α-Latrotoxin Induces Exocytosis by Inhibition of Voltage-dependent K⁺ Channels and by Stimulation of L-type Ca²⁺ Channels via Latrophilin in β-Cells

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The spider venom α-latrotoxin (α-LTX) induces massive exocytosis after binding to surface receptors, and its mechanism is not fully understood. We have investigated its action using toxin-sensitive MIN6 β-cells, which express endogenously the α- LTX receptor latrophilin (LPH), and toxin-insensitive HIT-T15 β-cells, which lack endogenous LPH. α-LTX evoked insulin exocytosis in HIT-T15 cells only upon expression of full-length LPH but not of LPH truncated after the first transmembrane domain (LPH-TD1). In HIT-T15 cells expressing full-length LPH and in native MIN6 cells, α-LTX first induced membrane depolarization by inhibition of repolarizing K⁺ channels followed by the appearance of Ca²⁺ transients. In a second phase, the toxin induced a large inward current and a prominent increase in intracellular calcium ([Ca²⁺]ᵢ) reflecting pore formation. Upon expression of LPH-TD1 in HIT-T15 cells just this second phase was observed. Moreover, the mutated toxin LTX⁴⁴(C), which is devoid of pore formation, only evoked oscillations of membrane potential by reversible inhibition of iberiotoxin-sensitive K⁺ channels via phospholipase C, activated L-type Ca²⁺ channels independently from its effect on membrane potential, and induced an inositol 1,4,5-trisphosphate receptor-dependent release of intracellular calcium in MIN6 cells. The combined effects evoked transient increases in [Ca²⁺]ᵢ in these cells, which were sensitive to inhibitors of phospholipase C, protein kinase C, or L-type Ca²⁺ channels. The latter agents also reduced toxin-induced insulin exocytosis. In conclusion, α-LTX induces signaling distinct from pore formation via full-length LPH and phospholipase C to regulate physiologically important K⁺ and Ca²⁺ channels as novel targets of its secretory activity.

The black widow spider venom α-latrotoxin (α-LTX) induces massive exocytosis of synaptic vesicles and of large dense core vesicles. This property has been extensively exploited to investigate the molecular mechanisms underlying exocytosis (1, 2). Toxin action requires first the binding to a surface receptor, and three distinct receptors for α-LTX have been identified: the latrophilins (LPHs), which contain a large extracellular adhesion molecule domain and a C-terminal portion bearing the signature of G-protein-coupled receptors (3, 4), neurexin Iα and β (2), and the receptor-like protein-tyrosine phosphatase σ (5). It is generally accepted that the toxin inserts subsequently as a tetramer into membranes to form a stable, cation-permeable pore (6), and the ensuing Ca²⁺ influx plays a major role in the activation of exocytosis. Indeed, expression of a C-terminally truncated form of LPH lacking all except the first transmembrane domain is sufficient to establish toxin-induced pores leading to calcium influx in epithelial HEK293 cells and sensitization of exocytosis in chromaffin cells (7–9).

Although these findings indicate that receptor-mediated signal transduction is not required for the action of α-LTX, other observations suggest that pore-mediated Ca²⁺ influx is not sufficient to explain the action of the toxin. α-LTX sensitizes exocytosis to Ca²⁺ in chromaffin cells and in synaptosomes (10, 11). Moreover, a point mutated toxin increases exocytosis in the absence of ion fluxes through a toxin-induced pore (12). The intracellular actions provoked by α-LTX to induce exocytosis are not fully resolved, apart from pore-mediated Ca²⁺ influx. Depending on the system, they may implicate phospholipase C with subsequent activation of protein kinase C and of release of Ca²⁺ from the intracellular stores (10, 11, 13–15).

We have previously demonstrated that α-LTX receptors are also expressed on primary β-cells and the toxin induces exocytosis of the peptide hormone insulin (16). Clonal β-cells cell lines provide a useful model for toxin-induced exocytosis of large dense core vesicles, because they differentially express LPH; whereas high levels of LPH are found in the toxin-sensitive MIN6 cells, HIT-T15 cells express only very low amounts and are toxin-insensitive (16). This situation is clearly distinct from PC12, chromaffin, or HEK293 cells, which are toxin-sensitive (7–9). β-Cells therefore provide a very suitable model and, moreover, ion channels, signal transduction events and the molecular mechanism underlying insulin exocytosis are relatively well characterized (17–19).

Using this model in combination with truncated or full-length receptors, we addressed the issue of receptor-mediated signaling and pore formation-dependent events in the action of latrotoxin on intracellular calcium, membrane conductances, and exocytosis. To obtain a better understanding of the underlying events, we also compared the effect of native α-LTX to the action of recombinant mutated latrotoxin, LTX⁴⁴(C), which is devoid of pore formation (12). Our data demonstrate for the first time a regulation of K⁺ channels and of L-type Ca²⁺ chan-
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defined by its receptor latrophilin via phospholipase C and contributes to the secretory effect.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal anti-Myc and anti-HA antibodies were purified from culture medium of myeloma cells generously provided by Dr. K. Matter (Université de Genève), polyclonal anti-Myc antibodies were obtained from Research Diagnostics (Flanders, NJ). Anti-Ca,1.2 and anti-Ca,1.3 antibodies were obtained from Alomone (Jerusalem, Israel). α-LTX was prepared and iodinated in Dr. Ushkaryov’s laboratory (12). Commercial preparations used (Calbiochem) gave qualitatively similar results. Recombinant LTX was produced and purified as described previously (12). Protein kinase and phospholipase C inhibitors, A23187, apamin, and ibotenic acid were from Calbiochem, calcein from Latoxan (Apt, France). The m9 subclone of MIN6 cells (20) was kindly provided by Dr. S. Seino (Chiba, Japan) and was used throughout this study. PC12 cells were generously provided by Dr. B. Rudkin (Ecole Nationale Supérieure, Lyon, France) and used between passages 2 and 9.

**Molecular Cloning**—The N-terminal HA-affinity epitope was obtained by inserting a sequence coding for HRQLPGDNDSTAGNSS between amino acids 24 and 25. To this end the adjacent sequences were amplified from full-length LPH using the primers pairs 1 (5’-GGGGATCCGTTGATAGCGGTTTGACTC-3’) and 2 (5’-GGGATCCGTTGATAGCGGTTTGACTC-3’). The amplicons were subcloned into pKS portion of LPH into the HindIII/KpnI sites. All sequences were verified by sequencing of both strands. The generation of LPH-TD1–7 and of LPH-TD1–5 has been described before (21). The fusion protein syt2-C2AB was constructed by PCR amplification of syt2-C2AB (amino acids 101–422) and insertion in-frame into peGFP. The construct was subsequently excised by restriction with HindIII and XhoI.

