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α-LTX and α-LTX^{N4C} induce $[Ca^{2+}]_i$ elevation through different mechanisms in pancreatic β cells

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Abstract α-latrotoxin (α-LTX), a neurotoxin from black-widow spider, causes vesicles release in presynapse of nerve terminal after binding to specific membrane receptors. α-LTX^{N4C} is an effective tool binding to calcium independent receptor for latrotoxin (CIRL), which is used to elucidate the mechanism of receptor-mediated signal pathway. In our study on the pancreatic β cells, we found that α-LTX inserts into the plasma membrane and forms stable non-selective cation channels. The influx of extracellular Ca^{2+} through the channels causes massive Ca^{2+}-dependent exocytosis of insulin-containing vesicles, whereas, α-LTX^{N4C}, binding with its receptor CIRL in extracellular divalent cation-dependent way, increases $[Ca^{2+}]_i$ by mobilization of the intracellular calcium stores.

Keywords: α-Latrotoxin, α-LTX^{N4C}, calcium.

α-latrotoxin (α-LTX) is the only neurotoxin from black-widow spider which has secretagogue effects in the vertebrates. It causes massive neurotransmitter and hormone release via two instinct mechanisms after binding with its high-affinity membrane receptors[1,2]. Several structurally and functionally unrelated membrane receptors for α-LTX have been reported. The first receptor discovered is neurexin[3,4], which binds with α-LTX only in the presence of extracellular Ca^{2+}. The fact that α-LTX preserves its stimulating effect on exocytosis in the absence of extracellular Ca^{2+} led to the discovery of its Ca^{2+}-independent receptor of α-LTX, calcium-independent receptor for latrotoxin (CIRL) or latrophilin[5,6]. CIRL is a plasmalemma protein with seven transmembrane segments and belongs to the secretin/calcitonin family of G protein-coupled receptors (GPCR)[7]. Recently, a third type receptor of α-LTX, protein tyrosine phosphatase (PTP_δ) which binds with the toxin in a Ca^{2+}-independent manner has been described[8]. It only plays a minor role in secretagogue effect of α-LTX.

In the presence of divalent cations, α-LTX, which is in presence of dimers in solution, forms Ca^{2+}-permeable non-selective pores in the plasma membrane via formation of tetramers or higher order oligomers and insertion into plasma membrane after binding with its receptor[9,10]. Evidence is accumulating that α-LTX is still active in the absence of extracellular Ca^{2+}, suggesting that it does not only act as an ionophore for Ca^{2+} and there is an additional Ca^{2+}-independent mechanism in its stimulation of secretion[11]. It is generally believed that the major Ca^{2+}-dependent effect is due to the Ca^{2+} influx through the toxin pore, whereas the Ca^{2+}-independent effect results from receptor-mediated signaling. It has been proposed that α-LTX activates latrophilin and induces generation of IP_3 thus mobilizing Ca^{2+} from intracellular stores via activation of phospholipase C (PLC)[12−14]. The mobilization of intracellular Ca^{2+} plays a pivotal role in mediating the effect of the toxin[14,15].

The effect of α-LTX on secretion via intracellular signal transduction pathways are overwhelmed by the effect via Ca^{2+} influx through the pore in the presence of extracellular Ca^{2+}. To avoid the complication and reveal α-LTX stimulating effect of secretion via its receptor-mediated actions and the possible mechanisms, we took the advantage of the recently developed mutant toxin, α-LTX^{N4C} which contains a small insert of 4 amino acids Val-Pro-Arg-Gly within the domain responsible for the formation of the ring-like tetramers. α-LTX^{N4C} binds to the receptors with affinity similar to the wild-type toxin, but neither forms pores nor penetrates into plasma membrane. Both α-LTX^{N4C} and α-LTX can trigger neurotransmitter release[12,16], so α-LTX^{N4C} is an effective tool to study the effects of receptor-mediated signaling. The present study focused

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on the different effects on inracellular Ca^{2+} changing induced by α-LTX and α-LTX^{N4C}. Our data showed that both α-LTX and α-LTX^{N4C} evoked $[Ca^{2+}]_i$ elevation in primary pancreatic β cells and insulin-secreting INS-1 cells via Ca^{2+} influx and mobilization of Ca^{2+} from intracellular store respectively.

