Alpha-latrotoxin Triggers Extracellular Ca^{2+}-dependent Exocytosis and Sensitizes Fusion Machinery in Endocrine Cells

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Abstract α-Latrotoxin from the venom of black widow spider induces and augments neurotransmitter and hormone release by way of extracellular Ca^{2+} influx and cellular signal transduction pathways. By using whole cell current and capacitance recording, the photolysis of caged Ca^{2+}, and Ca^{2+} microfluorometry and amperometry, we investigated the stimulating effect and mechanism of α-latrotoxin on exocytosis in rat pancreatic β cells, LβT2 cells and latrophilin plasmid-transfected INS-1 cells. Our data indicated that: (1) α-latrotoxin increased cytosolic Ca^{2+} concentration through the formation of cation-permitting pores and subsequent Ca^{2+} influx with the presence of extracellular Ca^{2+}; (2) α-latrotoxin stimulated exocytosis in normal bath solution and its stimulating effect on secretion was eradicated in Ca^{2+}-free bath solution; and (3) α-latrotoxin sensitized the molecular machinery of fusion through activation of protein kinase C and increased the response of cells to Ca^{2+} photolysed by a flash of ultraviolet light. In summary, α-latrotoxin induced exocytosis by way of Ca^{2+} influx and accelerated vesicle fusion by the sensitization of fusion machinery.

Key words α-latrotoxin; exocytosis; calcium; Ca^{2+}-sensitivity of fusion; protein kinase C (PKC); capacitance measurement; amperometry

Over the last 20 years, α-latrotoxin (α-LTX) from the venom of black widow spider has been widely used to study the molecular mechanisms of neurotransmitter and hormone release. α-LTX elicits robust neurotransmitter release in neurons, and stimulates hormone release in endocrine cells, including adrenal chromaffin cells, pituitary gonadotropes and secretory terminals of the posterior pituitary [1–4].

α-LTX can form non-selective cation pores on cell membrane and subsequently stimulate secretion though Ca^{2+} influx [3,5,6]. Evidence shows that the pores are large enough to conduct small compounds including neurotransmitters [7–9].

Two classes of α-LTX receptors have been identified: neurexin Iα and calcium-independent receptor for latrotoxin (CIRL)/latrophilin. Neurexin Iα, first discovered by Petrenko *et al*. [10], is a member of a highly polymorphic family of neuronal cell membrane proteins [11]. The binding of toxin to neurexin Iα is Ca^{2+}-dependent [12]. The ability of α-LTX to act in the absence of extracellular Ca^{2+} led to the discovery of another Ca^{2+}-independent receptor: CIRL/latrophilin. Latrophilin belongs to the G protein-coupled receptor protein family [13]. Studies have verified that α-LTX binds to two classes of receptors in tetramers or dimers [14].

Accumulated evidence indicates that α-LTX evokes secretion in the absence of extracellular Ca^{2+} by binding to latrophilin and activating the G protein-phospholipase C (PLC)-inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) signal transduction pathway [7,15]. Activation of PLC leads to the production of DAG and IP_3, two impor-

Received: August 15, 2005 Accepted: November 3, 2005
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This work was supported by the grants from the National Natural Science Foundation of China (30025023, 3000062, 30130230 and 30370647), the Major State Basic Research Program of China (G1999054000 and 2004CB720000), the Chinese Academy of Sciences Project (KSCX2-SW-224), and the National High Technology Research and Development Program of China (2002AA214061)
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DOI: 10.1111/j.1745-7270.2006.00129.x

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tant intracellular second messengers. Activation of protein kinase C (PKC) by DAG sensitizes the fusion molecular machinery and augments secretion [16,17]. IP_3 mobilizes the Ca^{2+} release from intracellular calcium stores to increase the local and global $[Ca^{2+}]_i$, which triggers and modulates exocytosis of vesicles [18]. However, in this study, in spite of the preservation of the secretagogue effect, we did not detect the elevation of cytosolic Ca^{2+} concentration by α-LTX in the absence of extracellular Ca^{2+}, suggesting that the IP_3 signal pathway did not play an important role in the stimulation effect on exocytosis of α-LTX, and there might be another pathway for α-LTX to regulate exocytosis, possibly by the activation of PKC.