**Cell Culture, Transient Transfection, Secretion, and Immunoblotting**—HIT-T15 cells grown on coverslips were used as control cells. HIT-T15 cells grown on coverslips were transiently transfected with LPH/CIRL constructs and a plasmid expressing DsRed fluorescent protein to identify transfected cells. 72 h after transfection, cells were loaded with 13 mM of the fluorescent probe indo-1/AM (Sigma) in KRB (3 mM glucose and 0.05% BSA) through a “pouring” pipette with a tip opening of 10–20 μm and positioned 2 cell diameters (40 μm) from the cell. Imaging) and deblurred by deconvolution (Autodeblur, Universal Imaging). The same set-up was used for imaging of living cells. In this case cells on coverslips were kept in 1 ml Krebs-Ringer buffer (KRB) (23) supplemented with 0.05% BSA (KRB-BSA) at 37 °C on a heated stage during acquisition. Toxin in KRB-BSA was pressure ejected (5 p.s.i. from a micropipette held at ~20 mm from the cell.

**Carbon Fiber Amperometry**—Pheochromocytoma (PC12 cells (50,000 cells per 35-mm dish) were transfected for 8 h using Lipofectamine and assayed 48 h later. Prior to recording, cells were loaded for 1 h (1 mM dopamine, 1 mM ascorbic acid, pH 7.4, in culture medium) and washed twice. During amperometry cells were kept at ambient temperature (23 °C) in modified Ringer buffer (145 mM NaCl, 5 mM KCl, 1.3 mM MgCl2, 3 mM CaCl2, 20 mM Hepes, pH 7.4, 10 mM glucose) on the stage of the inverted microscope described above and cotransfected cells identified by fluorescence of eGFP. Cells were stimulated by a 20-s suction (0.05% from a micropipette mounted on an Eppendorf microinjection (Femtojet) held at ~2 cell diameters (40 μm) from the cell to be recorded. Only single, round cells were used, and carbon fibers were positioned using a piezoelectric driver (PCS-1000, Burleigh Instruments). The micropipette and the carbon fiber (ProCFE, 5 μm, Axon Instruments) held at 700 mV were kept at approximately the same distance throughout experiments. Fibers were used only if the root mean square was <0.5 nA at 700 mV. Currents were recorded using an EPC9 at 2.5 kHz, low pass filtered at 1 kHz, and spikes were analyzed with a program generously provided by Dr. F. Borges (Universidad de La Laguna, Tenerife, Spain) (24).

**Measurements of [Ca2+]i**—HIT-T15 cell lines grown on coverslips were cotransfected with LPH/CIRL constructs and a plasmid expressing DsRed fluorescent protein to identify transfected cells. 72 h after transfection, cells were loaded with 13 mM of the fluorescent probe indo-1/AM (Sigma) in KRB (3 mM glucose) for 45 min at 37 °C, washed and maintained at room temperature in KRB (3 mM glucose and 0.05% BSA) before fluorescence measurements. MIN6 cells were handled identically except that transfection was omitted. [Ca2+]i measurements were carried out as already described (25). Test substances were applied in KRB (3 mM glucose and 0.05% BSA) through a “pouring” pipette with a tip opening of 10–20 mm and positioned at a distance of 50–100 mm from the cell. The absence of mechanical artifacts due to drug application was confirmed by applying external medium (KRB) to the cells.

**Electrophysiological Recordings**—The whole cell recording mode of the patch clamp technique was used as already described (25). Transiently transfected cells were identified as given above. KRB was used as standard extracellular solution contained, and the osmolality was...
adjusted to 300–310 mosm/kg with sucrose. The recording pipette was filled with an artificial intracellular saline containing (in mM): 150 potassium chloride, 2 MgCl₂, 1.1 EGTA, and 5 HEPES (pH 7.3 ± 0.01 with KOH; osmolality: 290 mosm/kg). Drugs were applied as described for calcium measurements, and all experiments were performed at room temperature (20–22 °C). To isolate voltage-dependent Ca²⁺ currents, K⁺ currents were eliminated by replacing K⁺ gluconate used to formulate the intracellular electrolyte with isomolar N-methylglucamine gluconate. The solution was then buffered to pH 7.3 with HEPES-glucosinate. Because Ca²⁺ currents were not always stable and often disappeared during whole cell recording, current-voltage (intensity-voltage) relations for calcium currents were constructed using cells that showed little or no rundown within 10–15 min after impalement. Results are expressed as mean ± S.E. Each experiment was repeated several times.

**Toxin Binding**—Cells were transiently transfected in 24 wells and detached 48 h later by the use of 10 mM EDTA in KRB at 37 °C. Cells were centrifuged (5 min, 2,000 × g, 4 °C) and resuspended in KRB containing 1.3 mM CaCl₂ but no EGTA. Aliquots were kept for cell counting and protein determination. 200 ml of cell suspension was transferred to 1.5-ml Eppendorf tubes and binding initiated by addition of radiolabeled toxin (26) for 15 min at 37 °C. The binding was stopped by the addition of ice-cold buffer and immediate centrifugation at 10,000 × g for 5 min at 4 °C. The pellets were washed once with ice-cold KRB, centrifuged at 10,000 × g for 2 min at 4 °C, and counted for radioactivity in a γ-counter. Nonspecific binding was determined in the presence of 50 nM native toxin.

**RESULTS**

**Surface Expression of Full-length and C-terminally Truncated LPH Constructs**—C-terminal truncations of LPH, which lack the domains homologous to G-protein-coupled receptors, have been reported to suffice for actions of α-LTX in several cell systems, although these constructs are not always efficiently expressed (7, 8, 21). In our attempt to test their function in toxin-mediated exocytosis of insulin-containing large dense core vesicles we transiently expressed full-length and truncated LPH in toxin-insensitive hamster HIT-T15, or in mouse MIN6 insulinoma cells, which express endogenous LPH and are toxin-sensitive (16). Three truncated constructs were made: LPH-TD1, terminated after the first transmembrane domain; LPH-TD1–5 (amino acids 1–1020), limited to five transmembrane domains; and LPH-TD1–7 (amino acids 1–1099), truncated after the last transmembrane domain.