1 Materials and methods

1.1 Solutions

The standard KRBB solution contained in mmol/L: NaCl 129, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 5.0, $CaCl_2$ 2.5, $MgSO_4$ $6H_2O$ 1.2, HEPES 10, BSA 0.1%, Glucose 3 (pH = 7.0 adjusted with NaOH, osmolarity = 300 mOsm). The normal extracellular bath solution contained in mmol/L: NaCl 150, KCl 2.8, $MgCl_2$ $6H_2O$ 2.0, $CaCl_2$ $2H_2O$ 2.6, Glucose 3.0 and HEPES 10 (pH = 7.4 adjusted with NaOH, osmolarity = 310 mOsm). The Ca^{2+} free bath solution consisted of components similar to the normal solution except that $CaCl_2$ was substituted by 1.0 mmol/L EGTA. Replacing 2.0 mmol/L NaCl by the same molar amount of $LaCl_3$, we got $LaCl_3$ containing solution. Unless otherwise stated, chemicals and reagents were purchased from Sigma (Sigma, St. Louis, MO, USA).

1.2 Cell culture

All experiments were performed on isolated rat pancreatic β cells with diameters of 12—14 μm at 30—32℃. Pancreatic β cells from adult male Wistar rats were prepared as described previously[17]. In brief, rats were killed by cervical sever, and pancreatic islets were isolated by collagenase digestion of the pancreas. The islets were further digested by dispase II to dissociate single â cells. The dispersed â cells were plated on glass coverslips and grown in DMEM (Dulbecco's modified Eagle's medium, Gibco, Carlsbad, CA) supplemented with 10% FBS (Gibco), 25 mmol/L HEPES, 2 g/L $NaHCO_3$, 100 IU/mL penicillin, 100 μg/mL streptomycin at 37℃ gassed with a humidified mixture of 5% CO_2 and 95% air. Cells cultured for 3—5 d were used in the experiments.

1.3 $[Ca^{2+}]_i$ measurement

To measure the $[Ca^{2+}]_i$ responses of β cells to the toxins, intact cells were loaded with Fura 2-AM (Molecular Probes, Carlsbad, CA, USA) by immersing the cells in 3 μmol/L Fura 2-AM containing bath solution for 30 min. $[Ca^{2+}]_i$ was then measured using illuminations alternating between 350 and 380 nm generated from the monochromator (Till Photonics, Planegg, Germany). The resulting fluorescence was acquired by a photodiode (Till Photonics). Considering that the concentration of Fura 2 which was introduced into cell by extracellular perfusion was unknown, we used directly the fluorescence ratio (R) as indicator of change of intracellular level.

1.4 Membrane capacitance (Cm) measurement

The Cm of β cells was measured in real time using an EPC9 amplifier (Heka Electronics, Lambrecht, Germany) in conventional whole-cell patch clamp configuration. A sine + DC protocol was applied using the Lockin extension of the Pulse program (Heka Electronics). β cells were voltage-clamped at a holding potential of –70 mV and a sine wave voltage command with an amplitude of 20 mV and a frequency of 1024 Hz was applied. Currents were filtered at 2.9 kHz and sampled at 15.6 kHz. The pipette solution contained in mmol/L: CsGlu 125, HEPES 40, MgATP 2, Na_2GTP 0.3, $MgCl_2$ $6H_2O$ 1, EGTA 0.3 (pH = 7.2 adjusted with CsOH, 300 mOsm). α-LTX (Cat. No. L-130, Alomone Labs, Jerusalem, Israel) and α-LTX^{N4C} (supplied kindly by Prof. Yuri Ushkaryov at Department of Biochemistry, Imperial College, London, UK) of 3 nmol/L contained in pipette solution were applied by local perfusion with pipettes (around 5 μm in diameters) pointing to the cells.

2 Results

2.1 α-LTX induced $[Ca^{2+}]_i$ elevation, which could be blocked by La^{3+}, via influx of extracellular Ca^{2+}

In Ca^{2+} free bath solution, application of α-LTX (3 nmol/L) at 60 s caused no significant fluorescence ratio ($F340/F380$) change in β cells. Using standard bath solution containing 2.6 mmol/L Ca^{2+} to replace the Ca^{2+} free solution, we could record significant $[Ca^{2+}]_i$ elevation induced by the toxin (Fig. 1). Flickering of inward currents accompanied by concurrent changes in the fluorescence ratio was observed under the whole-cell patch-clamp configuration in normal bath solution (holding potential, –70 mV), demonstrating that α-LTX inserted into plasma membrane and assembled into calcium-permeable pores or channels which increased $[Ca^{2+}]_i$ via Ca^{2+} influx (Fig. 2). Furthermore, application of α-LTX obviously elicited Ca^{2+}-depended exocytosis indicated by the increase of the membrane ca-