Challenging cells with α-LTX by extracellular perfusion in the Ca^{2+}-containing normal and the Ca^{2+}-free bath solution, we studied the effect of toxins on the intracellular Ca^{2+} level and exocytosis. Our data indicated that α-LTX directly evoked the robust secretion by way of Ca^{2+} influx, and augmented the response of the toxin challenged cells to the step-like $[Ca^{2+}]_i$ elevation elicited by a short flash of ultraviolet (UV) illumination. The mechanism underlying the latter effect was that α-LTX sensitized molecular fusion machinery through PKC activation, which was elicited by the latrophilin-hetero G protein-PLC-DAG-PKC signal transduction pathway.

Materials and Methods

Construction of latrophilin expression plasmid

The plasmid pcDNA3.1-latrophilin was kindly provided by Dr. Y. USHKARYOV (Department of Biochemistry, Imperial College, London, UK). The challenge of using latrophilin-enhanced green fluorescent protein (EGFP) fusion protein is that EGFP may alter the physiological function of latrophilin. We took advantage of the internal ribosome entry site (IRES)-EGFP cDNA vector, which contains the IRES of the encephalomyocarditis virus and the EGFP-coding region, to co-express latrophilin and EGFP for electrophysiological assay. The *Eco*RI/*Not*I-digested IRES-EGFP sequence of pIRES2-EGFP was ligated into *Eco*RI/*Not*I-digested pcDNA3.1-latrophilin vector to generate the pcDNA3.1-latrophilin-IRES-EGFP plasmid. All DNA cloning was performed using *Escherichia coli* DH5α competent cells. Construction integrity was verified by restriction enzyme analysis with *Hin*dIII (data not shown). Restriction enzymes and other standard molecular biology reagents were obtained from New England Biolabs (Beverly, USA).

Cell preparation

The pancreatic islets of male Wistar rats (150–200 g) were prepared by collagenase V digestion, and further digested by dispase II to dissociate single β cells in a Ca^{2+}-free Krebs-Ringer bicarbonate buffer, as described previously [19]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, USA) supplemented with 25 mM HEPES, 2 mg/ml $NaHCO_3$, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (Gibco) in 5% CO_2 at 37 ºC. The cells of insulin secreting insulinoma cell line INS-1 were grown in DMEM in the same conditions as used for β cells. Approximately 72 h before use, latrophilin was introduced into the endogenous receptor-lacking INS-1 cells by transfecting with pcDNA3.1-latrophilin-IRES-EGFP plasmid using Lipofectamine 2000 (Invitrogen, Groningen, Switzerland) according to the manufacturer's instructions. Cells expressing latrophilin were identified by green fluorescence (excitation wavelength 488 nm).

$[Ca^{2+}]_i$ measurement and Ca^{2+} uncaging

To measure the $[Ca^{2+}]_i$ response of primary pancreatic β cells, LβT2 and latrophilin-expressing INS-1 cells to α-latrotoxin (Alomone Labs, Jerusalem, Israel), the cells were loaded with fura-2/AM by incubation at 37 ºC for 20 min in normal bath solution supplemented with 3 μM fura-2/AM. $[Ca^{2+}]_i$ was measured by dual-wavelength excitation (340/380 nm) microfluorometry using either fura-2 or fura-6F as the Ca^{2+} indicator. $[Ca^{2+}]_i$ was calculated as follows:

$$[Ca^{2+}]_i=K_{eff}\times(R-R_{min})/(R_{max}-R)$$

where K_{eff}, R_{min} and R_{max} are constants and obtained from intracellular calibration as described previously [20]. Fura-2 and fura-6F were purchased from Molecular Probes (Eugene, USA). All other agents were purchased from Sigma (St. Louis, USA).

