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Trachynilysin mediates SNARE-dependent release of catecholamines from chromaffin cells via external and stored Ca^{2+}

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Summary

Trachynilysin, a 159 kDa dimeric protein purified from stonefish (*Synanceia trachynis*) venom, dramatically increases spontaneous quantal transmitter release at the frog neuromuscular junction, depleting small clear synaptic vesicles, whilst not affecting large dense core vesicles. The basis of this insensitivity of large dense core vesicles exocytosis was examined using a fluorimetric assay to determine whether the toxin could elicit catecholamine release from bovine chromaffin cells. Unlike the case of the motor nerve endings, nanomolar concentrations of trachynilysin evoked sustained Soluble *N*-ethylmaleimide-sensitive fusion protein Attachment Protein REceptor-dependent exocytosis of large dense core vesicles, but only in the presence of extracellular Ca^{2+}. However, this response to trachynilysin does not rely on Ca^{2+} influx through voltage-activated Ca^{2+} channels because the secretion was only slightly affected by blockers of L, N and P/Q types. Instead, trachynilysin elicited a localized increase in intracellular fluorescence monitored with fluo-3/AM, that precisely co-localized with the increase of fluorescence resulting from caffeine-induced release of Ca^{2+} from intracellular stores. Moreover, depletion of the latter stores inhibited trachynilysin-induced exocytosis. Thus, the observed requirement of external Ca^{2+} for stimulation of large dense core vesicles exocytosis from chromaffin cells implicates plasma membrane channels that signal efflux of Ca^{2+} from intracellular stores. This study also suggests that the bases of exocytosis of large dense core vesicles from motor nerve terminals and neuroendocrine cells are distinct.

Key words: Chromaffin cell, Trachynilysin, Exocytosis, Botulinum toxin, Caffeine, Ca^{2+} store, Catecholamine release

INTRODUCTION

Of the large number of neurotoxins that can promote neurotransmitter release, the majority selectively target ion channels and modulate their activity (reviewed by Molgó et al., 1997; Van der Kloot and Molgó, 1994), thereby, indirectly eliciting the exocytotic release of synaptic vesicles. Less common are neurotoxins that stimulate the release apparatus by acting upon cell surface signal-transducing receptors. One of the most studied toxins in this category is α-latrotoxin (α-LTX), a 120 kDa protein from the venom of the black widow spider *Latrodectus mactans tredecimguttatus*, which has been exploited successfully to decipher important steps of the exo-endocytotic cycle of synaptic vesicles (see review by Ceccarelli and Hurlbut, 1980; Rosenthal and Meldolesi, 1989; Henkel and Betz, 1995). Recent identification of synaptic targets for α-LTX has given important insights into novel mechanisms for modulating neuroexocytosis (Ushkaryov et al., 1992; Davletov et al., 1996; Petrenko et al., 1996).

Trachynilysin (TLY), a 159 kDa toxic protein isolated from the venom of the stonefish *Synanceia trachynis*, elicits regulated exocytosis via an unknown mechanism (Colasante et al., 1996). As has been established for α-LTX (reviewed by Rosenthal and Meldolesi, 1989), the application of nanomolar doses of TLY to frog neuromuscular junctions induces a dramatic increase in asynchronous acetylcholine release (Colasante et al., 1996). The latter effect lasts several hours, leading eventually to the selective depletion of clear synaptic vesicles from the swollen terminals; importantly, neither α-LTX (Matteoli et al., 1988) nor TLY (Colasante et al., 1996) diminishes the population of large dense core vesicles (LDCV). These data suggest that, in motor nerve terminals, α-LTX and TLY act exclusively on the neuroexocytosis pathway for the clear synaptic vesicles. However, recent studies have demonstrated that α-LTX triggers the release of catecholamine

from LDCVs of adrenal chromaffin cells (Barnett et al., 1996; Bittner et al., 1998) as well as the contents of LDCVs in pancreatic β-cells (Lang et al., 1998) and sympathetic noradrenergic neurons (reviewed by Depotter et al., 1997).

In view of these observations, it was pertinent to determine whether TLY can also stimulate secretion from neuroendocrine cells. Herein, it is shown that TLY does indeed elicit SNARE-dependent exocytotic release of catecholamines from chromaffin cells, but by a mechanism that apparently does not depend on voltage-activated Ca^{2+}-channels. However, measurement of the influence of TLY on intracellular fluorescence levels in fluo-3/AM-loaded chromaffin cells revealed that it induces secretion by causing a spatially-restricted elevation of the fluorescence that co-localizes with the one produced by the activation of caffeine-sensitive Ca^{2+} stores.

