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Expression of SNARE proteins in enteroendocrine cell lines and functional role of tetanus toxin-sensitive proteins in cholecystokinin release

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Abstract In neurons, synaptic vesicle exocytosis involves the formation of a core complex particle including syntaxin-1, synaptosomal-associated protein of 25 kDa (SNAP-25) and vesicle-associated membrane protein (VAMP)-2/synaptobrevin. The expression of these proteins was investigated in a panel of cell lines, including lines of endocrine and intestinal origin, by Western blotting and/or immunocytochemistry. The three core complex proteins were detected in the enteroendocrine, cholecystokinin (CCK)-secreting, cell lines STC-1 and GLUTag, and in the endocrine non-intestinal cell lines CA-77 and HIT-T15. In contrast, SNAP-25 and syntaxin-1 were undetected in the intestinal non-endocrine cell lines IEC-6, HT-29 and Caco-2, whereas a slight expression of VAMP-2 was documented in IEC-6 and HT-29 cells. Co-immunoprecipitation experiments indicated that syntaxin-1, SNAP-25 and VAMP-2 were present in a complex similar to that identified in brain. In the STC-1 cell line, treatment of streptolysin-O-permeabilized cells with tetanus toxin (Tetx) selectively cleaved VAMP-2 and VAMP-3/cellubrevin, and simultaneously abolished Ca^{2+}-induced CCK secretion ($IC_{50} \sim 12$ nM). These results show that endocrine cell lines of intestinal origin express syntaxin-1, SNAP-25 and VAMP-2, and suggest a key role for a Tetx-sensitive protein (for example VAMP-2 and/or VAMP-3) in the CCK secretion by STC-1 cells.

Key words: Syntaxin; Synaptosomal-associated protein of 25 kDa; Vesicle-associated membrane protein; Tetanus toxin; Enteroendocrine cell; Cholecystokinin exocytosis

1. Introduction

In neuronal and hormonal cells, release of the signaling substance depends on the precise targeting of transport vesicles to specific membrane compartments within the regulated exocytotic pathway. The SNARE hypothesis [1] proposes that this specificity is achieved through the mutual recognition of vesicle membrane (v-) and target membrane (t-) SNAREs [soluble NSF attachment protein receptors (where NSF = *N*-ethylmaleimide-sensitive fusion protein)] that assemble into stable docking and fusion complexes. According to this hypothesis, recent reports identified mammalian SNAREs that are implicated in endoplasmic reticulum to Golgi [2,3], and intra-Golgi transport [4]. In neurons, syntaxin-1 [5,6], SNAP-25 [7] (synaptosomal-associated protein of 25 kDa) and synaptobrevin II [8] [also known as vesicle-associated membrane protein 2 (VAMP-2)] are the three SNARE proteins that form the core complex involved in synaptic vesicle fusion with plasma membrane [9–11]. Recently, these core complex proteins were reported to be expressed in endocrine cells from the adrenal medulla [12,13], anterior pituitary [13–15] and pancreas [13,16–20]. Independent evidence for the crucial role of these SNAREs in neurotransmitter and hormone exocytosis came from studies using clostridial neurotoxins, tetanus toxin (Tetx) and botulinum toxins A to G [17–24], that selectively cleave syntaxin-1, SNAP-25 or VAMP-2 [22,23,25]. Insulin release, for instance, was shown to be impaired by Tetx [17,19,20] and botulinum toxin serotypes A [18,24], B [17], E [18] and F [24] in permeabilized insulinoma cells.

The gut epithelium includes a variety of endocrine cells derived, as endocrine pancreas, from the primary endoderm. The fusion machinery involved in peptide secretion from the diffuse endocrine system of the gut has not been identified. The aim of this work was therefore to determine in the enteroendocrine cell lines STC-1 and GLUTag the presence of syntaxin-1, SNAP-25 and VAMP-2, compared with endocrine and non-endocrine cells of different origin. We here report that STC-1 and GLUTag cells express both syntaxin-1, SNAP-25 and VAMP-2, and that these proteins form a SNARE complex. Additionally using the intestinal STC-1 cell line that harbors many features of native intestinal cholecystokinin (CCK)-producing cells [26,27], we show that treatment of permeabilized cells with Tetx results in a marked inhibition of Ca^{2+}-induced CCK exocytosis, thus emphasizing a functional role for a tetanus toxin-sensitive SNARE in a model of intestinal endocrine cells.