Step-like homogenous global $[Ca^{2+}]_i$ elevation was elicited by a flash of UV light generated by a Rapp flash lamp (Rapp Optoelektronik, Hamburg, Germany). The flash was followed by a series of illuminations alternating between 340 nm and 380 nm, which allowed radiometric determination of the Ca^{2+} concentration. The duration of these illuminations was adjusted to maintain relatively constant Ca^{2+} concentrations, as illumination at 340 nm or 380 nm also leads to the photolytic release of Ca^{2+}. Trains of light alternating at 340 nm and 380 nm were generated from a monochromator (Till Photonics, Planegg, Germany). The fluorescence was acquired by a photodiode (Till Photonics).

The DM-nitrophen-EGTA (DMNP-EGTA; Molecular Probes) containing pipette solution (110 mM Cs-glutamate, 2 mM MgATP, 0.3 mM GTP, 35 mM HEPES and 5 mM DMNP-EGTA) was adjusted to pH 7.2 using CsOH or HCl (osmolarity, 300 mOsm). The free Ca^{2+} concentration was measured to be ~200 nM *in vitro* by fura-2.

Membrane capacitance measurement and current recording

Cell capacitance measurement was carried out during whole cell recordings at 30 ºC–33 ºC using an EPC9 amplifier (Heka Electronics, Lambrecht, Germany). A sine+DC protocol was applied using the Lockin amplifier of the Pulse program (Heka Electronics). The cells were voltage-clamped at a holding potential of −70 mV and a sine wave voltage command with amplitude of 20 mV and frequency of 1024 Hz was applied. Currents were filtered at 2.9 kHz and sampled at 15.6 kHz. The currents induced by extracellular application of α-latrotoxin were recorded in the whole cell configuration using the EPC9 amplifier. Gö6983 (1 μM) was included in the pipette solution to block PKC activation, in addition, Gö6983 (500 nM in normal bath solution) was also incubated extracellularly for 10 min. The standard extracellular bath solution consisted of 138 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 5 mM D-glucose and 10 mM HEPES (adjusted to pH 7.4 with NaOH, osmolarity=310 mOsm). The Ca^{2+}-free external bath solution was similar to the standard bath solution, except that $CaCl_2$ was substituted by 1 mM EGTA.

Data analysis

Data analysis was performed using IGOR Pro 4.02 (WaveMetrics, Lake Oswego, USA) and the results were presented as mean±SEM. Statistical significance ($P<0.05$) was evaluated by Student's t test or the Mann-Whitney rank sum test according to the normality of datum distribution in SigmaStat 3.11 (Systat Software, Point Richmond, USA).

Results

α-LTX formed Ca^{2+} permitting channels on plasma membrane and induced elevation of global $[Ca^{2+}]_i$