MATERIALS AND METHODS

Materials

Tissue culture reagents were purchased from Gibco BRL (Paisley, Scotland), fluo3/AM from Molecular Probes Europe Bv (Leiden, The Netherlands); D600, ω-conotoxins (GVIA and MVIIC), caffeine from Sigma Chemical Co. (Dorset, UK), and purified ryanodine from Calbiochem (Nottingham, UK). Botulinum neurotoxin (BoNT) types A, B and C were purified as described previously (Shone and Tranter, 1995), and stock solutions were stored in 150 mM NaCl, 10 mM HEPES buffer (pH 7.4) at −20°C. TLY was purified from the venom of the stonefish (*S. trachynis*) by sequential anion-exchange, fast protein-liquid chromatography (FPLC) and size-exclusion FPLC (Colasante et al., 1996), and was stored at −60°C at a concentration of approximately 2 mg protein/ml.

Culture of bovine adrenochromaffin cells and their intoxication with BoNT/A, /B or /C

Chromaffin cells were prepared from bovine adrenal glands and maintained as primary cultures (Lawrence et al., 1994). Within 2-3 days the cells were incubated in the absence and the presence of BoNT/A, /B or /C (66 nM) in a low ionic strength buffer (37°C, 24 hours), to facilitate uptake of the neurotoxins as described previously (Marxen et al., 1991). The cells were then returned to their original medium to recuperate for 24 hours before further experimental manipulation.

Stimulation and quantitation of catecholamine secretion

Chromaffin cells were washed briefly once with buffer A (145 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM HEPES, pH 7.4) and processed in quadruplicate as stated in the figure legends. Aliquots (200 μl) of the medium were removed at the end of each experiment and cells were lysed with 1% (v/v) Triton X-100. Both set of samples were assayed fluorimetrically for catecholamine content, and the amounts released expressed as a percentage of the total quantity of catecholamines calculated to be present in the cells (Lawrence et al., 1994). Plotted data are representative of experiments carried out in quadruplicate and performed at least twice.

Electron microscopy

Control and TLY-treated chromaffin cells, grown in Petri dishes as monolayer cultures, were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature before being processed as previously described (Colasante et al., 1996) and embedded in Spurr's resin. Thin-sections cut from the plastic-embedded tissue using an ultramicrotome were counterstained with uranyl acetate and lead citrate, and examined using either a Hitachi-500 or a Phillips 201 electron microscope.

Calcium imaging

Chromaffin cells were loaded with fluo-3/AM by incubation (45 minutes, room temperature) with 5 μM fluo-3/AM and 0.02% pluronic acid (w/v) in Krebs-Ringer solution (containing in mM: NaCl, 154; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 5; glucose, 11). After washing the cells with fluo-3/AM-free solution, the coverslips were placed on the microscope stage and imaged either with an Olympus upright microscope equipped with an epifluorescence unit (Olympus, Japan) and an extended ISIS CCD, cooled video camera (Photonics Science, UK) or by confocal microscopy (see below). For the video experiments, illumination was provided by a quartz-halogen bulb (12 V/100 W) and fluorescence recorded through a Plan ×40 (0.7 NA) long-working-distance, water-immersion lens (Olympus, Japan), using an interference set of filters (excitation: 485 nm; emission: 535 nm) and a dichroic mirror (505 nm). Digitizing and analysis of fluorescence images were performed with a personal computer, using a DT3155 frame grabber and Imaging Workbench 2.1 software (Axon Instruments, Dipsi, France). Images from each experiment were processed identically before quantitation by outlining the chromaffin cells and determining its mean fluorescence before (Fo) and during TLY treatment (F). The index of the fluorescence variation ΔF/Fo was then calculated as (F-Fo)/Fo. For the confocal imaging, a Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, CA, USA) composed of an upright Nikon optiphot-2 microscope equipped with a single argon laser beam was used. The aperture setting of the confocal pinhole was 100 μm. Cells were visualized using a ×40 water immersion objective (Zeiss, 0.75 numerical aperture). The 488 nm band of the argon ion laser was used for excitation and the emitted light was collected through a 510 nm longpass. All experiments were performed at room temperature, and time images of cell sections were acquired with a frame interval of 5 seconds. A Silicon Graphics Personal Iris 4D/35G UNIX controlled the scanner module, and image analysis was done with ImageSpace 3.1 software from Molecular Dynamics. Sections were collected using a standard scanning mode format of 512 × 512 pixels. The fluo-3/AM loaded cells are presented using a pseudocolor scale.

Statistical analysis

Statistical analysis of data was performed using the Student's *t*-test (two tailed). Values are expressed as means ± s.e.m. Data were considered significant at $P<0.05$.