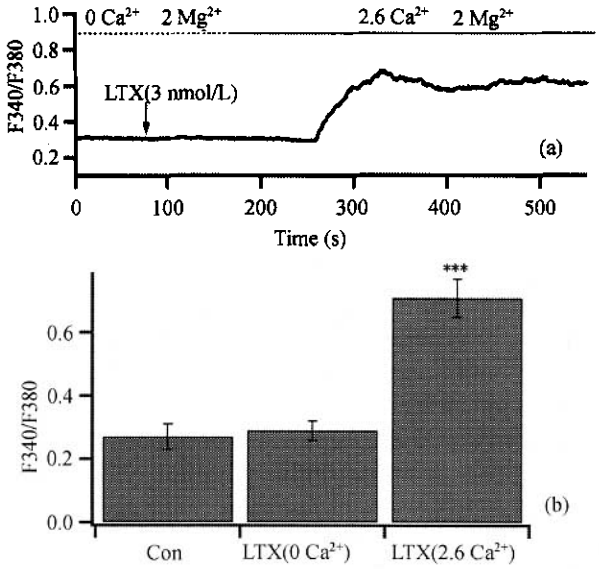

Fig. 1. α-LTX induced $[Ca^{2+}]_i$ elevation depending on extracellular Ca^{2+}. (a) As shown by a typical response of $[Ca^{2+}]_i$ elevation measured by fura-2 microfluoremetry, α-LTX failed to increase $[Ca^{2+}]_i$ in the absence of Ca^{2+}, whereas it caused robust $[Ca^{2+}]_i$ elevation in the presence of extracellular Ca^{2+} (2.6mmol/L). (b) Comparision of the amplitude of ratio (F340/F380) for control and LTX-treated cells ($n = 5$, $p < 0.01$).

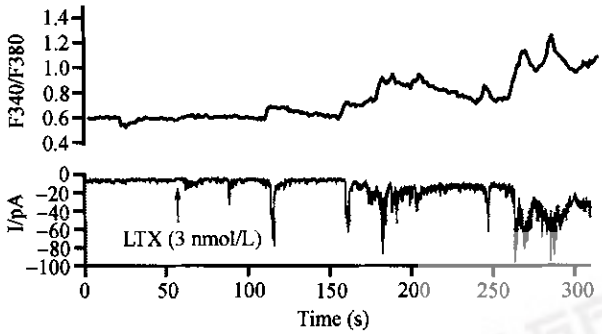

Fig. 2. α-LTX increased $[Ca^{2+}]_i$ via Ca^{2+} influx. Simultaneous measurement of $[Ca^{2+}]_i$ changes (reflected by the fluorescence ratio) and currents which were recorded from a single β cell in the whole-cell configuration. Small and brief bursts of α-LTX current elicited small $[Ca^{2+}]_i$ elevation ($n = 4$).

pacitance in pancreatic β cells (Fig. 3). The elevation of $[Ca^{2+}]$ was blocked by extracellular application of 2 mmol/L La^{3+}, which is a blocker of cation pore of α-LTX, as shown in Fig. 4.

2.2 The divalent cations were indispensable for α-LTX^{N4C} to evoke $[Ca^{2+}]_i$ elevation

As α-LTX^{N4C} cannot penetrate into the plasma membrane and form Ca^{2+} permeable pore to induce $[Ca^{2+}]_i$ elevation via extracellular Ca^{2+} influx, so it is a good tool to study the effect of exocytosis of α-LTX via

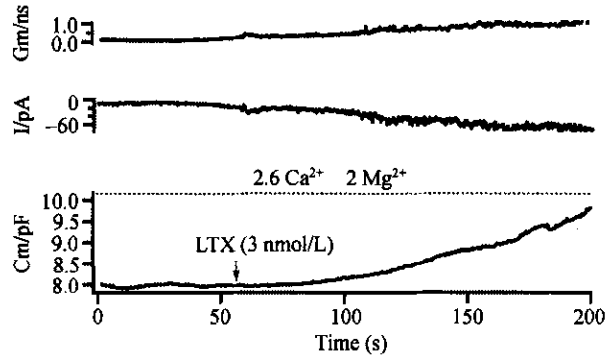

Fig. 3. α-LTX elicited excyotosis indicated by the membrane capacitance (C_m) increase. A significant increase in C_m (reflecting exocytosis) was detected when applying 3 nmol/L α-LTX. The cell was voltage clamped at **−70 mV.** α-LTX was applied at the time indicated by the arrow. The average increment of Cm was 1.6±0.4 pF ($n = 6$, $p < 0.05$).