We examined first their surface expression after transient transfection using constructs, which carry an HA tag at the extracellular N terminus and an Myc tag at their intracellular C termini (Fig. 1A). To identify surface expression, antibody binding to HA tags was conducted on ice prior to fixation. The results observed confirmed surface expression of LPH and LPH-TD1 in HIT-T15 cells as well as LPH-TD1 in MIN6 cells (Fig. 1B). Surface expression was also observed for LPH-TD1–7, whereas LPH-TD1–5 was visualized only in a small number of cells (data not shown). As shown in Fig. 1C, expression levels of total LPH and LPH-TD1 were comparable. The antibody used is directed against the N-terminal hemagglutinin epitope. Latrophilin is processed in the endoplasmic reticulum into two non-covalently bound subunits, N-terminal p120 and C-terminal p85 (25). Only the 120-kDa extracellular N-terminal form was detected on Western blots, which indicated that transiently expressed full-length LPH was completely cleaved in HIT-T15 cells.

To further quantify the expression, we analyzed binding of 125I-α-LTX to transiently expressed constructs in intact HIT-T15 cells (Fig. 2A). Similar affinities (Kₚ values of ~0.25 nM) were observed for LPH, LPH-TD1, LPH-TD1–7, and LPH-TD1–5, whereas only negligible binding was detected in control cells. Moreover, the amount of binding sites expressed was comparable for all constructs except for LPH-TD1–5. These observations indicated that LPH, LPH-TD1, and LPH-TD1–7 were efficiently expressed and bound the toxin with comparable characteristics in insulin-secreting cells. To assess whether these proteins are capable to mediate α-LTX-induced effects, we determined functional responses of transiently expressed LPH or its truncated forms first on exocytosis of large dense core vesicles.

**Full-length LPH Mediates Exocytosis Induced by α-LTX or LTX₇₋₅C**—Next we determined the effects of LPH and LPH-TD1 in α-LTX-induced exocytosis and transiently cotransfected these receptors in HIT-T15 cells with a plasmid coding for human prepro-insulin as reporter gene (Fig. 2A). Membrane depolarization by 48 mM KCl induced a 3- to 4-fold increase, which was not altered by expression of the different constructs. As expected, α-LTX up to 2 nM did not induce release of human C-peptide in control transfected HIT-T15 cells, whereas cells expressing full-length LPH increased human C-peptide release more potently than KCl to ~10-fold of basal secretion. The LPH construct LPH-TD1–7 behaved similarly though a slightly greater efficacy was constantly observed. Cells expressing LPH-TD1 demonstrated only a marginal increase in toxin-evoked hormone secretion (Fig. 2A). This observation stems from a large series of experiments done at different

![Figure 1. Full-length latrophilin and truncated latrophilin are expressed on the surface of HIT-T15 or MIN6 cells and bind α-LTX to comparable extent. A, scheme of constructs used (with N-terminal HA tags and C-terminal Myc tags). B, HIT-T15 or MIN6 cells were transiently transfected with plasmids encoding full-length LPH (LPH, LPH₁–1020) or LPH-TD1 (LPH₁–890). 48 h later, cells were immunostained at 4 °C with (PERM) or without permeabilization (NP) using anti-Myc (C terminus) or anti-HA (N terminus) antibodies. C, Immunoblot of control (CON) or transiently transfected HIT-T15 cells (with indicated constructs) using an antibody directed against the N-terminal HA-epitope. Molecular masses are given in kDa, and p120 is indicated by an arrow. D, Scatchard analysis of binding of 125I-α-LTX to HIT-T15 cells transfected with plasmids encoding following constructs: full-length LPH/CIRL (filled bars), LPH lacking the intracellular C terminus (LPH₁–1020, □), LPH truncated after the fifth amino acid (LPH₁–5, □), or after the first transmembrane domain (LPH₁–7, □). Mean values are given from three separate experiments. Inset: total binding (4 nM 125I-α-LTX, filled bars) and unspecific binding (in the presence of a 25-fold excess of unlabeled toxin, open bars).](image-url)
FIGURE 2. Effect of native α-LTX or recombinant LTXN4C on secretion from HIT-T15 cells and MIN6 cells expressing LPH or its truncated forms. A, HIT-T15 cells were transiently cotransfected with indicated constructs (or control plasmid) and a plasmid encoding human prepro-insulin and with a control plasmid (open symbols) or in the presence of 48 mM KCl (filled symbols) or in the absence of toxin. *, 2p < 0.05 as compared with control transfected cells. B, MIN6 cells were transiently cotransfected with indicated constructs and a plasmid encoding human growth hormone. The release of growth hormone was measured as in A. n = 3–8 for each point. *, 2p < 0.05 as compared with control transfected cells. C, effect of recombinant LTXN4C on secretion from HIT-T15 cells transiently cotransfected with a plasmid encoding human prepro-insulin and with a control plasmid (open circles) or with a plasmid encoding LPH (closed circles). n = 3–6 for each point; *, as in B. D, effect of recombinant LTXN4C (closed circles) or KCl (48 mM, open circle) on secretion from native MIN6 cells. n = 3–6 for each point; *, as in B.

LPH-TD1 has been shown to suffice for α-LTX binding and induction of exocytosis in neuroendocrine PC12 cells (7, 8). One major difference between PC12 and HIT-T15 cells resides in the expression levels of endogenous LPH. For this reason we also tested the effect of LPH-TD1 expression in MIN6 cells, known to express endogenous LPH receptor (16) (Fig. 2B). In control cells, α-LTX increased the release of reporter gene product. Co-expression of full-length LPH sensitized secretion to α-LTX, and this effect was also apparent in the case of expression of LPH-TD1.

We also tested whether a mutant toxin, LTXN4C, can induce secretion, because this protein does not assemble in pore-forming tetramers (21). As shown in Fig. 2 (C and D), LTXN4C induced insulin secretion in the subnanomolar range. Again transient expression of LPH was required in HIT-T15 cells to observe an effect (Fig. 2C), whereas responses similar to those induced by 48 mM KCl were observed in native MIN6 cells (Fig. 2D).