RESULTS

TLY evokes Ca^{2+}-dependent catecholamine release from chromaffin cells

Exposure of monolayer cultures of bovine chromaffin cells to TLY in the presence of external Ca^{2+} (2 mM) induced the release of catecholamines, as revealed using a fluorimetric assay (Fig. 1A,B). Maximal secretion was observed after 20-30 minutes exposure to 60 nM TLY only in a Ca^{2+}-containing medium (Fig. 1A). As shown in Fig. 1B, TLY gave a dose-dependent increase in catecholamine release reaching a plateau at 60 nM toxin with a 20 minutes incubation. These optimal conditions were employed for the following experiments. To ascertain if TLY needs Ca^{2+} as a co-factor to aid binding to an extracellular acceptor, chromaffin cells were pre-treated with TLY in the absence of Ca^{2+} and then extensively washed before adding Ca^{2+}. This resulted in a potent stimulation of secretion (Fig. 1C) comparable to that shown in Fig. 1A, indicating that TLY can bind to the cells independently of Ca^{2+} but this cation is required for maximal TLY-elicited catecholamine release. Examination of the Ca^{2+} concentration-dependency of TLY-induced release of catecholamine showed that the maximal

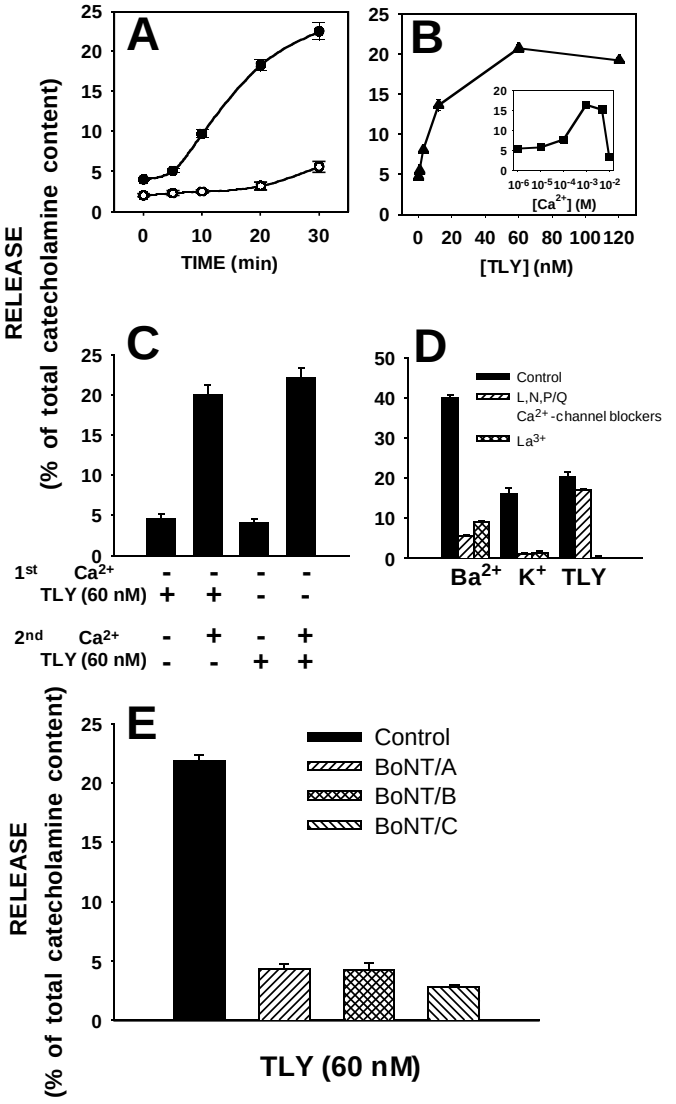

Fig. 1. TLY triggers Ca^{2+}- and SNARE-dependent catecholamine release. (A) Primary cultures of adrenal chromaffin cells bathed in buffer A in the presence (filled circles) or the absence (empty circles) of 2 mM Ca^{2+} were exposed for the indicated periods to 60 nM TLY before removal of an aliquot and assay of its catecholamine content by fluorometry. Amounts of catecholamine released (± s.e.m., n=4) are plotted as a % of the total content of the cells. (B) Chromaffin cells were incubated for 20 minutes with various concentrations of TLY (filled triangles) or 60 nM TLY in buffer A containing the indicated [Ca^{2+}] (filled square) before measuring secreted catecholamines. (C) Cells were incubated in buffer A (Ca^{2+}-free) with or without TLY (60 nM) for 15 minutes (1st phase), then extensively washed before exposure to fresh buffer A plus or minus Ca^{2+} (2 mM) and/or TLY for 20 minutes (2nd phase), as illustrated. Catecholamine release during the latter 20 minutes is plotted. (D) The secretion from cells which had been exposed for 20 minutes to Ba^{2+} (2 mM), TLY (60 nM) or K^+ (55 mM, Na^+ reduced to 95 mM) in the presence (patterned bars) or the absence (black bars) of Ca^{2+} channel blockers (mixture of D600 [100 μM], μ-conotoxin GVIA and MVIIC, [5 μM] diagonally striped bars) or La^{3+} (500 μM, cross-hatched bars). (E) Intact chromaffin cells which had been poisoned by prolonged exposure to BoNT/A, /B or /C (see Materials and Methods), and control cells treated with vehicle only, were exposed for 20 minutes to TLY (60 nM) in the presence Ca^{2+} (2 mM) before measuring the extent of catecholamine release.

effect was obtained using an external Ca^{2+} concentration of 1-5 mM (Fig. 1B insert). Notably, a strong inhibition of TLY release was detected at 10 mM extracellular Ca^{2+}; this agrees with previous observations that high Ca^{2+} concentrations lower catecholamine release from chromaffin cells (Knight et al., 1989; Bittner and Holz, 1992).

