a solution that lacked both Ltx and Ca^{2+} (Fig. 11B, lower trace). Channel formation occurred several seconds after removal of Ltx and Ca^{2+}, and the current re-

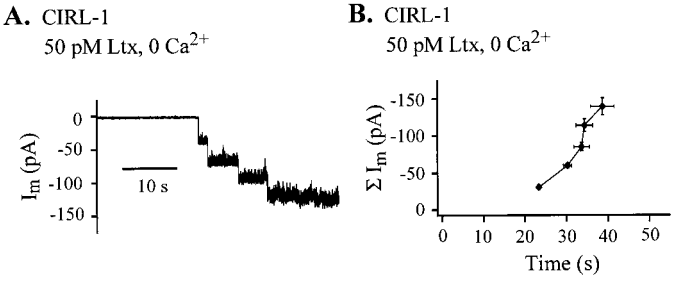

Fig. 9. Ltx-induced channel formation occurs in outside-out patches from CIRL-1-transfected HEK293 cells. Outside-out membrane patches excised from CIRL-1-transfected HEK293 cells were voltage clamped at −70 mV and current (I_m) was monitored before and during continuous application of 50 pM Ltx in the absence of extracellular Ca^{2+}. A, treatment with Ltx produced a large inward current in five of eight membrane patches (the current record shown represents an expanded time segment surrounding an initial I_m response and does not indicate the onset of toxin application). Note the appearance of large unitary current steps, which were similar to those seen in whole-cell recordings. B, plot of the average time course of Ltx-induced current responses of outside-out membrane patches ($n = 5$).

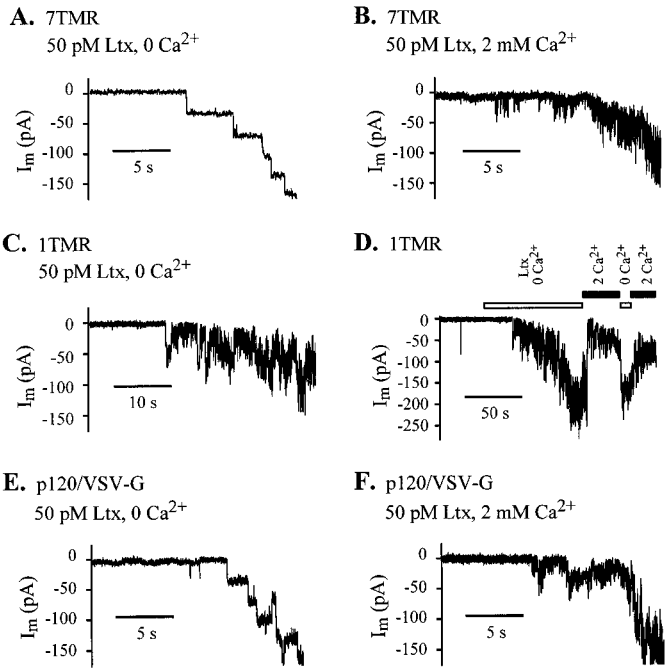

Fig. 10. Effects of mutations in the transmembrane domain and cytoplasmic tail of CIRL-1 on Ltx-induced channels in HEK293 cells. HEK293 cells were transiently transfected with plasmids encoding the extracellular domain (p120) of CIRL-1 and various mutations in the transmembrane or cytosolic domains. Transfected cells were then voltage clamped at a holding potential of −70 mV and whole-cell membrane current (I_m) was monitored. In all panels except D, the current records shown represent expanded time segments surrounding initial I_m responses and do not indicate the onset of each toxin application. A and B, 7TMR, the extracellular and transmembrane domains without the long cytoplasmic tail. (A) Ltx (50 pM) in the absence of extracellular Ca^{2+} produced discrete stepwise increases in current in a HEK293. B, Ltx (50 pM) application in the presence of extracellular Ca^{2+} produced rapidly fluctuating current increases. C and D, 1TMR, the extracellular domain with only the first transmembrane and cytosolic domains. C, Ltx (50 pM) application in the absence of extracellular Ca^{2+} caused rapidly fluctuating current increases in a HEK293 cell. D, the same cell as in C. Open and solid bars indicate periods of specified treatments in the absence and presence of extracellular Ca^{2+}, respectively. Ltx was first perfused in the absence of Ca^{2+}. Where indicated, solution containing 0 Ltx and either 2 mM Ca^{2+}or 0 $Ca^{2+}$was subsequently perfused. E and F, p120/VSV-G, the extracellular domain of CIRL-1 with all the transmembrane domains and the cytoplasmic tail replaced with the single transmembrane domain and cytosolic segment of VSV-G. E, Ltx (50 pM) application in the absence of extracellular Ca^{2+} produced discrete stepwise increases in current. F, Ltx (50 pM) application in the presence of extracellular Ca^{2+} produced rapidly fluctuating current increases.