Our LPH-TD1 construct differed by one amino acid from those truncated forms, which have been shown to enhance sensitivity to α-LTX in pheochromocytoma cell line PC12 cells (7, 8). To control further for expression and function of our construct we also measured exocytosis in PC12 cells. Exocytosis assessed by amperometry in non-transfected cells demonstrated a vigorous secretory response after application of 1 nM α-LTX (Fig. 3, A and C) but only a spurious response in the case of 0.1 nM toxin (concentrations in the pipette). We therefore used this concentration to test whether LPH or our LPH-TD1 construct may sensitize these cells to the effects of the toxin. Transient expression of either construct sensitized cells to the toxin to similar extent (Fig. 3, A and C). In addition, mutant toxin LTXN4C induced exocytosis albeit to a lesser extent than wild-type toxin (Fig. 3, B and C). This demonstrates that the construct used by us behaved similar to the ones previously reported in PC12 cells (7, 8) and that LPH-TD1 is indeed sufficient to sensitize exocytosis in cells expressing already endogenous LPH.

The requirement of full-length LPH for α-LTX-induced secretion in HIT-T15 cells as well as the effects of LTXN4C in clonal β-cells and PC12 cells clearly indicated the presence of signaling pathways distinct from pore-induced influx of calcium and other ions. We therefore investigated these putative pathways by examining first the effects of the toxins on free [Ca2+]i.

Full-length LPH, but Not C-terminally Truncated LPH-TD1, Mediates α-LTX-induced Oscillations in [Ca2+]i—We first measured whether transient expression of the constructs may alter basal levels of [Ca2+]i. A number of HIT-T15 or MIN6 cells showed spontaneous fluctuations of varying amplitude and frequency in basal [Ca2+]i (active cells). These oscillations were completely blocked in Ca2+-free extracellular medium or in the presence of Ca2+ channel inhibitors. Others exhibited a stable [Ca2+]i (silent cells) over a prolonged time period. The values of intracellular calcium were comparable in MIN6 cells (119.5 ± 2.4 nM; n = 30), to native HIT-T15 cells (115.5 ± 2.4 nM; n = 14), or HIT-T15 cells expressing full-length LPH (115.3 ± 2.5 nM, n = 45) or LPH-TD1 (114.7 ± 2.4 nM, n = 21). Therefore expression of full-length or truncated LPH did not affect basal [Ca2+]i.

Application of 2 nM α-LTX to native HIT-T15 cells did not alter [Ca2+]i (n = 14, data not shown). HIT-T15 cells expressing full-length LPH exhibited an increase in [Ca2+]i, upon exposure to 2 nM α-LTX (Fig.
In MIN6 cells nickel (Ni^{2+}, 0.5 mM), a calcium channel inhibitor, completely abrogated the phase I response and inhibited partially phase II (Fig. 4D). Ni^{2+} also reduced both KCl- and α-LTX-evoked insulin release by 97.1 ± 8.3 and 92.3 ± 6.1%, respectively (n = 6). As a comparison, the effects of KCl (48 mM) are also given for HIT-T15 and MIN6 cells (Fig. 4E).

**LTX^{NAC} Increases [Ca^{2+}], in MIN6 Cells by Calcium Mobilization and by Influx Through L-type Voltage-dependent Ca^{2+} Channels**—Our preceding observations suggested that the effect of α-LTX includes two distinct phases and that phase I differs from pore formation. We therefore examined the effects of LTX^{NAC} in MIN6 cells, because this recombinant toxin should not induce pore formation. As shown in Fig. 5A, the mutated toxin provoked a sharp, transient rise in [Ca^{2+}]i, in all cells tested (332 ± 8 nM above baseline; n = 46). In stark contrast to the native toxin, LTX^{NAC} never induced a plateau phase (compare Figs. 5A and 4C). We sometimes observed a slowly decreasing phase as shown in Fig. 5A (trace a; 14 out of 46 cells). In the absence of extracellular calcium ([Ca^{2+}]o), still increased by 91 ± 13 nM in 15 out of 28 cells (see traces a and b). This suggests that LTX^{NAC} is capable of inducing the mobilization of Ca^{2+} from intracellular stores.

To determine the implication of Ca^{2+} influx and mobilization we employed the L-type channel blocker calcisertine (27) and the inositol 1,4,5-trisphosphate-receptor antagonist xestospongin C (28). Both inhibited largely the rise in [Ca^{2+}]i (Fig. 5A; calcisertine 28 ± 7 nM, n = 7; xestospongin C 88 ± 8 nM, n = 8). Changes in [Ca^{2+}]i, induced by α-LTX, have been linked previously to the activation of phospholipase C (13). Phospholipase C can be inhibited in insulinoma cells by the compound U73122 (29, 30). Indeed, U73122, but not its inactive stereoisomer U73343 abolished the increase in [Ca^{2+}]i, evoked by LTX^{NAC} (32 ± 9 nM, n = 11 and 288 ± 10 nM, n = 6, respectively). We subsequently tested whether protein kinase C (PKC) may be implicated as a downstream target. Two reagents that inhibit PKC, BIS I and Gö 6983 (31, 32), considerably reduced the effect of LTX^{NAC} on [Ca^{2+}]i (56 ± 9 nM, n = 9 and 149 ± 40 nM, n = 14). Note that in the case of Gö 6983 we observed mainly shortening of the increase in [Ca^{2+}]i, in several cells (trace b, 4 out of 14 cells).

A rise in [Ca^{2+}]i, induces exocytosis, and binding of C2-domain-containing proteins, such as the cytosolic domain of synaptotagmin, to the plasma membrane is thought to be important for this process (33, 34). We employed this mechanism to compare further the effect of α-LTX and LTX^{NAC} in living MIN6 cells. To this end MIN6 cells were transiently transfected with a plasmid expressing the two cytosolic C2-domains of synaptotagmin 2 linked to eGFP (syr2-C_{2AB}-eGFP). As shown in Fig. 5B (upper panel), exposure of these cells to 1 nM α-LTX resulted in complete translocation to the plasma membrane, whereas in the case of LTX^{NAC} the extent of translocation was less prominent (n = 4 for each condition). Analysis of the time course revealed that α-LTX provoked first a partial translocation followed by complete translocation of syr2-C_{2AB}-eGFP. In contrast, LTX^{NAC} induced only partial translocation with clear oscillatory patterns (Fig. 5B, lower panel).