As extracellular Ca^{2+} appears to be a prerequisite for prompt stimulation of catecholamine release induced by TLY, it was of interest to study the possible involvement of voltage-activated Ca^{2+}-channels. In view of L, N and P/Q type Ca^{2+}-channels having been reported in chromaffin cells (Artalejo et al., 1994), the cells were pretreated for 15 minutes incubation with a mixture of D600 (100 μM) which specifically inhibits L-type and ω-conotoxin GVIA plus ω-conotoxin MVIIC (5 μM), which block N and N, P, Q-type Ca^{2+}-channels, respectively (Artalejo et al., 1994; Lopez et al., 1994; Albillos et al., 1996). The effectiveness of these inhibitors was revealed by their virtual abolition of K^+-depolarization or Ba^{2+}-evoked exocytosis (Fig. 1D). Nevertheless, this treatment had only a minimal effect (5-10% inhibition) on the stimulation by TLY of exocytosis from chromaffin cells (Fig. 1D), indicating that depolarization and consequential activation of voltage-activated Ca^{2+} channels play a minor role in the toxin's action. Such a lack of involvement of Ca^{2+} influx mediated via voltage-activated Ca^{2+}-channels raised the possibility that the stimulation of exocytosis is caused by activation of another type of channel or by formation of channels by TLY itself. Preliminary data indicates that TLY is indeed a pore-forming protein, as shown on differentiated neuroblastoma cells (Ouanounou et al., 1999) and planar lipid bilayer membrane (C. Mattei, C. Pompa and M. Thieffry, unpublished observations). The observation that the non specific Ca^{2+} channel blockers La^{3+} (Pruss and Stauderman, 1988) and Ni^{2+} (Fonteriz et al., 1992), respectively, abolished the toxin's stimulatory effect on secretion (Fig. 1D) and can prevent or reverse TLY-induced channel activity in neuroblastoma cells and planar lipid bilayer suggests that these pores are at least permeable to Ca^{2+}.

TLY induces SNARE-dependent exocytosis of LDCVs

Intact chromaffin cells can be intoxicated in vivo by various clostridial neurotoxins, using a low ionic strength medium which facilitates toxin internalization (Lawrence et al., 1994, 1996; Foran et al., 1996). Poisoning of these cells with BoNT/A, /B or /C results in the cleavage of SNAP-25 alone, synaptobrevin or both SNAP-25 and syntaxin, respectively. SNAP-25 and syntaxin, both associated predominantly with the plasma membrane, are implicated in the docking of large dense core granules by forming a ternary complex with the vesicular protein synaptobrevin (Rothman, 1995; see review by Morgan and Burgoyne, 1997). Therefore, it was pertinent to investigate whether this exocytotic machinery was required for TLY-induced catecholamine release. For this purpose, chromaffin cells were treated with BoNT/A, /B or /C before exposure to TLY. In the presence of external Ca^{2+} (2 mM), TLY-evoked secretion of catecholamines was drastically inhibited by each of the BoNT serotypes (Fig. 1E). Nevertheless, it is clear that, in the presence of Ca^{2+}, TLY

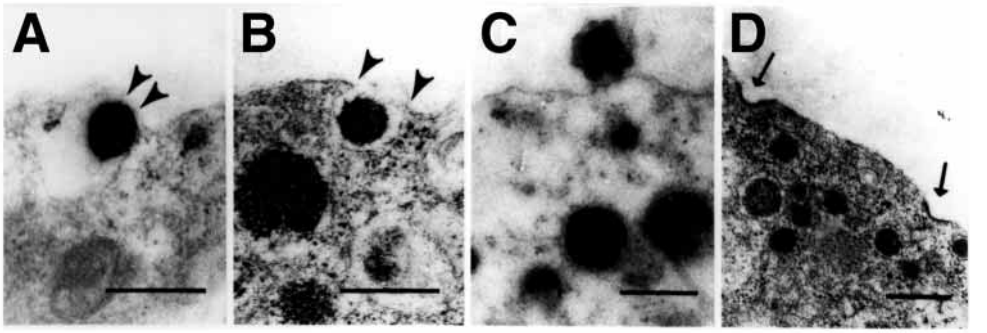

Fig. 2. TLY triggers LDCV exocytosis in chromaffin cells. Electron micrographs show various steps of exocytosis abundant in TLY-treated (60 nM, 20 minutes) but not in toxin-free preparations. Individual panels show docking (A), omega profile (arrowheads) characteristic of LDCV fusion (B) and expelled dense core material outside the chromaffin cell (C). (D) Two coated pits (arrows) which form at an early stage of endocytosis.

