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Synaptotagmin I is essential for Ca^{2+}-independent release of neurotransmitter induced by α-latrotoxin

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Abstract α-Latrotoxin causes massive release of norepinephrine from clonal rat pheochromocytoma PC12 cells, in the absence of external Ca^{2+}, by an unknown mechanism. The effect almost disappeared in PC12 variant cells deficient in synaptotagmin I, a synaptic vesicle protein, and was rescued by transfecting the synaptotagmin I gene. These results indicate that synaptotagmin I is essential for the Ca^{2+}-independent action of α-latrotoxin in PC12 cells.

Key words: Neurotransmitter release; α-Latrotoxin; Neurexin; Synaptotagmin; Exocytosis; Synaptic transmission

1. Introduction

α-Latrotoxin (α-LTX), a polypeptide neurotoxin from black widow spider venom, causes massive release of neurotransmitters from neurons and clonal rat pheochromocytoma PC12 cells [1–3]. In the presence of external Ca^{2+}, the toxin induces enormous Ca^{2+} influx, triggering Ca^{2+}-dependent exocytosis of synaptic vesicles. In the absence of external Ca^{2+}, it also induces neurotransmitter release by an unknown mechanism. Recently, α-LTX receptor was isolated, characterized as a neurexin [4,5] and its cytoplasmic domain was shown to bind to synaptotagmin I (p65) [6,7], a synaptic vesicle protein [8,9]. Previously, we have isolated three variant subclones of PC12 cells (PC12-F7, PC12-B3, PC12-D6) deficient in rat synaptotagmin I and II, as well as a normal subclone (PC12-G11) expressing synaptotagmin I [10]. In the present study, we examined the action of α-LTX on these PC12 subclones and found that synaptotagmin I is essential for the Ca^{2+}-independent neurotransmitter release induced by α-LTX.

2. Materials and methods

α-LTX was purified from black widow spider venom [11]. The PC12 cells were cultured as previously described [10]. Two days before experiments, cells were plated on polyethylenimine-coated plastic culture dishes (35 mm, Falcon) at a density of 10^6 cells per dish. The cells were incubated at 37°C for 60 min in a loading medium (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES, pH 7.3 with Tris-base) supplemented with [^{3}H]norepinephrine (New England Nuclear, 9.25 kBq/dish). The release was measured as described in [10] in an assay medium (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 5 mM $MgSO_4$, 11 mM glucose, 1 mg/ml bovine serum albumin, and 15 mM HEPES, pH 7.3 with Tris-base). After two successive 1-min incubation in the assay medium, the cells were incubated in the medium containing α-LTX. Membrane depolarization of the cells was evoked by raising the potassium concentration in the assay medium to 60 mM and decreasing the sodium concentration to 85 mM. The time when the assay medium was changed to the α-LTX-containing or high-K^+ medium was designated as 0 min. The amount of [^{3}H]norepinephrine released was measured in a scintillation counter (Walac, Pharmacia) and the release was expressed as a percentage of the total [^{3}H]norepinephrine stored in the cells at the beginning of each period. All the values are means of duplicate determinations.

pKNHrStg1 containing the entire protein-coding sequence of rat synaptotagmin I was transfected to PC12-B3 cells by a modified phosphate precipitation method using 10 mg of pKNHrStg1 per 10^6 cells [12]. Four independent clones expressing synaptotagmin I were isolated. The amount of synaptotagmin I expressed in these cells was estimated by probing immunoblots of the cellular proteins with a monoclonal anti-synaptotagmin antibody, mAb 1D12 [13,14] and ^{125}I-labeled protein A (Amersham). A Fuji Bioimage-analyzer BAS 2000 (Fuji Photo Film Co.) was used to quantify the radioactivity.

3. Results

In the presence of external Ca^{2+}, α-LTX induced a marked release of [^{3}H]norepinephrine from both synaptotagmin I-expressing (PC12-G11) (Fig. 1A) and synaptotagmin I-deficient PC12 cells (PC12-F7) (Fig. 1B). The action of α-LTX depended on its concentration and, from 0.01 μg/ml (76 pM) to 0.6 μg/ml (4.6 μM), the amount of [^{3}H]norepinephrine released was increased and the lag period was decreased. There were no significant differences in the concentration dependency between the cells with or without synaptotagmin I (Fig. 1C), indicating that the presence of synaptotagmin I has no effect on the affinity of the receptors for α-LTX.