Because inhibitors of L-type calcium channels and PKC abolished the effect of LTX^{NAC} on [Ca^{2+}]i, we asked whether these agents might alter toxin-induced insulin secretion. As expected, the Ca^{2+}E channel blockers nifedipine and calcisertine reduced secretion subsequent to membrane depolarization by KCl (Fig. 5C, upper panel). The blockers also reduced secretion evoked by LTX^{NAC} indicating the role of L-type channels in its action. Nifedipine did not alter hormone release induced by the calcium-ionophore A23187 underscoring the specificity of the inhibitors at the concentrations used. Similarly the PKC inhibitors Gö 6983, BIS I, and staurosporine reduced LTX^{NAC}-evoked insulin secre-
FIGURE 5. LTX<sup>4HCl</sup> evokes transient Ca<sup>2+</sup>-influx, Ca<sup>2+</sup>-release from intracellular stores, translocation of synaptotagmin C<sub>AB</sub>-domains, and secretion in MIN6 cells via phospholipase C and PKC. A, determination of cytosolic free calcium (in nanomolar) in individual MIN6 cells loaded with indo-1. Native MIN6 cell were exposed to LTX<sup>4HCl</sup> alone (0.2 mM; as indicated by horizontal bar) or in the presence of indicated agents (calciseptine, 1 μM; xestospongin C, 3.3 μM; U73122, 1 μM; U73343, 1 μM; Gö 6983, 5 μM; BIS I (1 μM). In certain conditions two types of responses were observed as indicated by a and b. B, MIN6 cells were transiently transfected with the calcium-sensing C<sub>AB</sub> domain of synaptotagmin 2 (linked to eGFP) and stimulated by 1 μM α-LTX or LTX<sup>4HCl</sup>. Images were recorded before and during stimulation (upper panel) and translocation measured (lower panel). C: upper panel, insulin secretion from MIN6 cells was stimulated by either KCl (35 mM), LTX<sup>4HCl</sup> (0.3 mM), or the calcium ionophore A23187 (calcimycin, 10 μM) in the absence or presence of nifedipine or calciseptine. Lower panel, MIN6 cells were kept in KRB or stimulated with LTX<sup>4HCl</sup> (0.3 mM) in the absence or presence of Gö 6983 (10 μM), BIS I (10 μM), or staurosporine (15 μM). n = 4–6 for each point. *p < 0.05 as compared with the absence of channel blockers or PKC inhibitors.

We investigated subsequently the time course in more depth in HIT-T15 cells expressing full-length LPH (Fig. 6B). To monitor membrane conductance and K<sup>+</sup> current, respectively, hyperpolarizing and depolarizing voltage steps of constant amplitude were repeatedly applied (every 10 s, Fig. 6B, trace a). During an initial period α-LTX did not induce any significant change on steady-state current (Fig. 6B, trace a, phase I) but slowly reduced the K<sup>+</sup> current evoked by electrical depolarization from −40 to +40 mV (Fig. 6B, trace b). The maximal effect was obtained 80 s after the beginning of toxin ejection. During this time no significant effect on basal membrane conductance could be detected (Fig. 6B, trace c). Second, 60–120 s after application of the toxin a huge inward current appeared (from 600 pA to >1 nA, Fig. 6B, trace a; phase II as indicated by vertical dashed line) and was associated with an increase in membrane conductance (from 1.1 ± 0.1 nS to 5.35 ± 0.2 nS, n = 7; Fig. 6B, trace c). This current slowly developed in stages and was not reversible within 15 min. It could be responsible for the phase II of the calcium response described above. Furthermore, the driving force (V<sub>m</sub> − E<sub>rev</sub>) underlying the long-lasting inward current (I) response to the toxin was estimated to obtain an indication on the nature of the ions implicated in this phase II event. Using Ohm’s law as applied to membranes (I = G(V<sub>m</sub> − E<sub>rev</sub>), where E<sub>rev</sub> is reversal potential), the conductance (G) can be derived by measuring throughout the time course changes in current responses to brief hyperpolarizing commands (20 mV from a holding potential, V<sub>hold</sub> of −40 mV). This in turn permitted repeated estimates of E<sub>rev</sub>. For example, the 24 estimates of the driving force calculated for the cell illustrated in Fig. 6B (trace d) yielded an average reverse potential E<sub>rev</sub> of +35 ± 2 mV. This value is consistent with the contribution of Ca<sup>2+</sup> ions to this current.

Because K<sup>+</sup> channels participate in the control of the membrane potential (31), we investigated the effect of α-LTX on these channels more closely (Fig. 6C). Cells were voltage-clamped at −40 mV, close to the mean resting membrane potential. Contamination of K<sup>+</sup> current recordings with Na<sup>+</sup> was avoided by the use of the Na<sup>+</sup> channel blocker tetrodotoxin (2 mM) in the external solution. Under these conditions, voltage steps of increasing intensity elicited an outward current of increasing amplitude. This outward current was greatly reduced by external application of 48 mM KCl (data not shown) confirming that it...
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represents a pathway for $K^+$ efflux. In HIT-T15 cells expressing LPH, a prominent inhibition of outward currents was observed (Fig. 6C, upper panels) in response to α-LTX (~70%), which was observed over the whole voltage range (~40 to +60 mV). In contrast, in cells expressing LPH-TD1 (Fig. 6C, lower panels), α-LTX was ineffective on $K^+$ currents elicited by depolarization from ~40 to ~10 mV, and it only slightly reduced $K^+$ current (~30%) for higher potentials. These data are in agreement with the current-clamp and $[Ca^{2+}]_i$ experiments showing that α-LTX produces phase I events only in HIT-T15 cells expressing full-length LPH.

**LTXN4C Induced Oscillation in Membrane Potential and Inhibited BK-type $K^+$ Channels in MIN6 Cells via Phospholipase C**—We next examined the behavior α-LTX and of LTXN4C in MIN6 cells. The $K^+$ channel blocker TEA (5 mM) is capable to elicit oscillations of the membrane potential as shown in Fig. 7. Application of wild-type α-LTX induced similar initial oscillations, and only later constant membrane depolarization was observed. The two events were clearly distinct and therefore again indicative for the presence of two phases. In stark contrast, the mutated toxin LTXN4C provoked only oscillations of the membrane potential of considerable amplitude (Fig. 7A).