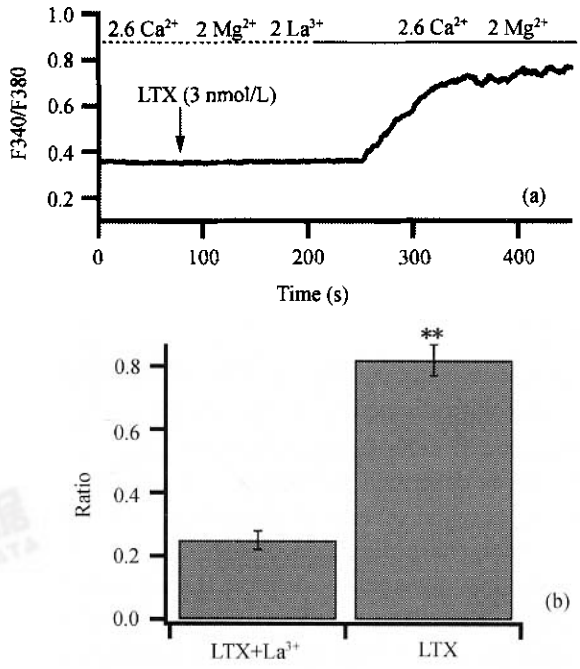

Fig. 4. La^{3+} blocked the effect of α-LTX on $[Ca^{2+}]_i$. (a) In the presence of 2 mmol/L extracellular La^{3+}, α-LTX failed to elicit elevation of $[Ca^{2+}]_i$ even in Ca^{2+} containing normal bath solution. When La^{3+} was omitted from the bath solution, α-LTX recovered the ability of elevating $[Ca^{2+}]_i$ in the same cell. (b) Comparision of the amplitude of the ratio (F340/F380). ($n = 5$, $p < 0.01$).

receptor-mediated signaling. Fig. 5(a) and (b) shows that α-LTX^{N4C} caused $[Ca^{2+}]_i$ elevation in both normal bath solution and Ca^{2+} free solution, demonstrating that α-LTX^{N4C} triggered $[Ca^{2+}]_i$ increase through intracellular Ca^{2+} store release. The pharmacological specificity of α-LTX^{N4C} was conformed by its wash effect. However, as shown in Fig. 5 (c), elevation of $[Ca^{2+}]_i$ was