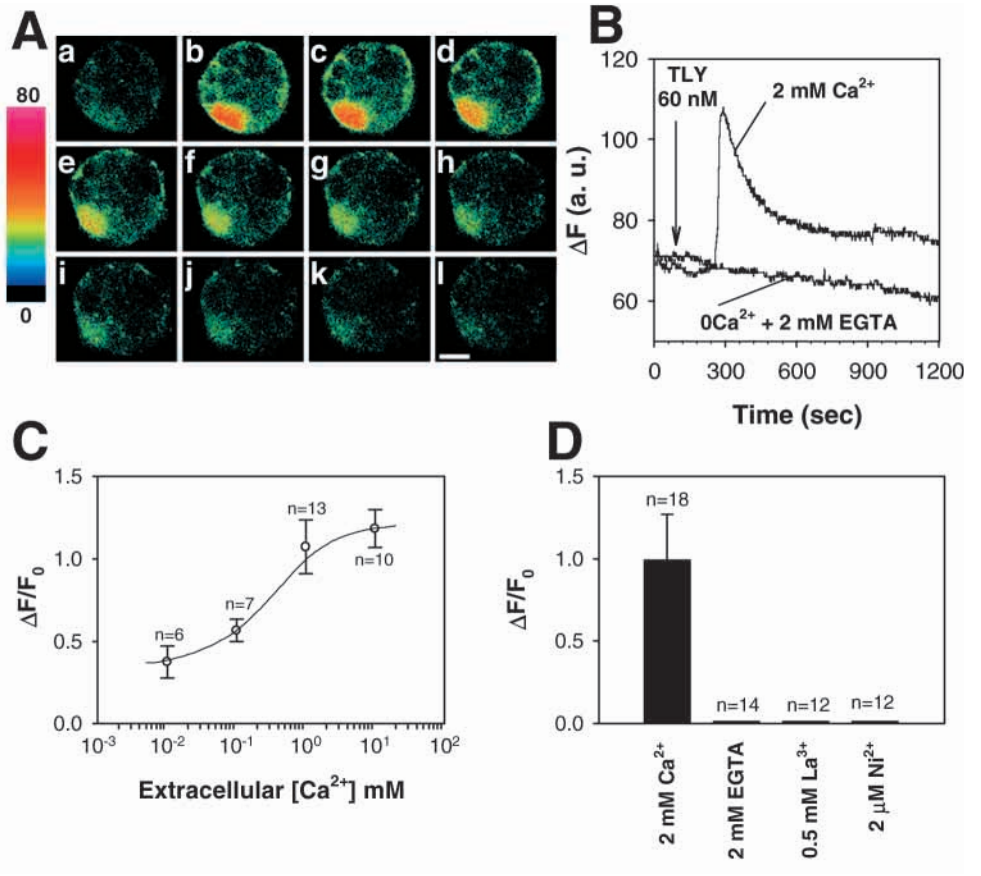

Fig. 3. TLY produced a localised increase in Ca^{2+} fluorescence signal. (A) Series of confocal images, acquired at 5 second intervals, of a fluo-3-labelled chromaffin cell treated with TLY (60 nM). (B) The kinetics of the changes in fluorescence triggered by TLY were monitored by measuring fluorescence intensity with a CCD video-camera; the experiments were performed both in the presence of Ca^{2+} (2 mM) and in the absence of Ca^{2+} (2 mM EGTA). (C) Fluorescence intensity was measured in fluo-3 loaded chromaffin cells bathed in buffers containing incremental concentrations of Ca^{2+} and exposed to TLY (60 nM). The values obtained from three independently conducted experiments were plotted as a ratio over the signal obtained in the absence of TLY; *n* represents the total number of cells examined. (D) Quantitation of the intracellular Ca^{2+} variations evoked by TLY in chromaffin cells bathed with buffer containing either 2 mM Ca^{2+}, with 500 μM La^{3+} or 20 μM Ni^{2+} which are nonspecific Ca^{2+} channel blockers or in the absence of Ca^{2+} (2 mM EGTA).

triggers the classical exocytotic process, possibly by increasing the intracellular Ca^{2+} level as described in the next section.

Ultrastructural experiments were performed to visualize exocytotic events in TLY-treated chromaffin cells. Toxin-treated cells exhibited a significant reduction in the number of LDCVs per section of chromaffin cell (Table 1). Notably, LDCVs at various stages of exocytosis were observed after 20 minutes exposure: initial 'docked' vesicles (Fig. 2A), classical omega (Ω) figures (Fig. 2B) indicative of fusion, exocytotically expelled dense core material (Fig. 2C), and coated pit formation (Fig. 2D) associated with endocytotic retrieval of the LDCV membrane. Interestingly, no coated vesicles were detected in the 105 profiles examined.