In the absence of external Ca^{2+}, a small but significant amount of [^{3}H]norepinephrine release was induced by α-LTX from synaptotagmin I-expressing PC12 cells (Fig. 2A). The concentration dependency of the effect in the absence of external Ca^{2+} was almost the same as that obtained in the presence of external Ca^{2+} (Figs. 1C and 2C). The cytosolic Ca^{2+} concentration, estimated by fura 2 fluorometry, was not changed by the addition of α-LTX (data not shown), indicating that [^{3}H]norepinephrine release in the absence of external Ca^{2+} involved a Ca^{2+}-independent mechanism. This result also indicates that the cells are alive after the α-LTX treatment and the

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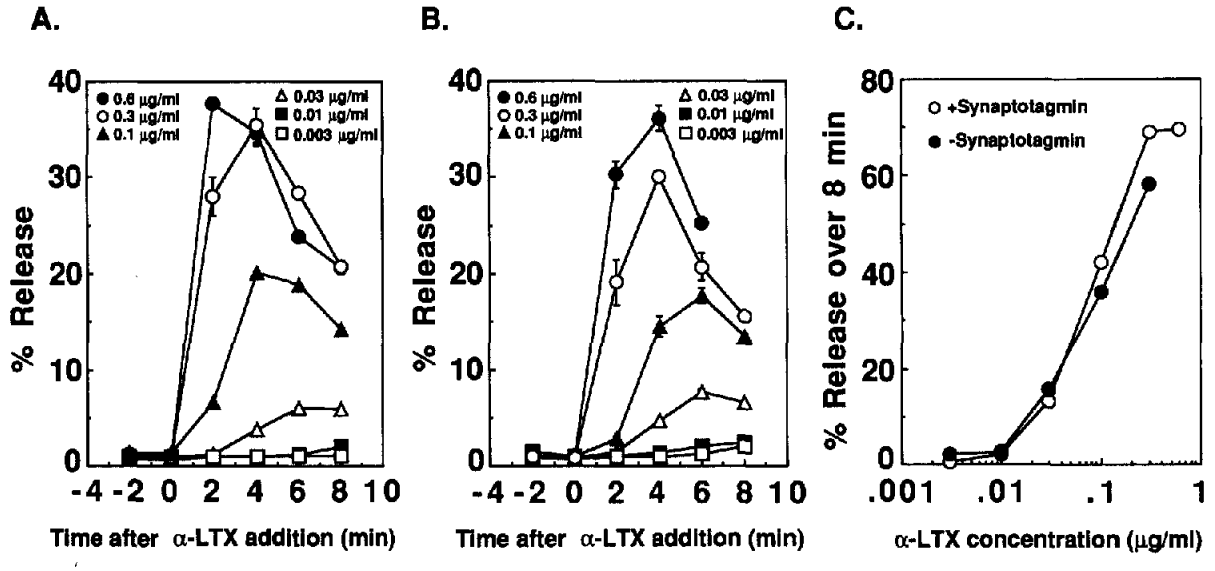

Fig. 1. α-LTX-induced [^{3}H]norepinephrine release from PC12 cells with or without synaptotagmin I in the presence of extracellular Ca^{2+}. The kinetics of [^{3}H]norepinephrine release induced by various concentrations of α-LTX from (A) synaptotagmin I-expressing PC12 cells (PC12-G11) and (B) synaptotagmin I-deficient PC12 cells (PC12-F7). Each point represents the mean of two experiments. (C) The dose–response curve for the effect of α-LTX on [^{3}H]norepinephrine release from synaptotagmin I-expressing PC12-G11 and synaptotagmin-deficient PC12-F7 cells. The amount of [^{3}H]norepinephrine released over 8 min is expressed as a percentage of the total [^{3}H]norepinephrine stored at the beginning of the experiments. Basal release was subtracted.

observed release is not due to cytolysis. In contrast, [^{3}H]norepinephrine release from the synaptotagmin-deficient cells was scarcely affected by α-LTX (Fig. 2B). Very slight release was induced by 0.1 μg/ml of α-LTX, however, secretion was not increased at higher α-LTX concentrations up to 0.3 μg/ml (Fig. 2C). Restricted release comparable to that from PC12-F7 was induced by α-LTX from the other two synaptotagmin-deficient variants, PC12-B3 and PC12-D6 (data not shown).