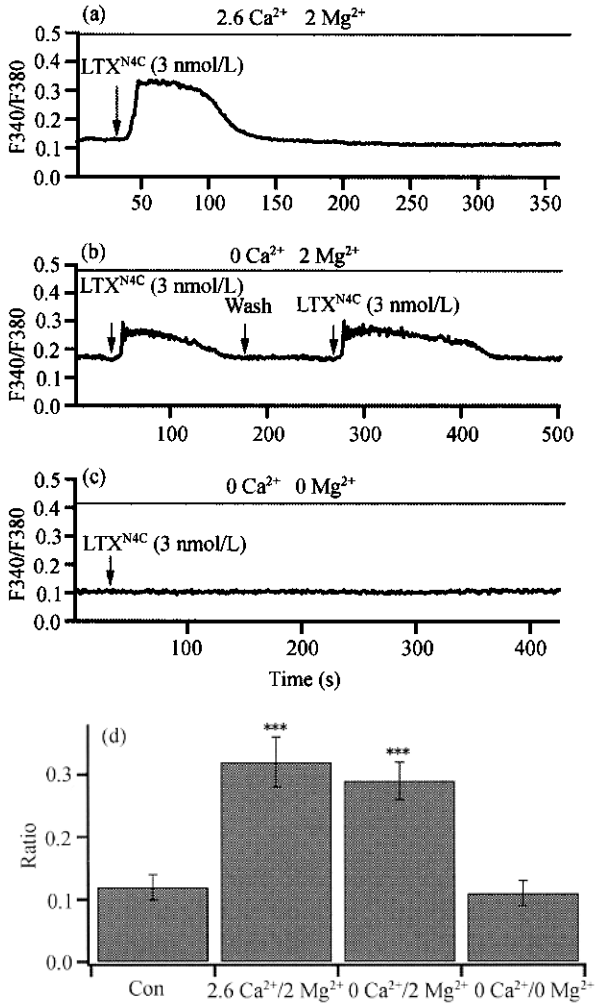

Fig. 5. α-LTX^{N4C} induced intracellular Ca^{2+} stores release. (a) α-LTX^{N4C} evoked a transient $[Ca^{2+}]_i$ elevation in the standard bath solution, whereas α-LTX elicited a sustained $[Ca^{2+}]_i$ increase (n = 6, p < 0.001, see Fig. 1). (b) α-LTX^{N4C} effect of $[Ca^{2+}]_i$ elevation was not abolished when extracellular Ca^{2+} was omitted. (n = 5, p < 0.001). (c) $[Ca^{2+}]_i$ rise triggered by α-LTX disappeared when both Ca^{2+} and Mg^{2+} from the extracellular were removed, indicating that α-LTX^{N4C} elevated $[Ca^{2+}]_i$ via mobilization of intracellular Ca^{2+} stores and its effect was dependent on the extracellular bivalent cation. (d) Comparision of the effects α-LTX^{N4C}-induced in three different bath solution.

eliminated by the further absence of extracellular Mg^{2+}. The result demonstrated that the effect of α-LTX^{N4C} depended on extracellular divalent cations. Based on this fact, we suggested that the binding of α-LTX^{N4C} with its receptor needed the presence of external bivalent cation.

2.3 Different effects on $[Ca^{2+}]_i$ induced by α-LTX and α-LTX^{N4C}

From the results above, we could draw the conclusion that α-LTX and α-LTX^{N4C} evoked $[Ca^{2+}]_i$ elevation via different mechanisms. To further validate our deduction, we used α-LTX and α-LTX^{N4C} to challenge the same β cell. Fig. 6(a) and (c) shows that α-LTX could not change $[Ca^{2+}]_i$, whereas α-LTX^{N4C} evoked obviously transient elevation of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. We obtained the same results in INS-1 cells by use of this two toxins. As shown by Fig. 6(b) and (c), in the same INS-1 cells immersed in Ca^{2+}-free bath solution, and α-LTX had no effect on $[Ca^{2+}]_i$.

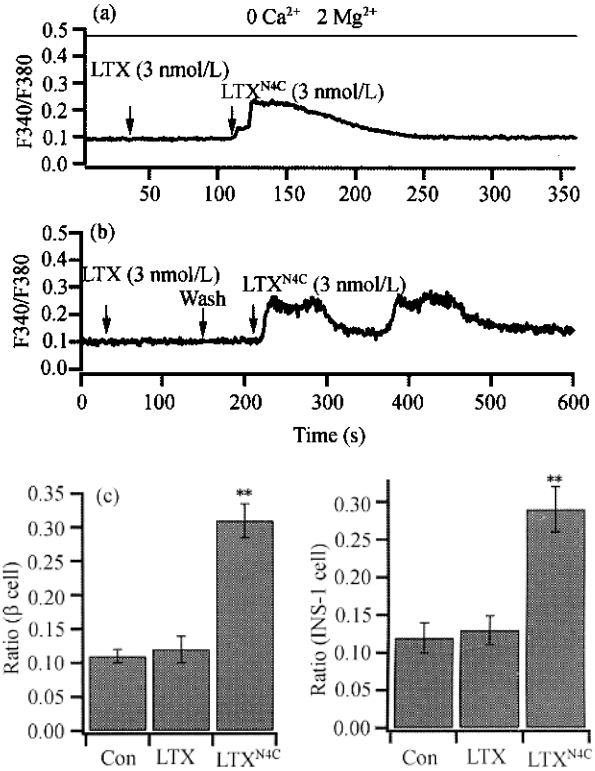

Fig. 6. The different effects of α-LTX and α-LTX^{N4C} on $[Ca^{2+}]_i$ in the absence of external Ca^{2+}. (a) By challenging the same β cell with α-LTX and α-LTX^{N4C}, we observed that α-LTX failed to evoke $[Ca^{2+}]_i$ elevation, whereas, α-LTX^{N4C} elicited robust $[Ca^{2+}]_i$ increase (n = 8, p < 0.01). (b) In the INS-1 cell, we obtained similar results, validating our data in primary pancreatic β cells (n = 4, p < 0.01). (c) Summary of the different effects induced by α-LTX and α-LTX^{N4C}.