As shown in **Fig. 1**, the extracellular application of 6

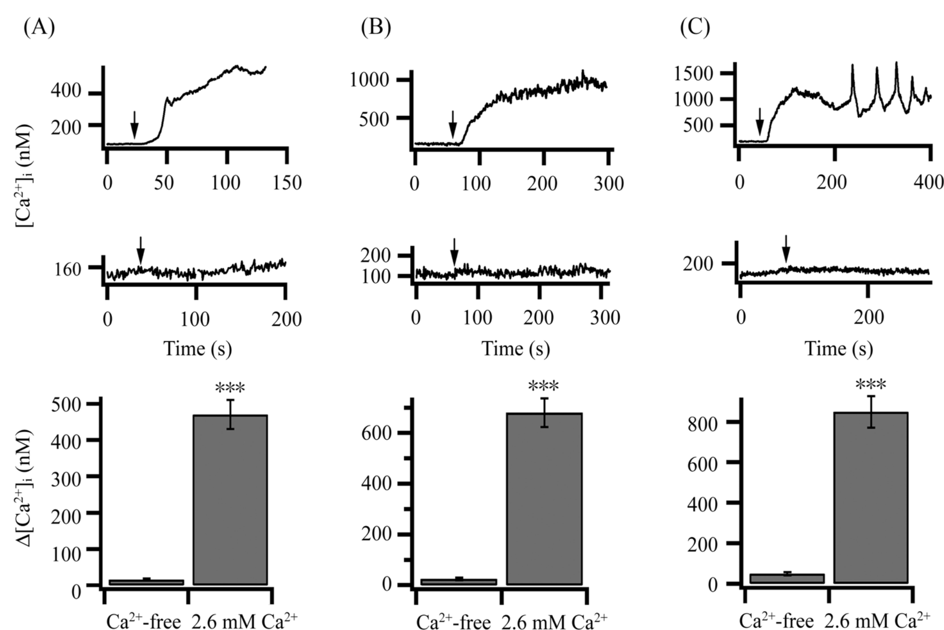

Fig. 1 α-Latrotoxin (α-LTX) induced $[Ca^{2+}]_i$ elevation in primary rat pancreatic β cells, LβT2 cells and latrophilin-expressing INS-1 (insulin secreting insulinoma line) cells by way of Ca^{2+} influx

The effect of α-LTX on $[Ca^{2+}]_i$ in cells in the normal and Ca^{2+}-free bath solutions. The upper panel shows the extracellular application of 6 nM α-LTX (start time indicated by arrows) by local perfusion with pipettes pointing to cell inducing $[Ca^{2+}]_i$ elevation in (A) rat pancreatic β cells, (B) latrophilin-expressing INS-1 cells, and (C) LβT2 cells, all immersed in the normal bath solution containing 2.6 mM Ca^{2+}. α-LTX did not markedly change $[Ca^{2+}]_i$ in the Ca^{2+}-free solution, as shown in the middle panel. The bottom panel summarizes the toxin-induced notable $[Ca^{2+}]_i$ elevation (***$P<0.001$ vs. $[Ca^{2+}]_i$ in Ca^{2+}-free bath solution) in three kind of cells in the presence of extracellular Ca^{2+} (β cells, n=6; latrophilin-expressing INS-1 cells, n=5; LβT2 cells, n=6).

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nM α-LTX by way of local perfusion induced remarkable $[Ca^{2+}]_i$ elevation ($\Delta[Ca^{2+}]_i$) in primary rat pancreatic β cells (*n*=6, 471.3±41 nM), latrophilin-expressing INS-1 cells (*n*=5, 681.2±56.3 nM) and LβT2 cells (*n*=6, 850.7±78.2 nM) in standard bath solution. However, α-LTX did not elicit $[Ca^{2+}]_i$ increase in these cells immersed in the Ca^{2+}-free extracellular solution (**Fig. 1**). These results suggested that α-LTX increased $[Ca^{2+}]_i$ by way of Ca^{2+} influx. To investigate the mechanism of Ca^{2+} influx, we measured the currents induced by α-LTX in LβT2 cells in whole cell configuration at different holding potentials in the normal (2.6 mM Ca^{2+}) and Ca^{2+}-free bath solution (**Fig. 2**). The data showed that α-LTX could evoke inward currents not only in the normal bath solution, but also in the Ca^{2+}-free extracellular solution [**Fig. 2(A)**]. The results suggested the formation of cation-permitting pores by α-LTX on plasmalemma was Ca^{2+}-independent and the conductance was not Ca^{2+} selective. By measuring and analyzing the current at three different holding potentials (−40, −70 and −100 mV), we estimated the characteristics of the conductance of pores or the channels formed by α-LTX. The histogram of current amplitudes versus frequencies, shown in **Fig. 2(B)**, shows that the whole cell currents in LβT2 cells at a holding potential of −70 mV had two distinct Gaussian distributions. Of the electrical events, those distributed around 0 pA (with amplitudes from −1.5 pA to +1.5 pA) were noise. The currents elicited by α-LTX had normal distribution around −6 pA (−4 pA to −8 pA). The voltage relationship of currents [**Fig. 2(C)**] demonstrated that the channel activity of α-LTX was unitary. Our results agree with previous reports that α-LTX induced inward current by forming pores or channels which have a unitary conductance [21].