In order to examine whether synaptotagmin I is indispensable for the Ca^{2+}-independent release of [^{3}H]norepinephrine, we transfected rat synaptotagmin I gene into the synaptotagmin I-deficient PC12-B3 cells and isolated four stable transfectants independently. The amount of synaptotagmin I expressed in these cells was estimated from the immunoblotting of cellular homogenates with an anti-synaptotagmin antibody (mAb 1D12) and was found to be variable (Fig. 3B). Fig. 3A shows

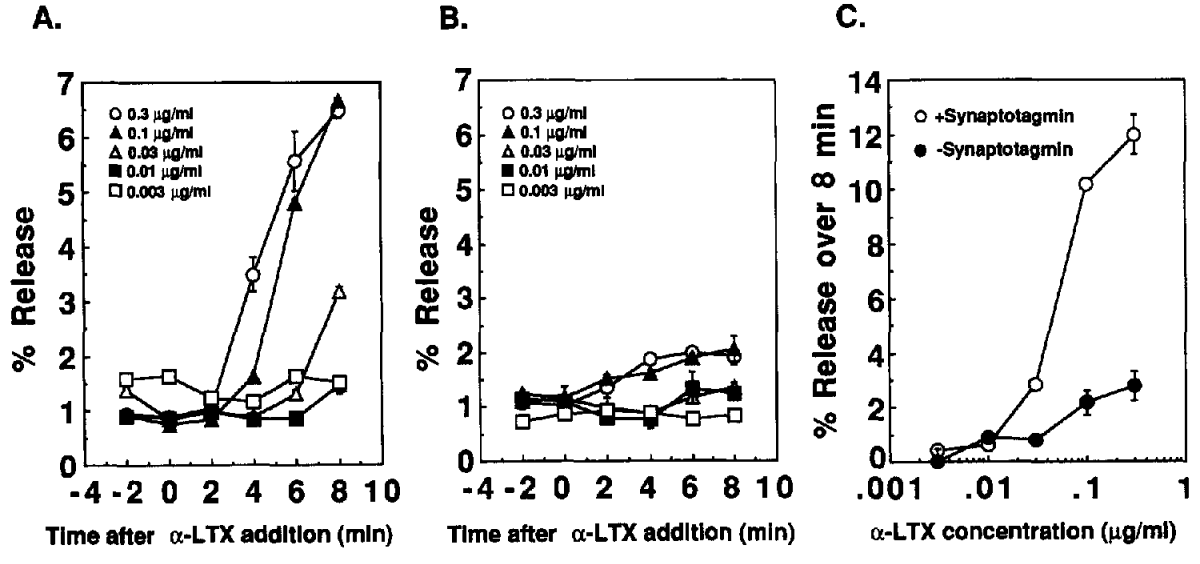

Fig. 2. α-LTX-induced [^{3}H]norepinephrine release from PC12 cells with or without synaptotagmin I in the absence of extra cellular Ca^{2+}. The kinetics of [^{3}H]norepinephrine release from (A) synaptotagmin I-expressing PC12 cells (PC12-G11) and (B) synaptotagmin I-deficient PC12 cells (PC12-F7) induced by various concentrations of α-LTX. [^{3}H]norepinephrine release was measured as described in section 2 except 2.5 mM $CaCl_2$ was replaced by 1 mM EGTA. (C) The dose–response curve for the effect of α-LTX on [^{3}H]norepinephrine release from synaptotagmin I-expressing PC12-G11 and synaptotagmin I-deficient PC12-F7 cells in the absence of external Ca^{2+}. The amount of [^{3}H]norepinephrine released over 8 min is expressed as a percentage of the total [^{3}H]norepinephrine stored at the beginning of the experiments. Basal release was subtracted.