2. Materials and methods

2.1. Materials

Monoclonal antibody against rat syntaxin-1 (6D2) was prepared as described [28]. Mouse monoclonal antibody against SNAP-25 (SMI 81) was from Sternberger Monoclonals Inc. (Baltimore, USA). Polyclonal antisera to SNAP-25 (residues 195–206, common to mouse, rat and human) and to rat VAMP-2 (residues 2–20) were prepared as previously described [7,13]. These antibodies cross-reacted with the different species used in this study, including human material (6D2 and anti-VAMP-2: Y. Shimazaki and M. Takahashi, unpublished;

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Abbreviations: CCK, cholecystokinin; SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; SL-O, streptolysin-O; Tetx, tetanus toxin

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anti-SNAP-25: S. Catsicas, unpublished). Polyclonal antisera to rat VAMP-1 (MC9; residues 1–15) and to rat VAMP-3 (MC16; residues 1–14) were kindly provided by Dr. P. De Camilli (Yale University, New Haven, USA). Horseradish peroxidase- and FITC-labeled secondary antibodies were obtained from Jackson Laboratories (West Grove, USA) and from Amersham (Les Ulis, France), respectively. Cell culture media, fetal calf serum (FCS) and additives were from Life Technologies (Cergy Pontoise, France). Rat brain extracts and bovine adrenal chromaffin cell lysates were generously supplied by Dr. A. Osen-Sand (Glaxo Institute for Molecular Biology, Geneva, Switzerland) and Dr. M.F. Bader (INSERM U338, Strasbourg, France), respectively. Streptolysin-O (SL-O) was produced and purified as described [29]. Tetx light chain was generously provided by Dr. U. Weller (University of Mainz, Germany). Protein G-Sepharose, bovine serum albumin (BSA), Triton X-100, sodium orthovanadate, aprotinin, leupeptin and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (Saint Quentin Fallavier, France). All other reagents were of analytical grade.

2.2. Cell lines and culture conditions

The following cell lines were used in this study: STC-1 (mouse small intestine endocrine cell line, [30]), GLUTag (mouse large bowel endocrine cell line [31]), HIT-T15 (hamster beta cell line, ATCC CRL 1777), CA-77 (rat medullary thyroid carcinoma [32]), IEC-6 (rat intestinal epithelial crypt cell line, [33]), HT-29 and Caco-2 [human colon adenocarcinoma cell lines (ATCC HTB 38 and HTB 37, respectively)]. The following cell culture conditions were used: STC-1 cells in RPMI with 5% FCS; HIT-T15 cells in RPMI with 10% FCS, 32.5 μM glutathione and 0.1 μM selenous acid; IEC-6 cells in DMEM with 10% FCS and 0.1 IU/ml insulin; CA-77 in Ham-F-10/DMEM (1:1) with 10% FCS; GLUTag, HT-29 and Caco-2 cells in DMEM with 10% FCS. All culture media were supplemented with 2 mM glutamine and antibiotics (100 IU/ml penicillin and 50 μM streptomycin) and cells were grown in a humidified CO_2/air (5:95%) incubator at 37°C.

2.3. Immunoprecipitation

Cells were solubilized in immunoprecipitation buffer (50 mM HEPES, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 10 mM EDTA, 2 mM Na_3VO_4, 100 mM NaF, 1% Triton X-100, 100 units/ml aprotinin, 20 μM leupeptin, 0.2 mg/ml PMSF, pH 7.5) for 15 min on ice. Cell extracts were clarified by centrifugation for 15 min at $14\,000 \times g$ and then incubated with monoclonal SNAP-25 antibody preadsorbed to protein G-Sepharose. Beads were subsequently washed twice in immunoprecipitation buffer, resuspended in Laemmli buffer and boiled before separation on 12% SDS-PAGE.

2.4. Immunoblots

After SDS-PAGE, proteins were transferred onto nitrocellulose sheets (Portran Ba 85, Schleicher and Schuell, Ecquevilly, France). Membranes were blocked using 5% non-fat dry milk in Tris-buffered saline containing 0.2% Nonidet P-40, and exposed to the antibodies overnight at 4°C in the same buffer. After incubation with appropriate secondary antibodies conjugated to horseradish peroxidase, blots were developed using the ECL method (Pierce, Rockford, USA). Protein concentrations were determined by the Bradford method (Bio-Rad, Ivry sur Seine, France).

