Latrotoxin Stimulates Secretion in Permeabilized Cells by Regulating an Intracellular Ca\(^{2+}\) - and ATP-dependent Event

A ROLE FOR PROTEIN KINASE C

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α-Latrotoxin, a component of black widow spider venom, stimulates transmitter release from nerve terminals and intact chromaffin cells and enhances secretion from permeabilized chromaffin cells already maximally stimulated by Ca\(^{2+}\). In this study we demonstrate that chromaffin cells contain a protein antigenically similar to the cloned Ca\(^{2+}\)-independent receptor for α-latrotoxin. Although this receptor has homology to the secretin family of G-protein-linked receptors, pertussis toxin has no effect on the ability of α-latrotoxin to enhance secretion, suggesting that neither G\(_i\) nor G\(_o\) is involved in the response. Furthermore, in the absence of Ca\(^{2+}\), α-latrotoxin does not stimulate polyphosphoinositide-specific phospholipase C. α-Latrotoxin specifically enhances ATP-dependent secretion in permeabilized cells. An in situ assay for protein kinase C reveals that α-latrotoxin augments the activation of protein kinase C by Ca\(^{2+}\), and use of protein kinase inhibitors demonstrates that this activation is important for the toxin’s enhancing effect. This enhancement of secretion requires Ca\(^{2+}\) concentrations above 3 μM and is not supported by Ba\(^{2+}\) or nonhydrolyzable guanine nucleotides, which do not stimulate protein kinase C. We conclude that α-latrotoxin stimulates secretion in permeabilized cells by regulating a Ca\(^{2+}\)- and ATP-dependent event involving protein kinase C.

α-Latrotoxin (α-Ltx), a component of black widow spider venom, is a potent stimulus for transmitter release from nerve terminals (1–4) and intact chromaffin cells (5, 6). The toxin binds to cell surface proteins in both a Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent manner (7), with distinct proteins responsible for each type of binding. The first Ca\(^{2+}\)-binding protein to be cloned, neurexin 1α, interacts with α-Ltx in the presence of Ca\(^{2+}\) (8–10). Synaptosomes prepared from mice that lack the gene for neurexin 1α do not exhibit Ca\(^{2+}\)-dependent α-Ltx binding and secrete less neurotransmitter in response to α-Ltx than synaptosomes from control mice (11).

More recently, a Ca\(^{2+}\)-independent receptor for α-Ltx, termed CIRL (6) or latrophilin (12, 13), has been cloned from rat and bovine brain. The protein sequence of CIRL shows significant homology to the secretin family of G-protein-linked receptors (6). Expression of this receptor in chromaffin cells (6, 14) or HIT-T15 cells (13) increases the sensitivity of the cells to α-Ltx, demonstrating that the protein is functional in coupling to Ca\(^{2+}\) influx and secretion. Experiments with mutant receptors truncated at the COOH terminus indicate that the intracellular domains of both neurexins and CIRL are unnecessary in mediating α-Ltx binding and subsequent neurotransmitter release in intact cells (15–17). This is consistent with the finding that neurexin 1α and full-length and truncated CIRL all facilitate α-Ltx-induced channel formation (18). Ca\(^{2+}\) influx through the toxin-induced pore is sufficient to support secretion, even in the absence of receptor signaling.

Although the effects of α-Ltx in intact cells appear not to require receptor signaling, we have identified a second receptor-mediated effect of α-Ltx which cannot be attributed to changes in Ca\(^{2+}\) permeability of the plasma membrane. Besides stimulating transmitter release in intact cells, α-Ltx enhances Ca\(^{2+}\)-dependent secretion in digitonin-permeabilized cells (14, 19), which are freely accessible to Ca\(^{2+}\) in the medium. The enhancement occurs when permeabilized cells are already maximally stimulated by Ca\(^{2+}\), indicating a function for α-Ltx at a step after Ca\(^{2+}\) entry.

In this study we investigated the effects of α-Ltx in chromaffin cells in more detail. We report a major new finding: α-Ltx stimulates secretion in permeabilized cells by regulating a Ca\(^{2+}\)- and ATP-dependent event that is mediated by the action of protein kinase C (PKC). These experiments provide the first illustrations of a signaling pathway for α-Ltx distinct from its ability to induce a Ca\(^{2+}\) permeability in intact cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Bovine adrenal chromaffin cells were prepared and maintained in culture as described previously (20), except that in some experiments the medium used was Dulbecco’s modified Eagle’s medium/Ham’s F-12 rather than Eagle’s minimal essential medium. HER293 cells were plated at a density of 2.4 × 10\(^5\) cells/well in 24-well plates and transfected with 0.25 μl of serum- and antibiotic-free Dulbecco’s modified Eagle’s medium/well containing 2 μl of LipofectAMINE and 0.55 μg of DNA. Medium containing 10% serum was added after 5 h and replaced by complete medium containing antibiotics and 10 μM cytosine arabinoside after 24 h. Cells were harvested after an additional 24 h. The plasmid encoding CIRL (pCDR7) has been described previously (6).