Using the same protocol as described for Fig. 6B, we investigated the time course and compared it to other inhibitors of $K^+$ channels. A run-down of the $K^+$ outward current was evident under our experimental conditions when comparing the earliest and latest time points in Fig. 7B (trace a). LTXN4C produces a pronounced and transient inhibition of the $K^+$ outward current from 455 ± 24 to 98 ± 8 pA ($n = 11$). This was similar to the first phase observed in LPH-expressing HIT-15 cells upon exposure to α-LTX (see Fig. 6B). However, LTXN4C did not produce a second phase as demonstrated by the return to the base line (Fig. 7B, trace a). A similar effect was produced by iberiotoxin, a peptide inhibitor of BK-type $K^+$ channels, whereas apamin, an inhibitor of SK-type $K^+$ channels, was ineffective in MIN6 cells (Fig. 7B, trace b). Indeed, iberiotoxin reduced outward currents to 94 ± 10 pA ($n = 10$). Moreover, inhibition of BK-type $K^+$ channels by iberiotoxin completely abolished the effect of LTXN4C (Fig. 7Bc; $n = 5$) indicating that the effects of LTXN4C and of iberiotoxin are mediated by the same type of channels.

We next examined whether phospholipase C is implicated in the action of LTXN4C on $K^+$ channels (Fig. 7D). Indeed, U73122 blocked the effect of the recombinant toxin on $K^+$ currents, whereas the stereoisomer U73343 was ineffective. Therefore, regulation of $K^+$ currents and of $[Ca^{2+}]_i$ (see above) implied phospholipase C.

The inhibition of the voltage-dependent $K^+$ outward current was further characterized in MIN6 cells (Fig. 7D) as before in HIT-T15 cells (see above). LTXN4C provoked a considerable inhibition of the currents and a right-shift of the I-V curve. We next asked whether inhibition of the $K^+$ outward currents are $per se$ sufficient to induce insulin secretion in MIN6 cells and may contribute to the secretory activity of the toxin. Indeed, TEA and iberiotoxin alone were capable to induce a significant increase in insulin secretion (Fig. 7E). TEA and iberiotoxin also augmented insulin secretion evoked by 35 mM KCl by 70.3 ± 8.8 and 95 ± 36%, respectively ($n = 4$).

**LTXN4C Transiently Increases L-type Voltage-dependent $Ca^{2+}$ Currents Independently from Its Action on Membrane Potential**—The LTXN4C-induced events on membrane potential are seemingly sufficient to induce opening of voltage-dependent $Ca^{2+}$ channels and to provoke influx of the cation. To our surprise, LTXN4C had still an effect on calcium currents when MIN6 cells were clamped to 0 mV thus obliterating any contribution from LTX-induced changes in membrane potential (Fig. 8A). LTXN4C enhanced inward currents both at the beginning and throughout the applied pulse. This led to a downward shift of the I-V curve indicating that the effect was not voltage-dependent (Fig. 8B). Note that this increase is considerable and amounts to some 50%. Time-course experiments using voltage jumps, shown in Fig. 8C, demonstrate the transient nature of the effect induced by LTXN4C as its application is accompanied by a short increase in $Ca^{2+}$ currents. Insulin-secreting cells express a variety of voltage-dependent $Ca^{2+}$ channels. In addition to the well established role of L-type channels, R-type channels may also control insulin exocytosis to a certain extent (35). To further determine the nature of the $Ca^{2+}$-current involved, we employed the L-type-specific blocker calcisepine, which abolished oscillations in $Ca^{2+}$ and in
Our analysis of α-LTX action in these cells has now revealed previously unidentified and physiologically important targets, i.e. K⁺ channels and L-type Ca²⁺ channels. Their regulation required the G-protein-coupling moiety of latrotoxin and stimulates exocytosis.

Various ionic events have been observed previously in native endocrine and neuroendocrine cells after application of α-LTX, including "spikes" and "plateaus" in primary chromaffin and B cells, but they were mainly interpreted in terms of pore formation (9, 37, 38). We have now been able to clearly define two distinct phases by the use of different cell lines, receptor constructs, and wild-type or mutated toxin. In phase I oscillatory membrane depolarizations appear as well as calcium spikes. In contrast, during phase II a large and mixed transmembrane conductance appeared representing known pore formation (39) and provoked a sustained increase in [Ca²⁺], as had been reported for secretory and non-secretory cells (7, 9, 40). Several observations demonstrated that phase I was independent from pore formation. First, this phase required the full-length receptor and was linked to the inhibition of voltage-dependent K⁺ fluxes. The inhibition of these K⁺ fluxes cannot be explained by pore formation, because it occurred prior to them (see Fig. 6B). Moreover, toxin-induced pores are known to be poorly selective and should induce increases but not reduction in ion fluxes (41). Most notably, phase I did not exhibit any significant effect on steady-state currents. The cells were held at −40 mV and depolarizing commands (450 ms/40 mV) were applied to elicit inward Ca²⁺ currents. LTXN⁴C (0.2 nM) enhanced the inward current at the beginning (Ipeak, circles) and at the end (Isteady state, squares) of the pulse. B, current-voltage (I-V) relationships for the cell shown in A of steady-state Ca²⁺ currents under control conditions (CON, open squares) and in the presence of 0.2 nM LTXN⁴C (filled squares). C, in another cell, we have repeatedly applied the same voltage jump (from −40 to 0 mV, every 20 s) to determine the time course of the LTXN⁴C (0.2 nM) effects. Note the transient increase of the inward current (arrow). D, as previously, a cell was held at −40 mV and depolarizing commands (450 ms/40 mV) were applied to elicit inward Ca²⁺ currents. Calciseptine (1 μM), an inhibitor of the L-type Ca²⁺ channel, completely and irreversibly blocked the inward current, and 2 nM LTXN⁴C was no longer capable of affecting this current. E, time course (as described in B) of the responses to calciseptine (1 μM) and LTXN⁴C (0.2 nM) of the cell shown in D. F, expression of Ca₁,2 and Ca₁,3. Post-nuclear supernatants from rat brain (80, 20 mg/lane), MIN6 (60 mg/lane), or HIT-T15 cells (60 mg/lane) were separated by SDS-PAGE, blotted, and incubated with corresponding antibodies. Molecular masses are given by bars (200 and 150 kDa). Preincubation of antibodies with the antigenic peptides completely abolished the immunoreactivity (data not shown).