TLY triggers Ca^{2+} influx into chromaffin cells

The requirement of external Ca^{2+} together with the inhibition by La^{3+} strongly suggests that TLY-induced Ca^{2+} influx into chromaffin cells underlies the toxin's stimulation of exocytosis. Thus, Ca^{2+} was monitored, using a CCD video camera, in chromaffin cells pre-loaded with the cell permeant Ca^{2+}-indicator fluo-3/AM. In the presence of 2 mM external Ca^{2+}, exposure of fluo-3/AM-preloaded chromaffin cells to TLY (60 nM) resulted in a transient increase in intracellular fluorescence

Table 1. Average number of LDCVs per chromaffin cell section before and after exposure to TLY (60 nM)

Exposure time (minutes)	0	5	10	20
Number of LDCV	268.9±13.1 *n*=40	223.3*±17.5 *n*=35	184.3*±13.6 *n*=40	146.8**±12.6 *n*=40

The values are expressed as means (± s.d.) and are significantly different from the control at $*P<0.05$ or $**P<0.001$. *n* represents the number of electron micrographs examined.

intensity (Fig. 3A,B). In all cases (n=18), the fluorescence intensity substantially increased (71.4±8.5%) with a rise-time of 15-30 seconds, but only after a consistently observed delay of about 2 minutes. The half-decay time was about 2 minutes, followed by an extended recovery (5-10 minutes) during which the Ca^{2+} fluorescence was notably higher than the resting level (Fig. 3B). Importantly, in Ca^{2+}-free medium supplemented with 2 mM EGTA, TLY exposure did not produce any change in intracellular Ca^{2+} fluorescence (n=11; Fig. 3B,D). It should be noted that La^{3+} and Ni^{2+}, nonspecific calcium channel blockers (Pruss and Stauderman, 1988, Fonteriz et al., 1992), potently inhibited TLY-evoked Ca^{2+} signals (Fig. 3D). TLY-induced intracellular changes in fluo-3 fluorescence, measured in the presence of incremental amounts of external Ca^{2+} revealed that only when the Ca^{2+} exceeded 100 μM were significant rises in Ca^{2+} fluorescence observed (Fig. 3C). Maximal changes were achieved with 1 mM external Ca^{2+} (in good agreement with the amount required for exocytosis); no further increase was observed at 10 mM (Fig. 3C), the concentration found to inhibit TLY-induced secretion of catecholamine (Fig. 1B, insert). These data, taken together with those of the release experiments, demonstrate that TLY triggers a large transient Ca^{2+} increase followed by a sustained intracellular Ca^{2+} level for at least 10 minutes, which are dependent on extracellular Ca^{2+} and potentially capable of eliciting catecholamine release by exocytosis.

In order to obtain high-resolution of the time-course of intracellular Ca^{2+} fluorescence variations triggered by TLY, confocal scanning microscopy was used. Fluo-3-loaded chromaffin cells exposed to TLY showed a transient increase in fluorescence throughout the overall cytoplasm but exhibited a higher intensity in a localized region of the cell (Fig. 3A). This Ca^{2+}-increase was confined to a restricted area of the chromaffin cell and faded without spreading (Fig. 3A), with the same time-course as observed previously (Fig. 3B).

The stimulatory effect of TLY on exocytosis is influenced by internal Ca^{2+} stores

In chromaffin cells, a localized rise in the cytosolic concentration of free Ca^{2+} signal is typically obtained when internal stores are mobilized (Burgoyne, 1991). Thus, the continuous monitoring of variations in the fluorescence of the Ca^{2+} signal was performed using confocal microscopy of a single identified fluo-3-loaded chromaffin cell treated sequentially with caffeine and TLY. Notably, the caffeine-induced restricted signal (situated in the middle of the cell in this confocal optical section), precisely co-localized with the one observed following subsequent application of TLY (Fig. 4A; n=10). It is worth noting that the intensity of the 5 mM caffeine-induced signal was much lower than that of TLY (Fig. 4A). To test whether TLY could induce Ca^{2+} release from caffeine-sensitive intracellular stores, the intensity of the fluorescence Ca^{2+} signal evoked by incremental amounts of caffeine was compared to that elicited by a subsequent application of TLY (60 nM) to identified fluo-3-loaded chromaffin cells, using confocal microscopy (Fig. 4B). The intensity of caffeine-induced Ca^{2+} signal increased dose-dependently, but the fluorescence intensity triggered by 60 nM toxin (applied 10 minutes after caffeine) exposure was progressively reduced as the caffeine concentration was raised (Fig. 4B). This raises the possibility that the toxin activates Ca^{2+} release from internal stores by stimulating the production of a second messenger. Indeed, pre-exposure of chromaffin cells to various concentrations of caffeine also resulted in a dose-dependent inhibition of the catecholamine release induced upon subsequent exposure to TLY (Fig. 4C). In a parallel experiment, it was observed that caffeine alone was unable to elicit significant catecholamine release, as has previously been reported (Burgoyne, 1991, and data not shown). Moreover, depletion of intracellular Ca^{2+} stores by thapsigargin (which inhibits Ca^{2+}-ATPase-mediated uptake of Ca^{2+} into the stores), or blockade of the ryanodine receptors (by 50 or 100 μM ryanodine) reduced TLY-induced catecholamine secretion (Fig. 4D), furthering the implication that the internal stores are necessary for the maximal effect of TLY.