Assays—Experiments were conducted with several α-Ltx preparations from two sources whose apparent optimal concentrations to enhance secretion in permeabilized cells were 50 pm, 0.4 nM, and 10 nM. Although the potencies of these toxin batches differed, experiments...
done with these preparations yielded comparable results. Physiological salt solution (PSS) contained 145 mM NaCl, 5.6 mM KCl, 5.6 mM glucose, 0.5 mM ascorbate, 15 mM HEPES (pH 7.4), and 2.2 mM CaCl₂ and 0.5 mM MgCl₂ unless otherwise indicated. In some experiments, the free Ca²⁺ concentration was buffered with 5 mM EGTA and 5 mM nitritro-
acetate acid at pH 7.0. The potassium glutamate solution (KGGEP) used in permeabilized cells contained 139 mM potassium glutamate, 20 mM PIPES (pH 6.6), 2 mM MgATP, and 5 mM EGTA with various amounts of CaCl₂ to yield buffered Ca²⁺ concentrations of 0–30 μM (20). In some experiments, 5 mM nitritroacetate acid was added to KGGEP (KGGEP), to yield buffered Ca²⁺ concentrations of 1–1,000 μM. Release was calculated as the amount of [³H]norepinephrine ([³H]NEm) released into the incubation medium divided by the total [³H]NEm (i.e. [³H]NEm released + [³H]NEm remaining in the cells). Stimu-
lated release was calculated as the difference between release in the presence and absence of agonist. Data are expressed as mean ± S.E. unless otherwise indicated. Significance was determined by Student’s t test. Error bars smaller than symbols were omitted from figures.

Materials—Reagents were received from the following sources. α-Ltx was from V. Krasnoprov and A. Petrenko (New York University Medical Center, New York) (8) or Alomone Laboratories (Jerusalem, Israel); [³H]NEm, [γ-³²P]ATP, and reagents for detection of enhanced chemi-
unemiscence (ECL) were from Amersham Pharmacia Biotech. Digitonin was from Fluka Chemical Corp. (Ronkonkoma, NY). Pertussis toxin was from BioWhittaker (Walkersville, MD). All other reagents were ob-
tained from Sigma.

RESULTS

The Enhancement of Secretion by α-Ltx in Permeabilized Chromaffin Cells Is Mediated by an Extracellular Protein Receptor—We have reported previously that α-Ltx enhances se-
cretion from permeabilized chromaffin cells in a dose-dependent manner (14). Because the effect of α-Ltx on permeabilized cells was readily observed after a brief incubation of intact cells with the toxin, toxin binding to an extracellular receptor rather than toxin entry seemed likely to mediate the enhancement of secretion. We confirmed the importance of the extracellular receptor in mediating the effects of α-Ltx in permeabilized cells by using two inhibitors of α-Ltx binding, trypsin and con-
canavalin A. Binding of α-Ltx to its receptors can be prevented by incubating tissues or membrane preparations with trypsin prior to exposure to α-Ltx (22). If the effects of α-Ltx in permeabilized chromaffin cells are mediated by receptors similar to those of the neuromuscular junction or in PC-12 cells, one would predict

![Fig. 1. Preincubation of intact cells with trypsin (panels A and B) or concanavalin A (panels C and D) blocks subsequent effects of α-Ltx on secretion from permeabilized chromaffin cells. Chromaffin cells were labeled with [³H]NEm, rinsed, and incubated with or without trypsin (panel A, indicated concentrations; panel B, 500 μg/ml) or 250 μg/ml concanavalin A (con A, panels C and D) for 10 min in PSS containing divalent cations. Panels A and C, cells were then incubated with or without 410 μM α-Ltx in PSS without Ca²⁺ or Mg²⁺ and with 0.1 mM EGTA. After 4 min, the toxin was removed, and cells were perme-
abilized with 20 μM digitonin in KGGEP without Ca²⁺ and with 200 μM/ml soybean trypsin inhibitor (panel A) or 4 min before the addition of 30 μM Ca²⁺ in KGGEP (also containing 200 μg/ml soybean trypsin inhibitor, panel A). Secretion was determined after 15 min. Panels B and D, cells were permeabilized with 20 μM digitonin in KGGEP without Ca²⁺ and with 200 μg/ml soybean trypsin inhibitor (panel B) for 4 min. Cells were then stimulated for 15 min in KGGEP with or without 30 μM Ca²⁺ with the indicated concentrations of α-Ltx. All KGGEP solutions also contained 2 mM MgATP. Stimulated secretion was calculated by subtracting the secretion in the absence of Ca²⁺ (usually −2%) from total secretion in the presence of Ca²⁺. n = 3 wells/group.