FIGURE 8. LTXN⁴C transiently increases L-type voltage-dependent Ca²⁺ currents in MIN6 cells independently from toxin-induced membrane depolarization. A, a cell was held at −40 mV and depolarizing commands (450 ms/40 mV) were applied to evoke inward Ca²⁺ currents. LTXN⁴C (0.2 nM; filled symbols) enhances the inward current at the beginning (Ipeak, circles) and at the end (Isteady state, squares) of the pulse. B, current-voltage (I-V) relationships for the cell shown in A of steady-state Ca²⁺ currents under control conditions (CON, open squares) and in the presence of 0.2 nM LTXN⁴C (filled squares). C, in another cell, we have repeatedly applied the same voltage jump (from −40 to 0 mV, every 20 s) to determine the time course of the LTXN⁴C (0.2 nM) effects. Note the transient increase of the inward current (arrow). D, as previously, a cell was held at −40 mV and depolarizing commands (450 ms/40 mV) were applied to elicit inward Ca²⁺ currents. Calciseptine (1 μM), an inhibitor of the L-type Ca²⁺ channel, completely and irreversibly blocked the inward current, and 2 nM LTXN⁴C was no longer capable of affecting this current. E, time course (as described in B) of the responses to calciseptine (1 μM) and LTXN⁴C (0.2 nM) of the cell shown in D. F, expression of Ca₁,2 and Ca₁,3. Post-nuclear supernatants from rat brain (80, 20 mg/lane), MIN6 (60 mg/lane), or HIT-T15 cells (60 mg/lane) were separated by SDS-PAGE, blotted, and incubated with corresponding antibodies. Molecular masses are given by bars (200 and 150 kDa). Preincubation of antibodies with the antigenic peptides completely abolished the immunoreactivity (data not shown).

FIGURE 7. LTXN⁴C induces oscillations in membrane potential and inhibits iberiotoxin-sensitive K⁺ currents in MIN6 cells via phospholipase C. A, effects of TEA (5 μM), α-LTX (2 μM), or LTXN⁴C (0.2 μM) on the membrane potential of native MIN6 cells. Applications of toxins or drugs are indicated by horizontal bars. Recordings were obtained under zero current conditions (current clamp), baselines (dotted lines) and phases (I and II) are indicated. B, time course of toxin effects on K⁺ currents. The cells were held at −40 mV, and depolarizing commands (450 ms/100 μV) were repeatedly evoked outward K⁺ currents. Panel a, LTXN⁴C (0.1 μM) reduced K⁺ current amplitude (arrow) from 455 ± 24 to 98 ± 8 pA (n = 11). The decline in the baseline is indicated by a dotted line. Panel b, iberiotoxin (arrows, 50 nM), an inhibitor of BK K⁺ channels, was ineffective. Panel c, in the presence of iberiotoxin (50 nM), LTXN⁴C (0.1 μM) did not induce any further inhibition (n = 5). Panel d, the phospholipase C inhibitor U73122 but not the inactive stereoisomer U73343 (both at 1 μM) inhibited LTXN⁴C effects on K⁺ current amplitude (U73122: 379 ± 40 pA, n = 9; U73343: 122 ± 15 pA, n = 8). In C, upper panels, a delayed outward current was elicited during a sustained depolarizing voltage step from −40 mV to various test potentials (10-mV increments). Patch clamp recordings were carried out under control condition (CON) and during application of toxin (LTXN⁴C, 0.1 μM). Lower panel, the amplitudes of the current responses at the end of voltage steps before (open squares) and after application of LTXN⁴C (filled squares) were plotted as a function of the step potential (I-V curves). D, insulin secretion from MIN6 cells was increased by KCl (35 mM), LTXN⁴C (0.3 μM), iberiotoxin (0.2 μM), or TEA (5 μM), n = 6 for each protein, * p < 0.05 as compared with KRB.

DISCUSSION

Pancreatic β-cells secrete the peptide hormone insulin by exocytosis and require a machinery similar to that of neuroendocrine cells (17). Insulin secretion evoked by LTXN⁴C (Fig. 5, A and C). Calciseptine completely blocked the voltage-evoked inward Ca²⁺ current and abrogated the effect of LTXN⁴C (Fig. 8D). This blockade of calcium currents and LTXN⁴C effects was observed throughout the time course (Fig. 8E). The molecular identity of L-type Ca²⁺ channels involved in insulin secretion is a matter of debate and may be formed by Ca₁,2 (α₁c) or Ca₁,3 (α₁d) (36). As the expression pattern has not been established in the MIN6 subclone used here (m9), we have performed immunoblots using isomorph-specific antibodies. As shown in Fig. 8F, both proteins are expressed and may be regulated by LTXN⁴C.
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release depending on pore formation. Therefore, not only mutant, but also wild-type toxin activates specific signaling pathways distinct from pore formation in neuronal and endocrine cells.

The specific events required for long-lasting effects of latrophilin, including its G-protein coupling domains in HIT-T15 cells that are naturally toxin-sensitive. LPH-TD1 did not suffice to support efficient toxin-induced stimulation of insulin exocytosis despite increases in [Ca\(^{2+}\)]. We do not think that reduced increases in [Ca\(^{2+}\)], in LPH-TD1-expressing cells (as compared with LPH-expressing ones, see Fig. 4) explain the absence of measurable exocytosis. Although changes in [Ca\(^{2+}\)], were observed only in one-third of the cells expressing LPH-TD1, their amplitude corresponded approximately to those induced by KCl over the stimulation period. Ca\(^{2+}\)-channels and exocytotic sites are coupled in β-cells (42), and it is feasible that pores induced during phase II in HIT-T15 cells expressing LPH-TD1 may not be coordinated with release sites.

Previous reports suggested that expression of the extracellular domain of LPH (LPH-TD1), if anchored to the plasma membrane, is sufficient for toxin-induced increases in [Ca\(^{2+}\)], or enhancement of exocytosis (4, 7, 8, 43). In contrast to HIT-T15 cells, the cellular systems used in these reports (PC12, HEK293, or chromaffin cells) express endogenous LPH and are sensitive to the toxin (7, 10, 44). Similar to those observations we have observed enhancement of α-LTX effects upon overexpression of our LPH-TD1 construct in cells that harbor endogenous LPH, such as PC12 and MIN6 cells. However, this was clearly not the case in HIT-T15 cells that do not contain functional endogenous toxin receptors. This suggests complementation between endogenous full-length and transiently expressed truncated receptor as the basis for LPH-TD1 effects. Indeed, G-protein-coupled receptors form oligomers, and truncated receptors can be salvaged by interaction with full-length receptors (45). Cooperativity is also indicated by co-immunoprecipitation of different toxin receptors provided that with full-length receptors (45). Cooperativity is also indicated by co-immunoprecipitation of different toxin receptors (45).