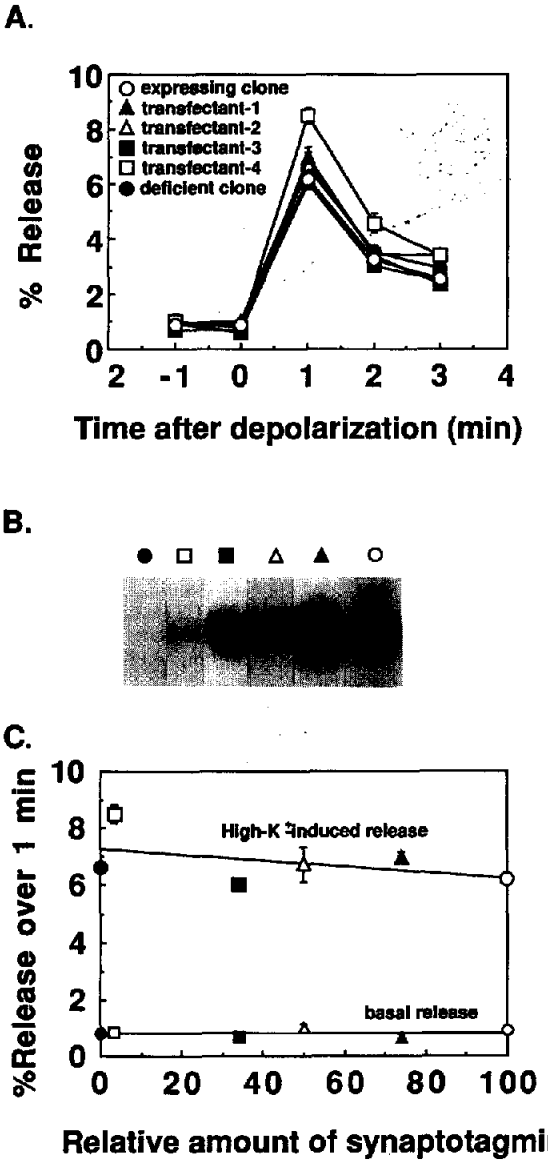

Fig. 3. High-K^+-induced [^{3}H]norepinephrine release in the presence of extra cellular Ca^{2+} from the four independent clones stably transfected with rat synaptotagmin I. (A) Kinetics of [^{3}H]norepinephrine release from the synaptotagmin I-expressing PC12 cells (PC12-G11), the synaptotagmin I-deficient PC12 cells (PC12-B3) and the four independent clones stably transfected with rat synaptotagmin I. (B) Immunoblotting of cellular proteins from PC12-G11 (○), PC12-B3 cells (●) and the transfectants (▲, △, ■, □). The symbols shown above each blot are used in Figs. 3 and 4 to identify the cells. (C) Lack of correlation between the amount of synaptotagmin I expressed and the extent of high-K^+-induced or basal release of [^{3}H]norepinephrine, over 1 min.

high-K^+-induced [^{3}H]norepinephrine release from the transfectants, synaptotagmin I-deficient PC12-B3 cells and synaptotagmin I-expressing PC12-G11 cells. All of the cells released [^{3}H]norepinephrine in response to high-K^+ stimulation and there was no correlation between the amount of synaptotagmin I expressed and percentage of [^{3}H]norepinephrine released in either basal or high-K^+ medium (Fig. 3C).

On the other hand, as shown in Fig. 4A, the α-LTX-induced secretion in the absence of Ca^{2+} was rescued in all of the transfectants and there was a good correlation between the extent of synaptotagmin I expression and the amount of [^{3}H]norepinephrine released (Fig. 4B). Thus, synaptotagmin I is essential for α-LTX-induced norepinephrine release from PC12 cells in the absence of external Ca^{2+}.

4. Discussion

The α-LTX receptor has been purified from bovine brain [4,5] and corresponding cDNA clones have been isolated from a rat brain cDNA library [15]. Sequence analysis revealed that α-LTX receptor was a member of the neurexin gene family. Neurexins have a single transmembrane segment and a large extracellular domain which contains repeated domains similar to sequences in proteins implicated in axon guidance and synaptogenesis, such as laminin A, slit, and agrin. Since more than 100 different neurexin transcripts may be generated by alternative splicing, neurexins may possibly be involved in the formation and stabilization of specific synaptic connections. The short cytoplasmic portion of neurexins displays no homology to consensus motifs involved in signal transduction, suggesting an association with other cytoplasmic proteins is necessary for α-LTX receptor function.