that trypsin should prevent the enhancement of secretion by the toxin. As expected, prior treatment of cells with trypsin specifically inhibited secretion stimulated by α-Ltx when Ltx was added before permeabilization (Fig. 1A). Incubation of cell monolayers with the indicated concentrations of trypsin for 10 min prior to incubation with α-Ltx, followed by permeabilization and stimulation by Ca²⁺, caused a dose-dependent decrease in the effects of α-Ltx on Ca²⁺-dependent secretion (Fig. 1A). The enhancement of secretion by α-Ltx was completely inhibited by a concentration of trypsin (500 μg/ml), which had no effect on secretion in the absence of α-Ltx.

In the experiment in Fig. 1A, cells were exposed to α-Ltx before permeabilization. It is possible that the function of the plasma membrane receptor is to allow the internalization of the toxin so that the toxin may interact with an intracellular target. In that case, adding α-Ltx to permeabilized cells should bypass the need for the extracellular receptor and restore to cells treated with trypsin the ability to respond to α-Ltx. Fig. 1B demonstrates that adding α-Ltx after the trypsinized cells were permeabilized did not restore the α-Ltx effect.

The lectin concanavalin A has also been reported to prevent binding of α-Ltx to membranes (22, 23). Indeed, a recently cloned Ca²⁺-independent receptor for α-Ltx (6) is extensively glycosylated (24, 25). Preincubating intact chromaffin cells with as little as a 100 nM concentration of the lectin abolished secretion stimulated by 150 μM α-Ltx (not shown). We asked whether a prior incubation of cells with lectin could prevent the effects of α-Ltx in permeabilized chromaffin cells when α-Ltx was added before (Fig. 1C) or after (Fig. 1D) permeabilization.
250 mM concanavalin A completely prevented the enhancement of secretion by α-Ltx in permeabilized cells (Fig. 1C). Again, adding α-Ltx to cells after permeabilization did not restore the enhancing effect of the toxin (Fig. 1D). The data indicate that a functional extracellular receptor is required to mediate the enhancement by α-Ltx of secretion in permeabilized cells.

A receptor that binds α-Ltx in the absence of Ca²⁺, termed CIRL or latrophilin, has recently been cloned from rat (6) and bovine (12) brain. The receptor is comprised of two subunits: an extracellular domain containing the α-Ltx binding site (p120), and a membrane-spanning and intracellular domain (p85). An antibody generated against a peptide from the intracellular COOH-terminus of p85 (CEGPPDGDGQMQLVTSL) was used to probe a blot of chromaffin cell proteins (Fig. 2). The antibody recognized a protein in bovine brain synaptosomes (lane 1), bovine chromaffin cells (lane 2), and HEK293 cells transiently transfected with a plasmid for CIRL (lane 3). Lanes 3 and 5 contained chromaffin and transfected HEK293 cells, respectively, probed with antibody that had been preblocked with peptide, demonstrating the specificity of the antibody binding. Thus, chromaffin cells possess a protein of slightly lesser mobility than the brain isoform of CIRL, which is also recognized specifically by an antibody to the COOH terminus of CIRL.

Lack of Effect of G Protein Reagents—The cloned receptor for α-Ltx (6) is predicted to have seven membrane-spanning domains homologous to membrane-spanning domains of the secretin receptor family (27), suggesting that CIRL may be a G-protein-linked receptor. ADP-ribosylation of Gαi or Gαo by pertussis toxin effectively uncouples Gβ or Gγ-linked receptors from their downstream effects. We asked whether the effects of α-Ltx in permeabilized or intact cells might be inhibited by pertussis toxin. Cultured chromaffin cells were incubated for 12 h with or without pertussis toxin, at concentrations sufficient to fully ADP-ribosylate Gαi and Gαo (28). Pertussis toxin treatment did not inhibit subsequent α-Ltx-induced secretion in either intact or permeabilized cells (not shown). Thus, if the effects of α-Ltx in permeabilized cells are mediated through a G-protein, the G-protein is not a pertussis toxin-sensitive member of the Gαi or Gαo families.