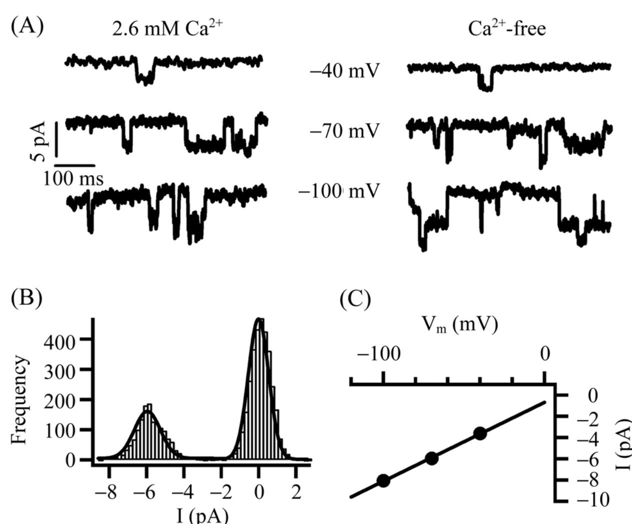

Fig. 2 α-Latrotoxin (α-LTX) assembled into cation channels
(A) α-LTX elicited inward currents in LβT2 cells holding at −40, −70 and −100 mV in the presence and absence of extracellular Ca^{2+}. The results suggest α-LTX was able to assemble into non-selective cation-permitting pores or channels. (B) α-LTX induced currents in LβT2 cells holding at −70 mV. There were two distinct fitted Gaussian distributions. The electrical events with amplitudes from −1.5 pA to +1.5 pA were noise. The currents elicited by α-LTX (left peak) had a normal distribution, around −6 pA. (C) Current-voltage relationship of the currents. The currents recorded at different holding potentials in five cells were linear with voltages (real line with a slope of 0.07 nS), indicating that currents of α-LTX were unitary. I, membrane current; V_m, membrane voltage.

α-LTX induced robust secretion by way of Ca^{2+} influx

We examined the effect of α-LTX on exocytosis using the whole cell capacitance measurement and amperometry with the EPC9 patch amplifier. In the normal bath solution (2.6 mM Ca^{2+}), the application of α-LTX by local perfusion with pipettes pointing to the cells elicited robust secretion in the primary pancreatic β cells (*n*=5) and LβT2 cells (*n*=6) [**Fig. 3(A)**]. However, the stimulatory effect