sponses were characterized by sustained individual steps that had an average amplitude of 28.8 ± 0.7 pA (n = 14). These data suggest that toxin binding to neurexin 1α, but not channel formation, was dependent on extracellular Ca^{2+}. Once formed, the channel produced from Ltx interaction with neurexin 1α exhibited characteristics nearly identical with that of the channel produced by Ltx interaction with CIRL-1.

Discussion

Ltx Produces High Conductance Channels on Interaction with CIRL-1 or Neurexin 1α. This study demonstrates that the interaction of Ltx with transiently expressed CIRL-1 produced 400-pS channels in chromaffin and HEK293 cells. An analysis of CIRL-1 mutant receptors indicated that the transmembrane structure of CIRL-1 is not critical for channel formation. Ltx interaction with neurexin 1α transiently expressed in HEK293 cells produced channels that were nearly identical with those produced by Ltx interaction with CIRL-1, demonstrating that channel formation does not require a specific extracellular binding domain of the Ltx receptor. As discussed below, our findings suggest that the Ltx receptors CIRL-1 and neurexin 1α act to recruit and tether Ltx to the membrane to facilitate its insertion into the membrane and formation of high conductance channels and that the properties of the conductance pathway itself are determined primarily by Ltx.

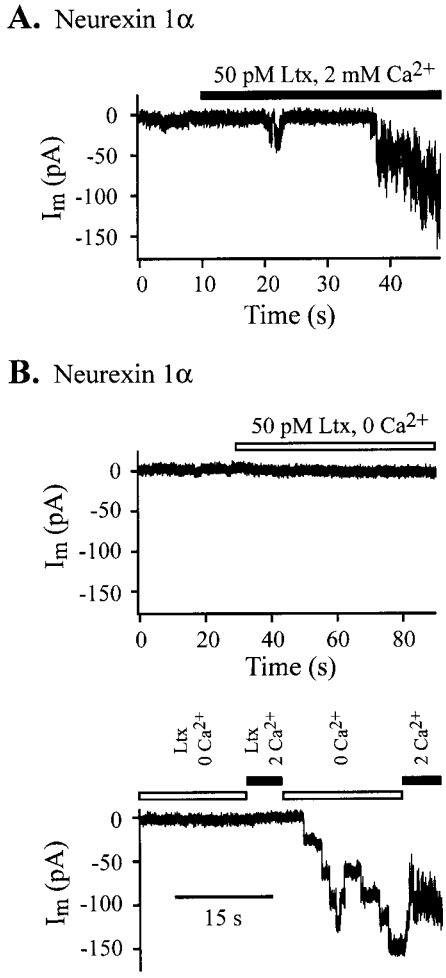

Fig. 11. Expression of neurexin 1α in HEK293 cells facilitates Ltx-induced channel formation. HEK293 cells were transiently transfected with plasmid encoding neurexin 1α. Cells were then voltage clamped at a holding potential of −70 mV and whole-cell membrane current (I_m) was monitored. A, continuous Ltx (50 pM) application in the presence of extracellular Ca^{2+} produced an inward current characterized by rapid fluctuations. B (upper trace), Ltx (50 pM) in the absence of extracellular Ca^{2+} was applied continuously. No current response was observed after 60 s of Ltx application. B (lower trace), the same cell as in B, upper trace. After a period of Ltx (50 pM) application in the absence of extracellular Ca^{2+}, a 5 sec pulse of Ltx (50 pM) was applied to the cell in the presence of extracellular Ca^{2+}. Immediately following this pulse, the cell was perfused with a solution that lacked both Ltx and Ca^{2+}. Discrete inward current steps were observed. At the indicated time, the cell was perfused with a solution containing 2 mM Ca^{2+}. Extracellular Ca^{2+} reduced the amplitude of the current response. Open and solid bars indicate periods of specified treatments in the absence and presence of extracellular Ca^{2+}, respectively.