One possible effector system for G-protein-linked receptors is the activation of polyphosphoinositide-specific phospholipase C, leading to the production of IP₃ and diacylglycerol. A complicating factor is that chromaffin cells possess a Ca²⁺-activated phospholipase C, which can be stimulated by micromolar Ca²⁺ in permeabilized cells (29), or by Ca²⁺ influx through voltage-sensitive Ca²⁺ channels (30). Therefore, to ensure that any effect was caused by a direct activation of the α-Ltx receptor and not secondary to Ca²⁺ influx through a toxin-induced pore, the experiment in Fig. 3A was conducted in the absence of extracellular Ca²⁺ with 1 mM EGTA. α-Ltx (200 pM) had no effect on IP₃ levels at either 10 s or 2 min, whereas angiotensin II (Ang II, 100 nM, 10 s) increased IP₃ by 3-fold (Fig. 3A). In other experiments, α-Ltx had no effect at other times (1–4 min, not shown).

Because changes in IP₃ levels were only determined at selected time points, this result was confirmed by examining one of the consequences of IP₃ generation, the release of Ca²⁺ from intracellular stores. Chromaffin cells loaded with fura-2/AM were incubated with 50 pM α-Ltx, in PSS without Ca²⁺, and with 1 mM EGTA. In the absence of extracellular Ca²⁺, α-Ltx was unable to stimulate a rise in intracellular free Ca²⁺ (Fig. 3B, C). Binding of α-Ltx to its receptor was verified by the fact that addition of 2 mM Ca²⁺ to the bath resulted in an immediate and profound rise in intracellular Ca²⁺ (Fig. 3B). In addition, the intracellular Ca²⁺ stores were competent to undergo release because adding Ang II after α-Ltx elicited the expected Ca²⁺ response (Fig. 3C). This result is in full agreement with the direct measurement of IP₃ levels above. There is no evidence to suggest that α-Ltx interaction with the endogenous chromaffin cell receptor directly activates a G-protein-linked phospholipase C.

**ATP Dependence of the Enhancing Effects of α-Ltx in Permeabilized Cells**—When chromaffin cells were pretreated with α-Ltx and then permeabilized for 4 min before stimulation with Ca²⁺, the degree of enhancement was not uniform over the time course of the incubation with Ca²⁺ (Fig. 4A). Little or no effect was seen at 2 or 4 min, but at 8 and 18 min, secretion was enhanced by 49% and 101%, respectively. In permeabilized cells, the rate of secretion typically declines with time, even in the presence of a continuing Ca²⁺ stimulus (20). α-Ltx had no effect on the initial rates of secretion but markedly prolonged the secretory response.

We have demonstrated previously that during the first few minutes of a Ca²⁺ stimulus, two components of secretion are present. One component does not require the presence of MgATP, and has been termed “primed” secretion (20, 31). Such primed secretion probably reflects the prior action of ATP in
This was indeed the case. When cells pretreated with while having little effect on secretion that was already primed. tentively enhance the ATP-dependent component of secretion cells were incubated with 2
a nonspecific kinase inhibitor, staurosporine (Fig. 5
2m M MgATP (Fig. 5
Panels A–C, cultured chromaffin cells labeled with [3H]NE were incubated with or without inhibitors (panel A, 2 μM staurosporine; panel B, 5 μM Ro31-8220; panel C, none) for 4 min in PSS with divalent cations. Cells were then permeabilized with 20 μM digitonin in KGEP with 2 mM MgATP and 0.1 mM EGTA. Cells were then permeabilized for 4 min with 20 μM digitonin in KGEP without Ca2+, and with MgATP. The solution was removed, and the cells were incubated for 12 min with or without 30 μM Ca2+, with either 2 mM MgATP (+ATP) or 0.35 mM MgCl2 (−ATP). n = 3 wells/group.