2.5. Immunocytochemistry

STC-1 and GLUTag cells were grown for two days before immunofluorescence staining. Cells were washed in PBS and fixed for 10 min in PBS, 4% paraformaldehyde. After washing twice in PBS, cells were permeabilized in PBT (PBS containing 0.3% Triton X-100 and 0.5% BSA) and then incubated with anti-syntaxin-1 (6D2, 1/200), anti-SNAP-25 (SMI 81, 1/1000) and anti-VAMP-2 (1/250) antibodies for 1 h. Following incubation with FITC-conjugated anti-mouse or anti-rabbit IgG antibody (1/100), immunofluorescence was visualized with a Leitz fluorescence microscope or with a Zeiss LSM10 laser confocal microscope. No staining was observed in cells treated identically but without addition of primary antibodies.

2.6. Streptolysin-O permeabilization, neurotoxin treatment and CCK determination

Permeabilization experiments were performed as described previously [18,34] with slight modifications according to [35]. Briefly, STC-1 cells were washed once in a Ca^{2+}-free Krebs buffer and resuspended in ice-cold KG buffer (in mM: 140 K^+-glutamate, 5 NaCl, 7 $MgSO_4$, 20 HEPES, 0.4 EGTA, pH 7.0) containing 1 IU/ml SL-O. After 5 min, the cells were washed free of unbound SL-O by centrifugation and resuspension in KG buffer at 4°C. STC-1 cells ($2.5\ 10^5$ cells/60 μl final volume) were then incubated at 37°C for 10 min in KG buffer supplemented with 2.5 mM Na_2ATP in the presence of the indicated amount of light chain Tetx [21,36,37]. The efficiency of permeabilization (more than 95% of cells permeabilized) was checked with 0.04% Trypan blue. Cells were stimulated by addition of 60 μl KG buffer containing 2.5 mM Na_2ATP, EGTA (10.2 mM, final concentration) and $CaCl_2$ to regulate the level of free Ca^{2+} [18] and incubated for 7 min at 37°C. The reaction was then quenched by the addition of 200 μl ice-cold KG buffer and cells were sedimented. The amount of CCK released into the supernatant and the remaining immunoreactive material in the pellets were determined by RIA with antiserum 39A as previously described [38]. The detection limit and ID_{50} of the assay were 0.5 and 4.0 pM, respectively. Results were expressed as a percentage of total cell content [(peptide released in medium × 100)/(peptide released in medium+cell content at the end of the incubation)].

2.7. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) followed by post hoc comparison of Fisher. Differences between two means with a P value < 0.05 were regarded as significant. All values were expressed as means ± S.E.M. of at least three experiments.

3. Results and discussion

3.1. Expression of syntaxin-1, SNAP-25 and VAMP-2 in intestinal endocrine cell lines

The presence of syntaxin-1, SNAP-25 and VAMP-2 was investigated by immunoblotting in the enteroendocrine cell lines STC-1 and GLUTag, and for comparison in the intestinal non-endocrine cell lines IEC-6, HT-29 and Caco-2, and in the non-intestinal endocrine cell lines HIT-T15 and CA-77 (Fig. 1A). Syntaxin-1, SNAP-25 and VAMP-2 were expressed in the intestinal CCK-producing cell lines STC-1 and GLUTag, as well as in CA-77 medullary thyroid carcinoma cells that transcribe the CCK gene [27]. The three SNAREs were also detected in the beta cell line HIT-T15 (this report and [17,19,20,24]). Surprisingly, a slight expression of VAMP-2 appeared in the non-transformed intestinal epithelial cell line IEC-6, and in the colon adenocarcinoma cell line HT-29 but not Caco-2. In contrast, SNAP-25 and syntaxin-1 expression was not detected in IEC-6, HT-29 and Caco-2 cells. As expected [5–8,12], the expression of these proteins was detected at a high level in bovine adrenal chromaffin cells and in rat brain extracts (Fig. 1A).

The 6D2 anti-syntaxin-1 antibody used in this study recognizes both syntaxin-1A and -1B isoforms. These two isoforms were present in the brain (Fig. 1B and [1,6]), as well as in neuroendocrine chromaffin cells (Fig. 1B). In contrast, the syntaxin-1A isoform, which shows a faster electrophoretic mobility [1], was found to be the most abundant isoform in the endocrine cell lines STC-1, GLUTag, CA-77 and HIT-T15. A similar pattern of syntaxin-1 expression has been described in pancreatic and anterior pituitary endocrine cells [14–16].

Subcellular localization of syntaxin-1, SNAP-25 and VAMP-2 in the enteroendocrine cell lines STC-1 (Fig. 2) and GLUTag (data not shown) was examined by immunocytochemistry and confocal laser microscopy analysis. Essentially all STC-1 and GLUTag cells were stained with the anti-SNARE protein antibodies. Syntaxin-1 (Fig. 2A) and SNAP-25 (Fig. 2B) staining was localized to the periphery

of the cells, while very little cytoplasmic labeling was observed. In contrast, confocal laser microscopy showed a patchy intracellular distribution for VAMP-2 (Fig. 2C). Overall, these results suggest that syntaxin-1 and SNAP-25 could act as plasma membrane t-SNAREs in the enteroendocrine cells STC-1 and GLUTag, while the cytoplasmic distribution of VAMP-2 suggests a v-SNARE status for this protein.