Receptor Tethers Ltx to the Membrane to Facilitate Channel Formation. The structural basis by which CIRL-1 facilitates Ltx-induced channel formation and regulates channel behavior was investigated by determining the effects on Ltx-induced conductances of mutations in the membrane domain and cytosolic tail of CIRL-1. One of these mutants, 7TMR, consisting of the extracellular and the entire transmembrane domains but lacking the long cytoplasmic tail, produced current responses identical with those produced by Ltx interaction with wild-type CIRL-1. The results suggest that the cytoplasmic tail does not contribute to channel behavior.

Importantly, a mutant receptor, p120/VSV-G, consisting of the extracellular domain of CIRL-1 anchored to the membrane by a single transmembrane domain of VSV-G, exhibited conductance properties that were nearly identical with those of the channels produced by toxin interaction with CIRL-1 or 7TMR. These results indicate that the conductances caused by the interaction of Ltx with CIRL-1 do not require its specific transmembrane structure and do not result from the specific interaction of the transmembrane domain with other cellular proteins, including G-proteins (see below). Instead, the results suggest that CIRL-1 tethers Ltx to the membrane to facilitate channel formation.

Although the study focused on the function of CIRL-1, the ability of the Ca^{2+}-dependent receptor for Ltx, neurexin 1α, to support Ltx-induced channel formation in HEK293 cells was also investigated. As expected, the productive interaction of Ltx with neurexin 1α required extracellular Ca^{2+}. Subsequent channel formation could be studied in the presence or absence of Ca^{2+}. Stable, 400-pS channels were observed in the absence of Ca^{2+}. Membrane currents became noisy and were decreased by extracellular Ca^{2+}. These characteristics were indistinguishable from those resulting from the interaction of Ltx with transiently expressed CIRL-1 or p120/VSV-G. Neither the extracellular domains that bind Ltx nor the membrane anchoring domains of neurexin 1-α and CIRL-1 share sequence homology (Ushkaryov et al., 1992; Krasnoperov et al., 1997). The similar conductances resulting from the interaction of Ltx with CIRL-1, p120/VSV-G, and neurexin 1α indicate that the channels resulting from interaction of Ltx with a membrane-bound receptor require neither specific extracellular binding domains nor specific membrane anchoring domains of the receptor. The results suggest that the toxin molecule itself plays a primary role in determining channel characteristics.

Although a specific transmembrane domain is not critical for channel formation, the nature of the transmembrane

binding domain can affect channel characteristics. Ltx interaction with 1TMR, which consists of the extracellular domain of CIRL-1 together with its first transmembrane and cytosolic segments, produced transient current responses that obscured identification of discrete current steps in the absence of Ca^{2+}. The instability of channels produced by Ltx interaction with 1TMR may reflect instability of the conformation of the moiety in the membrane.

The Ltx-Induced Channel and the Rise in Cytosolic Ca^{2+} Do Not Require G-Protein Activation or Cytosolic Components. The prediction that CIRL-1 is a G-protein-coupled receptor raised the possibility that the channel produced by Ltx interaction with CIRL-1 results from a G-protein-requiring transduction cascade. As indicated above, such a mechanism in HEK293 cells is unlikely because of the similarity of the channels resulting from the interaction of Ltx with CIRL-1 and p120/VSV-G. Although the conductance effects are not caused by activation of a G-protein-linked pathway, the interaction of Ltx with CIRL-1 could, nevertheless, activate a G-protein-linked pathway that would be responsible for other cellular effects. It has also been suggested that Ltx interaction with CIRL-1 activates phospholipase C by a G-protein-linked mechanism, causing Ca^{2+} release from IP_3-sensitive Ca^{2+} stores (Davletov et al., 1998). However, we found that Ltx interaction with CIRL-1 in HEK293 cells in Ca^{2+}-free medium did not alter $[Ca^{2+}]_i$. This result was not simply due to an inability of our cells to couple transiently transfected G-protein-coupled receptors and phospholipase C, because application of carbachol under Ca^{2+}-free conditions to cells transfected with muscarinic M_3 receptor produced a rise in $[Ca^{2+}]_i$. It is likely that the Ltx-induced rise in $[Ca^{2+}]_i$ in Ca^{2+}-containing medium is a direct effect of Ca^{2+} influx through the channel with a secondary effect of Ca^{2+}-activated phospholipase C activity and IP_3 production. This conclusion is consistent with investigations in COS7 cells (Krasnoperov et al., 1997) and chromaffin cells that failed to demonstrate an increase in IP_3 production in response to Ltx in Ca^{2+}-free medium (M. A. Bittner and R. W. Holz, submitted).