PKC Is Involved in the Enhancement of Secretion by a-Ltx—
The ability of a-Ltx to enhance specifically the ATP-dependent component of secretion suggests the possibility that a-Ltx affects an ATP-requiring reaction that mediates the secretory pathway. We found that the effect of a-Ltx could be inhibited by a nonspecific kinase inhibitor, staurosporine (Fig. 5A). When cells were incubated with 2 μM staurosporine while having little effect on secretion that was already primed. This was indeed the case. When cells pretreated with a-Ltx were then permeabilized and stimulated with Ca2+ in the absence of MgATP, a-Ltx had no effect on secretion (Fig. 4B, open bars). The enhancement by a-Ltx was seen only when MgATP was included in the incubation with Ca2+ (Fig. 4B, filled bars). In this experiment, a-Ltx doubled the ATP-dependent secretion (the difference between secretion in the presence of ATP (filled bars) and secretion in the absence of ATP (open bars), from 9.0 to 18.1%.

One kinase that has been particularly well characterized in its effects on secretion is PKC (32–34). We asked whether PKC might be involved in the effects of a-Ltx. Preincubation with Ro31-8220 (an inhibitor of PKC which blocks the ATP binding site) inhibited by 63% the effect of a-Ltx to enhance secretion (Fig. 5B). Chelerythrine (another PKC inhibitor that blocks the substrate binding site) similarly inhibited the effect of a-Ltx (Fig. 5C). The inhibition of a-Ltx effects on secretion by Ro31-8220 was partial rather than complete, under treatment conditions that abolished the phosphorylation of an exogenous substrate (discussed below, Fig. 6). It is likely that these inhibitors are simply more effective at blocking phosphorylation of an exogenous rather than an endogenous substrate. Indeed, Ro31-8220 was similarly unable to block fully the enhancement of secretion by phorbol ester (not shown). Alternatively, a mechanism in addition to PKC activation could contribute to a-Ltx effects on secretion.

In contrast to the effects of PKC inhibitors, the enhancement of secretion by a-Ltx was not inhibited by H-89 (an inhibitor of PKA) or calmodulin-dependent protein kinase (cAMP-dependent protein kinase) or calmodulin-dependent protein kinase inhibitors KN-62, KN-93, and calmidazolium (Table I). The result suggested that PKC activation is required for the enhancement of secretion in permeabilized cells by a-Ltx. If this were the case, then one would predict that preactivation of PKC with phorbol ester (which itself enhances secretion) might prevent an additional enhancement of secretion by a-Ltx. In the absence of 12-O-tetradecanoylphorbol 13-acetate (TPA), a-Ltx caused a substantial enhancement of secretion (Fig. 5D).
However, incubation of cells with 100 nM TPA for 20 min before the addition of α-Ltx completely prevented the enhancement of secretion by the toxin.

If PKC is involved in mediating the effects of α-Ltx, then loss of the enzyme by down-regulation should prevent the enhancement by α-Ltx. Incubation of cells for 18 h with 1 μM TPA, a treatment that depletes cellular PKC by 90–95% (33), completely abolished the ability of α-Ltx to enhance secretion in permeabilized cells (not shown). The data implicated PKC in regulation of the enhancement of secretion in permeabilized cells by α-Ltx. Inhibiting the enzyme or reducing its levels by prolonged activation blocked the enhancement of secretion by α-Ltx. Similarly, activating PKC before the addition of α-Ltx prevents the toxin’s effects.

We thus investigated whether PKC was activated by α-Ltx using an in situ assay for the enzyme. PKC activity was measured directly in permeabilized cells by determining the extent of phosphorylation of an exogenous peptide substrate (21). Chromaffin cells were permeabilized for 4 min in KGEP containing digitonin and then stimulated with or without 30 μM Ca²⁺ and with or without 250 μM α-Ltx with 3 mM MgATP, in the continuing presence or absence of inhibitor. Concentrations of α-Ltx used in the three experiments were 410, 300, and 50 μM, respectively.

α-Ltx has two effects on chromaffin cells. The toxin interacts with an endogenous receptor to increase Ca²⁺ influx in intact cells, thus stimulating secretion. This effect occurs via the production of a high conductance channel (18) rather than through the activation of G-protein-coupled signaling pathway because it occurs with truncated receptors lacking transmembrane and intracellular domains (15, 17). This paper focuses on a second effect of α-Ltx and presents a major new finding: α-Ltx, through its interaction with its extracellular receptor, enhances secretion in permeabilized chromaffin cells by a mechanism involving PKC. We have studied this phenomenon in permeabilized cells because the interpretation of α-Ltx effects on secretion in intact cells is complicated by the toxin’s effects on Ca²⁺ permeability. The result reported here is the first indication of an intracellular signaling pathway for the Ca²⁺-independent α-Ltx receptor, a G-protein-linked receptor whose endogenous ligand remains unknown. We additionally demonstrate that the enhancement of secretion is entirely Ca²⁺-and ATP-dependent and is not observed when Ba²⁺ or guanine nucleotides are used to stimulate secretion. These results are discussed below.