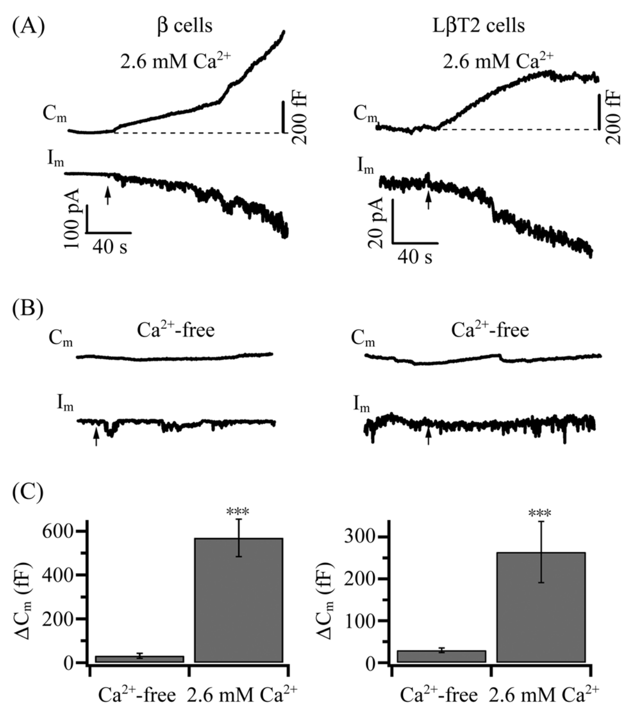

Fig. 3 α-Latrotoxin (α-LTX) induced Ca^{2+}-dependent exocytosis
(A) Extracellular application (start time indicated by arrows) of 6 nM α-LTX elicited exocytosis indicated by the membrane capacitance (C_m) increase in primary rat β cells and LβT2 cells in extracellular solution containing 2.6 mM Ca^{2+}. (B) In the Ca^{2+}-free extracellular solution, α-LTX had no effect on the capacitance of cells. (C) Summary of the amplitude of exocytosis triggered by α-LTX in Ca^{2+}-free and 2.6 mM Ca^{2+} bath solutions in β cells (***P<0.001, *n*=6) and LβT2 cells (***P<0.001, *n*=5). I_m, membrane current.

on the secretion of α-LTX was eliminated in the Ca^{2+}-free bath solution [**Fig. 3(B)**]. The results of the capacitance measurement were further confirmed by our amperometry in primary β cells. The cells were preloaded with serotonin (5-hydroxytryptamine, 5-HT) for 4–16 h and sensitized by incubation in 10 μM forskolin, which induces a big increase in the cytosolic cAMP level and sensitizes the secretory apparatus by way of the activation of protein kinase A, as reported previously [22]. Extracellular application of 6 nM α-LTX elicited numerous spikes of 5-HT in normal bath solution [**Fig. 4(A)**], but very few spikes in the Ca^{2+}-free solution [**Fig. 4(B)**]. 5-HT is taken up by insulin-secreting vesicles and co-released with insulin. The quanta spikes, recorded with 5 μm carbon fiber electrodes, coincided with that reported previously (**Fig. 4**) [22]. Our results indicated that α-LTX induced robust secretion by way of Ca^{2+} influx through the cation-permitting pores formed by α-LTX [23].

α-LTX sensitized the molecular machinery of fusion

To examine and identify the possible effect and underlying mechanism of α-LTX on secretion in the absence of extracellular Ca^{2+}, we used weak flash stimuli to evaluate whether α-LTX has any sensitization effect on fusion machinery. The photolysis of Ca^{2+}-caging compound by a flash of UV light of about 800 microseconds releases its caged Ca^{2+} and leads to homogenous global calcium elevation in the cytosol. The Ca^{2+} stimulus triggers vesicles to fuse with plasmalemma. After the flash photolysis, exocytosis proceeds with an initial, rapid exocytotic burst followed by a slower, sustained phase. The initial burst component represents the fusion of the readily releasable vesicles [24,25]. The kinetics of the burst component may reflect the processes of Ca^{2+} binding and unbinding to the so-called Ca^{2+}-sensor and the final fusion. The maximum rate of release is a reliable indicator for evaluation of Ca^{2+} sensitivity of fusion at a certain calcium level. **Fig. 5(A)** shows the flash response in the α-LTX-treated and control latrophilin-expressing INS-1 cells. α-LTX increased the amplitude of the exocytotic burst and the rate constant of release (7.3 s^{-1} for α-LTX-treated cells and 3.3 s^{-1} for control) at similar post-flash calcium levels. The kinetics of the response in the α-LTX+Gö6983-treated cells was similar to that in control cells [**Fig. 5(B)**]. **Fig. 5(C)** summarizes the maximum fusion rates of exocytotic bursts of the control, α-LTX and α-LTX+Gö6983 challenged cells. Our data showed that α-LTX markedly increased the maximum fusion rate of latrophilin-expressing INS-1 cells in response to photolysed Ca^{2+} stimuli, when compared to the control (175±68 fF/s, n=10) and the α-LTX treated INS-1 cells (590±131 fF/s, n=8, P<0.01). The maximum fusion rates of the α-LTX+Gö6983 challenged cells (189±24 fF/s, n=8) were not significantly different to that of control cells (P=1), but were notably different to that of α-LTX treated cells (P<0.01), demonstrating that the exocytosis effect of α-LTX was completely blocked by application of Gö6983.