3.2. *Complex formation of syntaxin-1, SNAP-25 and VAMP-2 in intestinal endocrine cell lines*

During neurotransmitter exocytosis, the targeting of synaptic vesicles to plasma membrane has been proposed to occur through the formation of a stable ternary complex composed of VAMP/synaptobrevin (v-SNARE), syntaxin-1 and SNAP-25 (t-SNAREs) [1,9]. This complex could be isolated from detergent extracts by immunoprecipitation with syntaxin-1 or SNAP-25 antibodies [1]. The formation of this synaptic core complex was also described in adrenal chromaffin cells [12], PC12 pheochromocytoma cells [13] and RINm5F insulinoma cells [13]. To further examine the role of the SNARE proteins present in enteroendocrine cell lines, we investigated the formation of stable ternary complexes containing syntaxin-1, SNAP-25 and VAMP-2 by immunoprecipitation with the SNAP-25 antibody SMI 81 (Fig. 3). Immunoblotting of syntaxin-1, SNAP-25 and VAMP-2 demonstrated that proteins that are part of the SNARE complex in brain (Fig. 3 and [1,9]) could also be detected in the immunoprecipitate from STC-1, GLUTag, HIT-T15 and CA-77 cells (Fig. 3). No non-specific binding was observed when immunoprecipitation was performed without SNAP-25 antibody (not shown) or with detergent-extracts from Caco-2 cells (Fig. 3), that do not express any of the three SNARE proteins investigated here (Fig. 1). Similar results were obtained when using the anti-SNAP-25 polyclonal antiserum for immunoprecipitation (not shown).

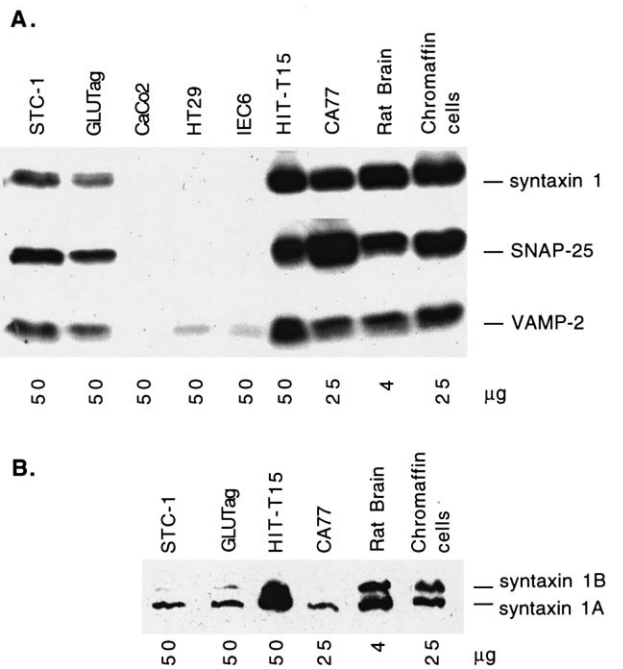

Fig. 1. Immunoblot analysis of SNAREs proteins in cells and tissue of different origin. Indicated amounts of solubilized extracts were subjected to SDS-PAGE and transferred to nitrocellulose blot. A: SNARE proteins were visualized with monoclonal anti-syntaxin-1 (6D2), monoclonal anti-SNAP-25 (SMI 81) and polyclonal anti-VAMP-2 antibodies. B: Extracts were loaded onto a 6 M-urea SDS-polyacrylamide gel [1]. After electrophoresis, proteins were transblotted onto nitrocellulose and immunostained with anti-syntaxin-1 (6D2) antibody. Results are representative of three independent experiments.