**DISCUSSION**

α-Ltx Effects in Permeabilized Cells—We reported previously that chromaffin cells exhibit α-Ltx binding, the majority of which is Ca²⁺-and independent (14). Preincubation of intact cells with α-Ltx without divalent cations and with EGTA led to enhanced secretion in cells subsequently permeabilized and stimulated by Ca²⁺. Here we show that the effect of α-Ltx is blocked by prior incubation of intact cells with trypsin and concanavalin A (Fig. 1), indicating a role for an endogenous glycoprotein receptor for α-Ltx. If, as has been suggested recently (36), the function of the plasma membrane receptor is to facilitate toxin entry into the cytoplasm, then permeabilization with digitonin should reverse the effects of agents which prevent α-Ltx binding, by allowing α-Ltx access to the cell’s interior. However, α-Ltx had no effect following trypsin or concanavalin A treatment, even when the toxin was added after permeabilization (Fig. 1, B and D). This result underscores the importance of the plasma membrane receptor in mediating the effects of α-Ltx in permeabilized as well as intact cells.

The enhancement of secretion in permeabilized cells is mediated by a cloned, Ca²⁺-independent receptor for α-Ltx, whose transient expression increases the sensitivity of both intact and permeabilized cells to stimulation by α-Ltx (14). The effects of
high α-Ltx concentrations acting on endogenous receptors in nontransfected cells are virtually identical to those of very low concentrations of α-Ltx acting on transiently expressed CIRL. Moreover, an 85-kDa protein in nontransfected chromaffin cells is recognized by an antibody to the COOH terminus of CIRL. Taken together, the data indicate that the effects of α-Ltx on intact and permeabilized cells are mediated by an endogenous receptor identical to or closely resembling CIRL.

Although CIRL appears to be a G-protein-linked receptor, the effects of α-Ltx were insensitive to pertussis toxin, suggesting that neither Gi nor Gq is required for the stimulatory effects of α-Ltx. Furthermore, binding of α-Ltx to its receptor does not directly activate phospholipase C (Fig. 3), as determined by both production of IP3 and release of Ca2+ from intracellular stores. The inability of α-Ltx to elicit Ca2+ release from intracellular stores is consistent with its inability to stimulate secretion in the absence of extracellular Ca2+ (14).

The Role of PKC in Mediating the Effects of α-Ltx in Permeabilized Cells—The α-Ltx-induced enhancement occurred late in the time course of secretion (Fig. 4A), with α-Ltx having no effect during the first minutes of the Ca2+ stimulus. This is consistent with a requirement for ATP (Fig. 4B) because the later phase of secretion is completely ATP-dependent. In permeabilized cells, Ca2+ and guanine nucleotides activate phospholipase C, whereas α-Ltx activates neither phospholipase C (35) nor PKC. 2 Both Ca2+ and guanine nucleotides activate phospholipase C, but only Ca2+ activates PKC. Thus, the inability of α-Ltx to enhance secretion stimulated by Ba2+ and guanine nucleotides corroborates a role for PKC in mediating the toxin’s effects in permeabilized cells. Moreover, the sensitivity of the α-Ltx effect to Ca2+ is not identical to the Ca2+ sensitivity of the regulated pathway. α-Ltx was unable to enhance secretion at Ca2+ concentrations below 10 μM, despite the fact that this secretion is almost entirely ATP-dependent. The data suggest that Ca2+ itself is specifically required in the signaling pathway by which α-Ltx effects are mediated.

α-Ltx-induced secretion in intact cells depends strongly upon the ability of the toxin to facilitate divalent cation entry through ion channel formation. We conclude that α-Ltx effects in permeabilized cells occur through an α-Ltx-enhanced activation of PKC which requires both high micromolar Ca2+ that directly enters permeabilized cells and a separate receptor-

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mediated event. This receptor-mediated effect is likely to be present in intact cells, but it is experimentally difficult to distinguish its contribution to the secretory response. In one study using patch-clamped rat adrenal chromaffin cells, low doses of α-Ltx which did not produce channel activity or a rise in intracellular Ca\(^{2+}\) were able to enhance depolarization-induced exocytosis (26). The receptor-mediated action of α-Ltx may reflect the physiological function of the still unknown endogenous ligand for CIRL.

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