DISCUSSION

It has been demonstrated herein, for the first time, that TLY is a potent secretagogue for neuroendocrine cells. The elicited catecholamine release resulted from the exocytotic fusion of LDCVs, as shown by electron microscopy and by its sensitivity to cleavage of SNAREs by BoNT/A, /B and /C. The presence of external Ca^{2+} is essential for TLY-evoked secretion, suggesting that Ca^{2+} influx is required. Importantly, selective inhibition of voltage-activated Ca^{2+} channels had minimal effect on TLY-induced catecholamine release. However, La^{3+} and Ni^{2+}, nonselective Ca^{2+} channel blockers (Pruss and Stauderman, 1988; Fonteriz et al., 1992), abolished this vesicular secretion. Since preliminary data show that TLY can indeed form channels in a planar lipid bilayer (C. Mattei, C. Pompa and M. Thieffry, unpublished results) and in the membrane of neuroblastoma cells (Ouanounou et al., 1999), it is suggested that TLY could directly, or through an interaction with an unknown acceptor, elicit a pore permeable to Ca^{2+} in the chromaffin cell plasma membrane responsible for initiating Ca^{2+} influx. Interestingly, Ca^{2+} imaging experiments showed that TLY causes a transient increase in the fluorescent Ca^{2+} signal, that was spatially restricted. Such localized Ca^{2+} signals are typical of chromaffin cells' response to agonists, like caffeine, which mobilize Ca^{2+} from internal stores (see review by Burgoyne, 1991). The observed precise co-localization of the intracellular Ca^{2+} signals that were evoked by TLY and caffeine suggested a role for the mobilization of Ca^{2+} stores in cellular responses to the toxin. Although, mobilization of such stores by caffeine itself does not stimulate catecholamine secretion (Burgoyne, 1991), it is possible that TLY, by stimulating Ca^{2+} influx across the plasma membrane and thereby triggering Ca^{2+}-induced Ca^{2+}-release (CICR) from endoplasmic reticulum (ER) stores, may raise cytosolic Ca^{2+} to a level capable of supporting exocytosis. In a recent study, endoplasmic reticulum Ca^{2+} has been shown to be released both via the IP_3 receptor or CICR in chromaffin cells (Alonso et al., 1999). TLY-induced Ca^{2+} efflux from the caffeine-sensitive stores is shown herein to be highly dependent on the Ca^{2+} content within those stores, since it is sensitized in a dose-dependent manner by caffeine. Therefore, TLY could induce a long-lasting Ca^{2+} rise composed of a combination of Ca^{2+} influx and CICR from