Our data also suggest that neurexins may not compensate for truncations in LPH as HIT-T15 cells, but not MIN6 cells, express considerable levels of endogenous neurexin Ia and IB (16).

Our data demonstrate for the first time that two types of ion channels are regulated as downstream effectors by binding of toxin to latrophilin: inward-rectifying K\(^{+}\) channels and voltage-dependent Ca\(^{2+}\) channels (VDCCs). As inhibition of phospholipase C abolished membrane depolarization and increases in [Ca\(^{2+}\)], the following sequence of events can be established. Activation of phospholipase C leads to closure of inward-rectifying K\(^{+}\) channels, and the ensuing membrane depolarization will induce opening of VDCCs followed by Ca\(^{2+}\) influx. In addition, toxin binding to LPH further stimulated VDCCs independently from its action on membrane depolarization. Clearly, combining both effects will considerably enhance the efficiency of the toxin in terms of exocytosis.

Pharmacological approaches using specific agents identify the VDCC as L-type, which constitutes the major but not only VDCC in β-cells in terms of exocytosis (35, 36). It is currently still a matter of debate whether Ca\(_{1.2}\) or Ca\(_{1.3}\) constitutes the major molecular form implicated in insulin secretion (46). Similar to primary cells, HIT-T15 and MIN6 cells express Ca\(_{1.2}\) and Ca\(_{1.3}\) channels (see Ref. 36 and our study). Thus, both may be targets of LPH-mediated signaling, although Ca\(_{1.3}\) seems to be expressed in these cells to a greater extent.

The observed increase in [Ca\(^{2+}\)], required the activation of protein kinase C according to pharmacological criteria. L-type VDCCs are subject to phosphorylation by PKC subsequent to the activation of receptors coupled to the G-protein G\(_{\alpha}\), and the functional outcome depends on the splice variant present (47, 48). Such a mechanism could eventually underlie the stimulation of L-type VDCCs by LTX\(^{NAC}\) observed here, although other pathways may apply (36). In β-cells an intriguing parallel to LPH-mediated signaling is given by the recent description on fatty-acid-induced insulin secretion via the G-protein-coupled receptor GPR40 (29). Similar to LPH, GPR40 regulates phospholipase C and L-type VDCCs.

Different K\(^{+}\) currents with specific characteristics and roles at distinct stages modulate secretion in β-cells, mainly the ATP-sensitive K\(_{ATP}\), the voltage-dependent K\(_{v}\), and the calcium- and voltage sensitive K\(_{Ca}\) (49). Glucose metabolism alters the ATP/ADP ratio, which leads to the closure of K\(_{ATP}\) channels, membrane depolarization, Ca\(^{2+}\) influx via VDCCs, and insulin exocytosis (18). Repolarization of β-cells implies mainly voltage-dependent (K\(_{v}\)) and perhaps Ca\(^{2+}\)-activated K\(_{Ca}\) channels (50, 51). Our observations of voltage-dependent effects and their resistance to diazoxide exclude the K\(_{ATP}\) Channel as target. Genetic or pharmacological inhibition of K\(_{v}\) leads to increased stimulated insulin secretion in primary, HIT-T15, and MIN6 cells (52, 53). In addition, HIT-T15 and primary cells express functional voltage-dependent Ca\(^{2+}\)-activated K\(_{Ca}\) channels (54). As demonstrated here for MIN6 cells by the use of iberiotoxin and apamin, these cells express the BK-\(^{-}\), but not the SK-type of these K\(_{Ca}\) channels. The observed inhibition of latrotoxin effects on K\(^{+}\) currents by iberiotoxin suggests that latrotoxin specifically inhibits BK-type channels. Moreover, inhibition of repolarization by BK channels using iberiotoxin was sufficient to induce insulin exocytosis. Regulation of BK channels by α-LTX therefore contributes to the stimulation of insulin exocytosis and may well explain the enhancement of depolarization-induced secretion by the native toxin in chromaffin and primary β-cells (37, 38, 55).

Using pharmacological approaches and taking advantage of the mutant toxin LTX\(^{NAC}\), we have in part delineated the downstream signaling of latrophilin in clonal β-cells. The increase in [Ca\(^{2+}\)], depends on activation of phospholipase C, and similar pathways have been described previously in synaptosomes, in neurons, in neuroblastoma cells transiently expressing latrophilin, as well as in the case of the recently identified latrophilin-like orthologue in Caenorhabditis elegans (12, 13, 15, 56). In concordance with recordings from pyramidal neurons, the observed release from intracellular stores in MIN6 cells implied the inositol 1,4,5-trisphosphate receptor (13). Latrotoxin-induced inhibition of K\(^{+}\) channels or Ca\(^{2+}\) influx through VDCCs has not been reported in these neuronal preparations. L-type VDCCs are of physiological importance in neurons, however, they are not directly linked to neuroexocytosis in contrast to exocytosis in endocrine cells (57). As far as BK channels are concerned, their potential role in neuro-exocytosis is not fully established (58, 59).

Inhibition of calcium influx only partially inhibited toxin-induced exocytosis of insulin. This suggests that LPH-mediated stimulation implies downstream targets in addition to ion channels. Indeed, subnanomolar concentrations of α-LTX stimulate exocytosis even in the absence of changes in [Ca\(^{2+}\)], (60) and on top of Ca\(^{2+}\) in permeabilized cells (10). Protein kinases A and C are known to augment exocytosis by increasing the size of the releasable pool of vesicles in insulin-secreting cells (61). A most recent detailed analysis demonstrated that both α-LTX and LTX\(^{NAC}\) increase exocytosis in β-cells by sensitizing the release process to calcium via PKC (62).

Collectively, our data support the view that α-LTX activates exocytosis via latrophilin employing specific signaling mechanisms in addition to formation of a cation-permeable pore. Most importantly, the


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Receptor-mediated mechanisms are mediated by phospholipase C and impinge on targets relevant for the physiological regulation of insulin exocytosis, namely repolarizing K+ currents and L-type calcium channels.

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