3 Discussion

α-latrotoxin (α-LTX) is a neurotoxin from black-widow spider which causes neurotransmitter and hormone release[1,19]. Expression of the α-LTX receptor in endogenous receptor-lacking PC12 cells can also induce cells to become sensitive to the toxin. This suggests that the receptors are responsible for the function

of α-LTX[7,20]. In this study, we found that α-LTX evokes $[Ca^{2+}]_i$ elevation in 2.6 mmol/L Ca^{2+} containing bath solution, whereas it has no effect on elevation of $[Ca^{2+}]_i$ in the absence of Ca^{2+}. These results suggest that α-LTX induced $[Ca^{2+}]_i$ elevation via extracellular Ca^{2+} influx, which are blocked by trivalent ion La^{3+}, through the cation pores formed by the toxin in pancreatic β cells[21−23]. The mechanisms underlying La^{3+} of Ca^{2+} influx may be as follows: (i) La^{3+} may block the formation of Ca^{2+} permeable pore of the toxin in plasma membrane via blocking the toxin to assemble into tetramer; (ii) La^{3+} may make the pore structure unstable and block the channel[21].

It is reported that mobilization of intracellular Ca^{2+} store plays a key role on the function of α-LTX in the absence of extracellular Ca^{2+}[14,15] via binding to one of its receptors CIRL/latrophilin which is a G protein-coupled receptor linking to $G\alpha_{q/11}$. The downstream effecter of $Ga_{q/11}$ is phospholipase C (PLC). Activation of PLC leads the production of IP_3 and DAG, which are two important intracellular second messengers. IP_3 induces release of Ca^{2+} from the intracellular stores[12]. However, the α-LTX is still active even in the presence of thapsigargin which blocks IP_3-sensitive Ca^{2+} stores[24]. Furthermore, there are few α-LTX-sensitive intracellular Ca^{2+} stores in the synapses[25]. In our present study, we did not observe $[Ca^{2+}]_i$ elevation evoked by the wild type α-LTX in the absence of extracellular Ca^{2+}, indicating that intracellular Ca^{2+} stores probably play less prominent role in the function of α-LTX.

In the presence of extracellular divalent cations, α-LTX can insert itself into the plasma membrane and form stable non-selective cation channels[26]. The action of α-LTX via extracellular Ca^{2+} influx dominates that via intracellular signal transduction pathways. To exclude the possible interference of cellular function by La^{3+} which is often used to block the channel of α-LTX, we used a mutant toxin $\alpha\text{-LTX}^{N4C}$ to reveal the effect of $[Ca^{2+}]_i$ elevation of the toxin. $\alpha\text{-LTX}^{N4C}$ contains a small insert (4 amino acids: Val-Pro-Arg-Gly) within the domain responsible for the formation of the ring-like tetramers, so $\alpha\text{-LTX}^{N4C}$ has not big change in structure, but it can not assemble further into tetramers any more. As a result, $\alpha\text{-LTX}^{N4C}$ is unable to form pores[10,16,21], but its affinity with the receptors remains unchanged. $\alpha\text{-LTX}^{N4C}$ evoke $[Ca^{2+}]_i$ elevation in both normal and Ca^{2+} free solution. Our data are consistent with the reports that $\alpha\text{-LTX}^{N4C}$ mobilizes the intracellular IP_3-sensitive Ca^{2+} via the CIRL- $G\alpha_{q/11}$-PLC signal transduction pathway[12,14], In Ca^{2+} and Mg^{2+} free bath solution, $\alpha\text{-LTX}^{N4C}$ effect of $[Ca^{2+}]_i$ elevation is abolished, suggesting that divalent cations are essential for $\alpha\text{-LTX}^{N4C}$ binding to its receptors.

The different mechanisms underlying the effects of α-LTX and $\alpha\text{-LTX}^{N4C}$ are demonstrated by the facts that α-LTX can only elicit $[Ca^{2+}]_i$ elevation in the presence of extracellular Ca^{2+}, whereas $\alpha\text{-LTX}^{N4C}$ elevate $[Ca^{2+}]_i$ in both presence and absence of extracellular Ca^{2+}. Our results disagree with the reports that α-LTX and $\alpha\text{-LTX}^{N4C}$ had the same effects on intracellular Ca^{2+} level[26]. From our results, we suggest that α-LTX evoke $[Ca^{2+}]_i$ elevation mainly via formation of Ca^{2+} permeable channels after binding with the receptor neurexin, and $\alpha\text{-LTX}^{N4C}$ induce Ca^{2+} from intracellular stores via production of the second messenger IP_3 mainly by binding with the receptor CIRL and cellular signal transduction. However, many details of mechanisms of the toxin remain elusive and need to be further elucidated.

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