In the present study, we have demonstrated that synaptotagmin I is essential for Ca^{2+}-independent release from PC12 cells induced by α-LTX. Synaptotagmin I has been shown to bind to the cytoplasmic region of neurexins and thus is likely to play a key role in coupling neurexins to intracellular processes. Recently, we have isolated a cDNA clone encoding the third isoform of rat synaptotagmin, synaptotagmin III [16]. By Northern blotting, we found that synaptotagmin III gene was expressed in both synaptotagmin I-expressing and synaptotagmin I-deficient PC12 cells which were used in the present study (data not shown). The C-terminal region of synaptotagmin is essential for binding to neurexins [17] and C-terminal sequences vary among rat synaptotagmin isoforms. Thus, it is likely that the different synaptotagmin isoforms have different affinities for neurexins and play different roles in the synapse.

In the presence of extracellular Ca^{2+}, α-LTX induces massive Ca^{2+} influx into both synaptotagmin-expressing and synaptotagmin-deficient PC12 cells. Since α-LTX forms a cation-selective channel after incorporating into artificial lipid bilayers [18–20], it is possible that the ion channel formed by α-LTX induces Ca^{2+} influx and neurexins solely help the toxin to bind close to the presynaptic plasma membrane.

In the absence of external Ca^{2+}, α-LTX induces Ca^{2+}-independent release of neurotransmitters from a variety of neurons [1]. Since neurexins are localized in presynaptic nerve terminals and have large extracellular domains, they may interact with proteins in the postsynaptic membrane. These trans-synaptic contacts may activate neurexins, inducing a small but continuous release of neurotransmitter. It is possible that the persistent secretion may modify the properties and/or strength of synaptic connections. Thus, the signaling role of neurexins in synaptic function (or in the secretory process) may have more subtle implications than previously thought.

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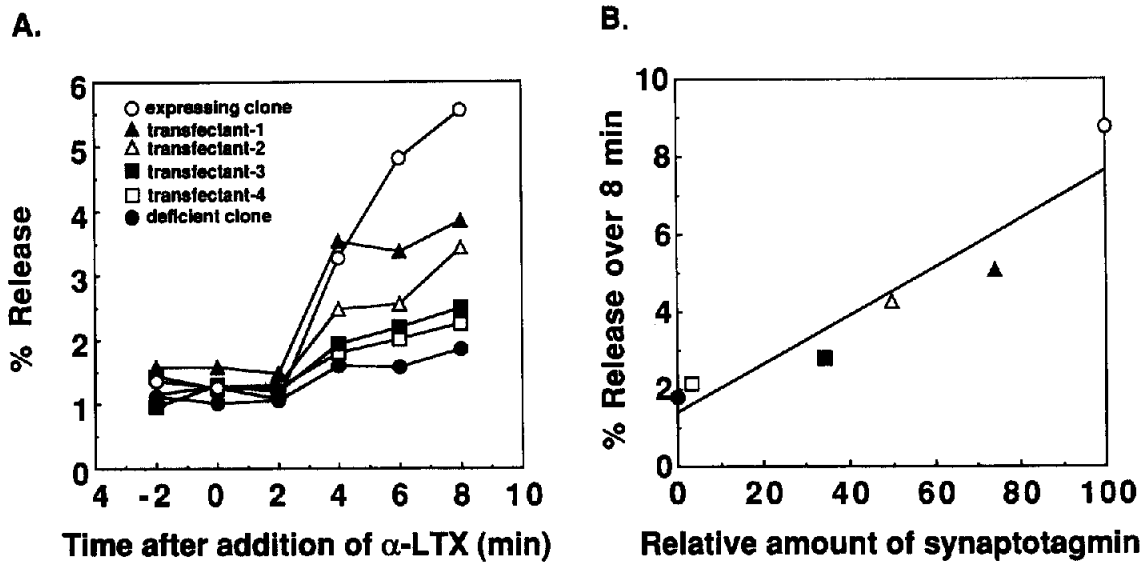

Fig. 4. Recovery of α-LTX-induced [^{3}H]norepinephrine release in the absence of extracellular Ca^{2+} by the expression of synaptotagmin I. (A) Kinetics of [^{3}H]norepinephrine release induced by 0.3 μg/ml of α-LTX from the synaptotagmin I-expressing PC12 cells (PC12-G11), the synaptotagmin I-deficient PC12 cells (PC12-B3) and the four independent clones stably transfected with rat synaptotagmin I. (B) Correlation between the amount of synaptotagmin I expressed and the amount of α-LTX-induced [^{3}H]norepinephrine release over 8 min from the synaptotagmin I-expressing PC12 cells, synaptotagmin I-deficient PC12 cells and the four independent clones stably transfected with rat synaptotagmin I.

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