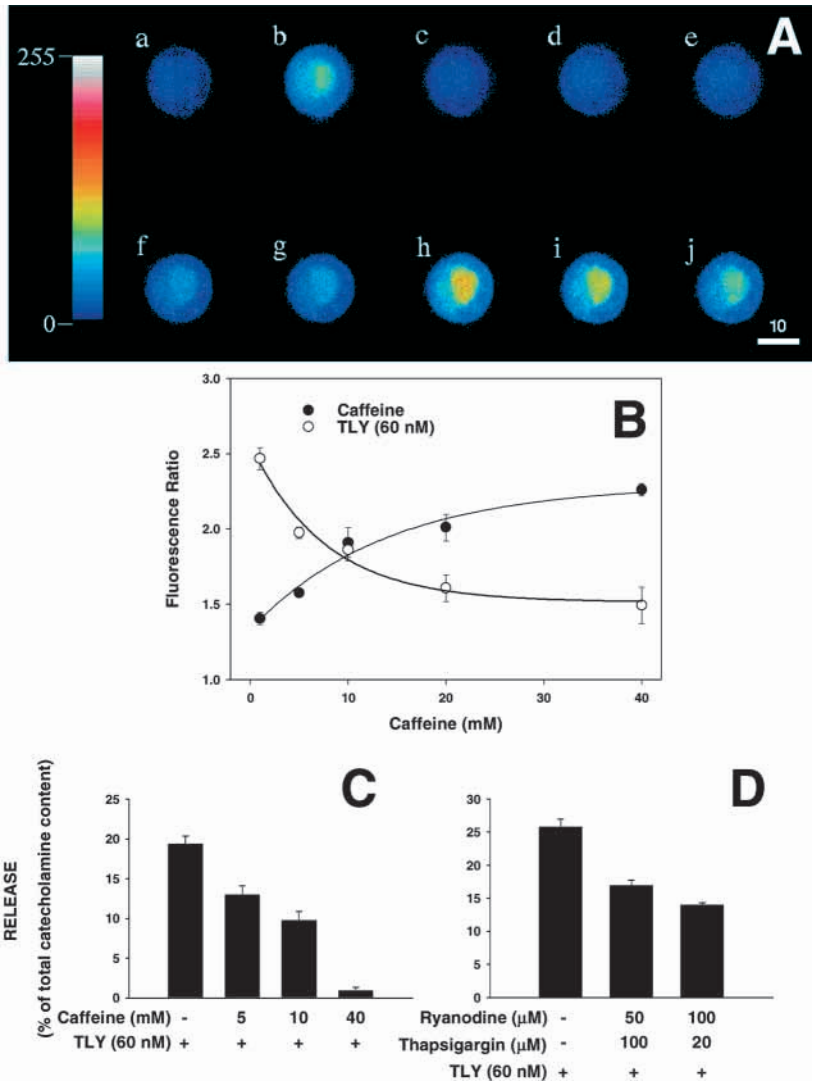

Fig. 4. Co-localisation of caffeine- and TLY-induced increase in Ca^{2+} signal: influence of Ca^{2+} stores on TLY-evoked release of catecholamines. (A) Confocal images were acquired every 5 seconds of a single fluo-3 loaded chromaffin cell first treated with caffeine (a-e), then washed and exposed to 60 nM TLY (f-j). Note that the Ca^{2+} transient evoked by caffeine is mainly distributed at a restricted area of the cell and that subsequent treatment with TLY induced a rise in Ca^{2+} fluorescence that precisely co-localized with the latter. (B) Confocal imaged fluo-3-loaded cells were treated for 10 minutes with caffeine (1, 5, 10, 20 or 40 mM, filled circles) and subsequently subjected to TLY (60 nM, open circles). The ratio of the evoked peak amplitude signal and the initial resting fluorescence value were calculated for a fixed region. The dose-dependent increase in signal elicited by caffeine is associated with a decrease in the following TLY-evoked fluorescent signal. (C) Chromaffin cells bathed in buffer A containing 2 mM Ca^{2+} were pre-treated (20 minutes) with or without the indicated concentrations of caffeine and subsequently exposed to TLY (60 nM, 20 minutes), before measuring the extent of catecholamine release. The measured release evoked by caffeine was negligible (not shown). (D) Chromaffin cells were incubated for 20 minutes with or without ryanodine and thapsigargin, at concentrations shown, in buffer A containing 2 mM Ca^{2+}, then washed before exposure to TLY (60 nM, 20 minutes) and measuring the catecholamine secretion.

the Ca^{2+}-stores, both being required for catecholamine secretion as revealed by their respective inhibition by La^{3+} and caffeine (Figs 3D and 4B). In this connection, it has been previously reported that K^+-induced depolarization elicits less secretion in chromaffin cells depleted by caffeine of their Ca^{2+} stores than from untreated cells (Lara et al., 1997). These data suggested that caffeine-sensitive Ca^{2+} stores could have a double role, acting as a sink or as a source of Ca^{2+}, depending on their filling state. Accordingly, pre-exposure of the cell to the combination of both ryanodine and thapsigargin also decreased TLY-evoked release of catecholamine. However, it cannot be ruled out that the inhibition of TLY-induced secretion by high doses of caffeine is due to some other effect, as recently discussed (Taylor and Broad, 1998); the store-operated Ca^{2+} channels in the plasma membrane of PC12 cells that are responsible for the refilling of Ca^{2+} stores are blocked by exposure to such high doses of caffeine (Bennett et al., 1998). Finally, we propose that TLY-induced Ca^{2+} initial influx near the Ca^{2+} stores triggers a Ca^{2+} release from the endoplasmic reticulum by a CICR mechanism responsible for most of the catecholamine secretion.

In chromaffin cells, the majority of the catecholamine is stored within the LDCV-like granules (Burgoyne et al., 1994). It is notable that α-LTX only induces the exocytosis of catecholamine from chromaffin cells if extracellular Ca^{2+} is present (Bittner et al., 1998). Exocytotic responses to TLY or α-LTX are also alike in that they are both sensitive to agents which perturb intracellular Ca^{2+} stores (Fig. 4 and Davletov et al., 1998). Notably, for the first time, compelling evidence is shown for Ca^{2+} efflux from the caffeine-sensitive store in response to TLY. Despite the similar effects of TLY and α-LTX on neurons and neuroendocrine cells, the former did not compete with the latter for binding to synaptosomal membranes; also, chromaffin cells transiently transfected with latrophilin did not exhibit significantly higher sensitivity to TLY compared to control cells (data not shown). In conclusion, our results indicate that TLY, purified from *Synanceia trachynis* venom, stimulates Ca^{2+}-dependent exocytosis of LDCV from neuroendocrine cells, in contrast to its inability to elicit the release of neuropeptides from motor nerve terminals. Binding and activation of a yet to be discovered receptor for TLY seems likely. Interaction of TLY with such a receptor would result in Ca^{2+}-influx, which in turn activates Ca^{2+}-induced Ca^{2+}

release from caffeine-sensitive stores and, ultimately, LDCV exocytosis.

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