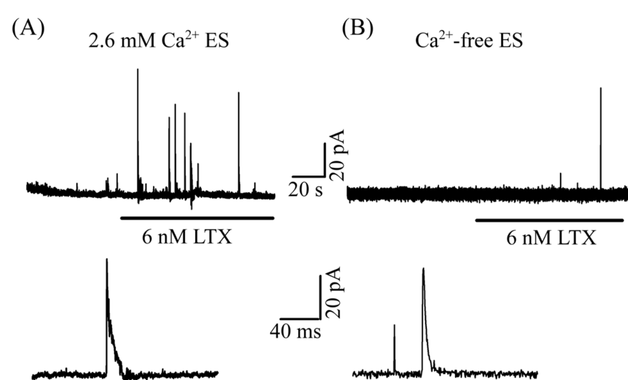

Fig. 4 α-Latrotoxin (α-LTX) evoked Ca^{2+}-dependent insulin secretion in primary rat pancreatic β cells measured by amperometry

(A) Application of 6 nM α-LTX evoked serotonin (5-HT) (co-releasing with insulin) spikes recorded by 5 μm carbon fibers in pancreatic β cells in 10 μM forskolin containing normal bath solution. Before amperometric measurement, the β cells were loaded with 5-HT by immersing them in cell culture medium containing 1 mM 5-HT for 4–16 h. α-LTX elicited robust vesicle release in normal bath solution. A single spike is shown in the lower panel. (B) The effect of α-LTX on insulin secretion was almost eradicated in Ca^{2+}-free extracellular solution containing 10 μM forskolin.

Discussion

α-LTX is capable of stimulating neurotransmitter and hormone release, and it has been used widely in the study of exocytosis as a potent toxin tool [26,27]. It is reported that there are two pathways in the mechanism underlying the effect of α-LTX: (1) by way of extracellular influx; and (2) by way of cellular signal transduction [2,3,6]. Our data indicate that very low dosage of the toxin can induce the robust intracellular Ca^{2+} level increase in primary pancreatic β cells, latrophilin-expressing INS-1 and LβT2 cells in the presence of extracellular calcium. The $[Ca^{2+}]_i$ elevation induced by the toxin is attributable to the formation of the Ca^{2+}-permeable pores or channels and the resultant Ca^{2+} influx. The characteristics of ion channels formed by the toxin demonstrated that these channels are non-selective cation channels with a huge unitary conductance (up to 200 pS). The channel activity remains in the absence of

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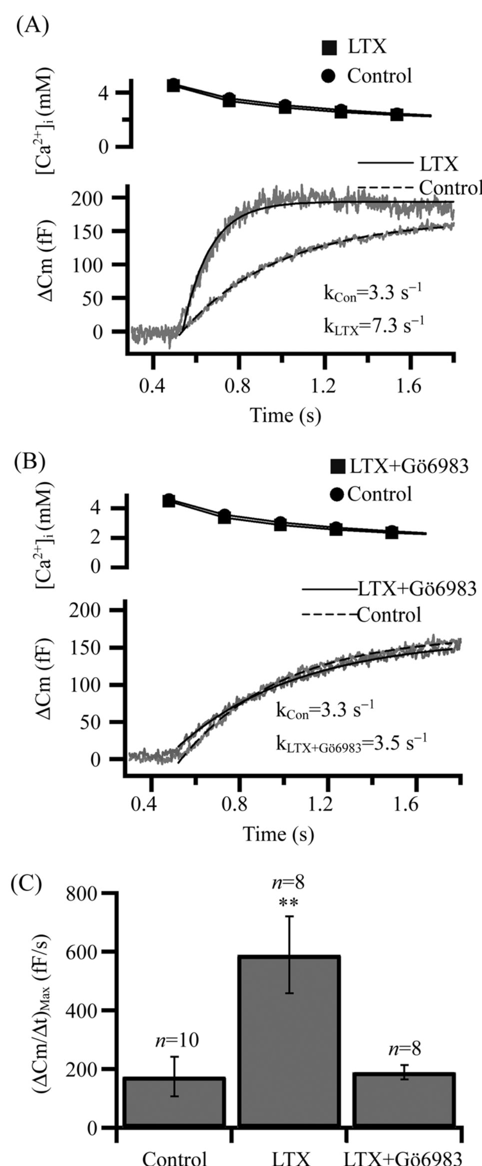