Evidence for Multiple Steps between Ltx Binding and Channel Formation. The kinetics of Ltx-CIRL-1-dependent channel formation revealed distinct steps in channel formation. Following onset of perfusion of 5 or 50 pM Ltx to CIRL-transfected HEK293 cells, the onset of channel formation had latencies of 37 or 16 s, respectively. Importantly, following brief application of Ltx, channels first appeared many seconds after extracellular Ltx had been washed away. Thus, following binding of toxin to CIRL-1, events that can require many seconds lead to channel formation. Indeed, there is evidence that suggests that there are two states of binding of Ltx to receptor. The dissociation of prebound Ltx from synaptosomes (Tzeng and Siekevitz, 1979) or from PC 12 cells (Rosenthal et al., 1990) is biphasic with half-times of dissociation of the order of 5 to 10 min (Tzeng and Siekevitz, 1979) and several hours. It has been suggested that the binding data reflect a sequence of steps in which Ltx binds with relatively low affinity followed by a higher affinity association (Tzeng and Siekevitz, 1979). This change in the state of binding of Ltx to its receptor could correspond to channel formation.

Although binding of Ltx to receptors occurs without evidence of cooperativity (Tzeng and Siekevitz, 1979), our data suggest that subsequent events leading to channel formation may reflect cooperative interaction between Ltx-CIRL-1 complexes. There were concentration-dependent delays before channels were observed. In every case, once an initial channel was formed, more channels rapidly appeared in CIRL-1- and control-transfected chromaffin cells and in CIRL-1-transfected HEK293 cells. Furthermore, in five of eight patches excised from HEK293 cells transfected with CIRL-1, application of 50 pM Ltx produced 400-pS channels. Once the channel cascade began, the rates of channel addition in the presence of 50 pM Ltx were similar to those in whole-cell recordings (compare Figs. 8A and 9B). Because outside-out patches have less than 0.1% of the whole-cell area, the results support the notion that recruitment of additional channels is a local membrane event. The patches that did not respond to 50 pM Ltx also did not respond to subsequent application of 500 pM Ltx, probably because the patches lacked CIRL-1. It is possible that if higher concentrations of Ltx were applied to these patches, channel formation due to the direct effects of Ltx (independent of CIRL-1) would have been observed.

Because the successive conductance steps were identical in size, each conductance step probably reflected a discrete new channel. Once a channel is formed it may rapidly recruit other Ltx-CIRL-1 complexes to form additional channels. Because Ltx is a large protein (120 kDa) with multiple repeats (Kiyatkin et al., 1990), the toxin molecule itself could directly recruit more receptors or more toxin molecules.

Acknowledgments

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Erratum

An error was made in the title of the article by Hlubek et al. [Hlubek MD, Stuenkel EL, Krasnoperov VG, Petrenko AG and Holz RW (2000) Calcium-Independent Receptor for α-Latrotoxin and Neurexin H Facilitate Toxin-Induced Channel Formation: Evidence That Channel Formation Results from Tethering of Toxin to Membrane. *Mol Pharmacol* **57:**519–529]. The correct title is "Calcium-Independent Receptor for α-Latrotoxin and Neurexin 1α Facilitate Toxin-Induced Channel Formation: Evidence That Channel Formation Results from Tethering of Toxin to Membrane." We regret any inconvenience caused by this error.