Fig. 5 α-Latrotoxin (α-LTX) increased Ca^{2+} sensitivity of fusion in latrophilin-expressing INS-1 (insulin secreting insulinoma line) cells
Averaged $[Ca^{2+}]_i$ (upper panel) and membrane capacitance changes (ΔC_m, lower panel) of exocytotic bursts in response to weak photolysis of caged Ca^{2+} of control, (A) 6 nM α-LTX treated, and (B) α-LTX+Gö6983 challenged latrophilin-expressing INS-1 cells. Superimposed curves were single exponential fitting trace with the rate constants (k) indicated. α-LTX accelerated the rate constants of exocytotic bursts from 3.3 s^{-1} to 7.3 s^{-1}. (C) Summary of the effects of α-LTX and α-LTX+Gö6983 on the maximum rate of exocytosis [$(\Delta C_m/\Delta t)_{max}$, fF/s]. The maximal rate was obtained by dividing the change of capacitance (ΔC_m) during time of ι/2 by Δt of ι/2 ($\Delta C_m\iota/2/\Delta t\iota/2$) from each exocytosis trace, where ι is the time constant from the exponential fit of the exocytotic burst (**P<0.01).

extracellular Ca^{2+}. But when other divalent cations such as Mg^{2+} are omitted, the currents induced by α-LTX disappear (data not shown). Our results are identical with former reports [5,23,28]. These indicate that the Ca^{2+} influx is efficient to evoke robust exocytosis.

α-LTX binds with CIRL/latrophilin and activates the receptor-mediated pathway [7]. Latrophilin is a G protein-coupled receptor which links with $G\alpha_{q/11}$ [6,13]. The downstream effector of $G\alpha_{q/11}$ is PLC. Activation of PLC leads to the generation of IP_3 and DAG, two important intracellular second messengers. IP_3 mobilizes intracellular calcium stores to release Ca^{2+} and induces the exocytosis [29]. However, we failed to observe that α-LTX increases $[Ca^{2+}]_i$ in primary rat β cells, latrophilin-expressing INS-1 cells or LβT2 cells when the Ca^{2+} was omitted from the extracellular solution, arguing against the hypothesis that α-LTX mobilizes intracellular calcium stores.

The Ca^{2+} sensitization of fusion machinery by PKC is an important and effective way to increase the release of neurotransmitters and hormones [29–32]. PKC is able to increase Ca^{2+} sensitivity of the molecular machinery of fusion and to accelerate secretion [16,29]. As endocrine cells share similar secretory apparatus with neurons, we used INS-1 cells as a model for secretion. Using global homogenous Ca^{2+} to stimulate secretion in latrophilin-expressing INS-1, we demonstrated that α-LTX elicits a much faster secretory response compared with the control, and the effect of toxin on exocytosis is completely eradicated by the application of Gö6983, a specific PKC blocker (**Fig. 5**). The results indicate that α-LTX increases the Ca^{2+} sensitivity of fusion machinery by way of activation of PKC, and helps to explain the long-recognized extracellular Ca^{2+}-independent effect of α-LTX on exocytosis. In addition, our results argue against the hypothesis that α-LTX directly regulates some pivotal proteins of fusion machinery after insertion into the membrane [33].

Acknowledgements

We thank Mrs. X. P. XU for the skilled technical support in cell preparation and plasmid transfection, W. ZHOU for the support of carbon fiber electrodes (CFE), and the Partner Group Scheme of the Max Planck Institute for Biophysical Chemistry (Goettingen, Germany).

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