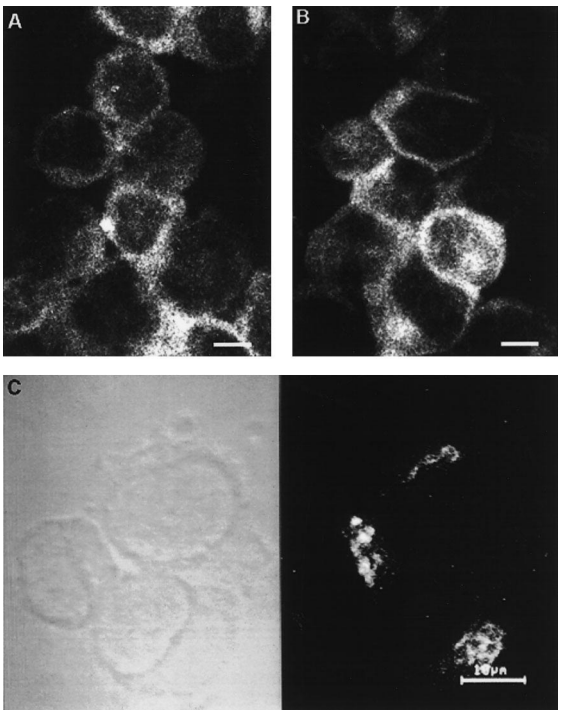

Fig. 2. Immunofluorescence location of SNARE proteins in the intestinal endocrine STC-1 cells. Immunocytochemistry was done as described in Section 2 with (A) anti-syntaxin-1 (1/200), (B) anti-SNAP-25 (1/1000) and (C) anti-VAMP-2 (1/250) antibodies. Primary antibodies were detected with FITC-coupled secondary antibodies and visualized by indirect fluorescence microscopy (A–B), or by laser confocal, in phase contrast (C, left panel) and fluorescence (C, right panel), microscopy. Scale bars = 10 µm.

3.3. *Tetanus toxin cleaves VAMP-2 and VAMP-3, and inhibits Ca^{2+}-induced CCK release in streptolysin-O-permeabilized STC-1 cells*

Tetanus toxin is a zinc-dependent protease that cleaves VAMP-2 [22,23,25] and VAMP-3/cellubrevin [39] in a highly selective manner. The toxin was shown to inhibit synaptic vesicle exocytosis [22,23] as well as catecholamine [21] and insulin [17,19,20] release from adrenal chromaffin and insulinoma cells, respectively. Here, we permeabilized STC-1 cells with streptolysin-O to allow Tetx to enter cells. The effects of the Tetx treatment were analyzed on both VAMPs cleavage, and Ca^{2+}-induced CCK secretion (Fig. 4). Tetx treatment of SL-O-permeabilized cells was found to cause the cleavage of VAMP-2 in a dose-dependent manner, but not of syntaxin-1, SNAP-25 or VAMP-1, the latter being expressed in STC-1 cells (Fig. 4, upper panel). The CCK secretion evoked by a high Ca^{2+} concentration ($4.2 \pm 0.6\%$ vs. $2.1 \pm 0.2\%$ of total cell content for 40 µM vs. 0.1 µM free Ca^{2+}, respectively) was inhibited when cells were treated with Tetx (Fig. 4, lower panel). These observations argue for the involvement of tetanus toxin-sensitive protein(s) in CCK exocytosis from enteroendocrine cells. This protein could be the SNARE protein

VAMP-2 identified in STC-1 and GLUTag CCK-producing cells, and involved in insulin secretion from pancreatic beta cells [20]. However, a functional role of the ubiquitously expressed tetanus toxin-substrate VAMP-3/cellubrevin [39], also present in STC-1 cells (Fig. 4, upper panel), cannot be excluded. Indeed, this protein, that was shown to participate with VAMP-2 in insulin exocytosis from HIT-T15 cells [20], was cleaved by Tetx treatment of STC-1 cells (Fig. 4, upper panel). In addition, the inhibition of CCK secretion correlated with the cleavage of VAMP-2 and of VAMP-3, the half-maximum and maximum concentrations of Tetx being about 12 and 50 nM for both VAMPs cleavage and inhibition of CCK release. These concentrations were comparable to those required for VAMP-2 and VAMP-3 cleavage and insulin exocytosis inhibition in insulin-secreting cells [17]. Whether VAMP-2 or VAMP-3, or both VAMPs, are required in the exocytotic process of CCK remains to be elucidated.

In conclusion, this study indicates that the three synaptic core complex proteins, syntaxin-1, SNAP-25, and VAMP-2, are expressed in enteroendocrine CCK-secreting cell lines. In addition, a Tetx-sensitive protein (for example, VAMP-2 and/or VAMP-3) is required for Ca^{2+}-induced CCK exocytosis in the STC-1 cell line. A next challenge will be to understand how physiological stimuli, such as nutrients, hormones and neurotransmitters, are able to modulate the exocytotic machinery of intestinal CCK-producing cells, and how this organization could be disrupted in states of neuroendocrine secreting tumors.

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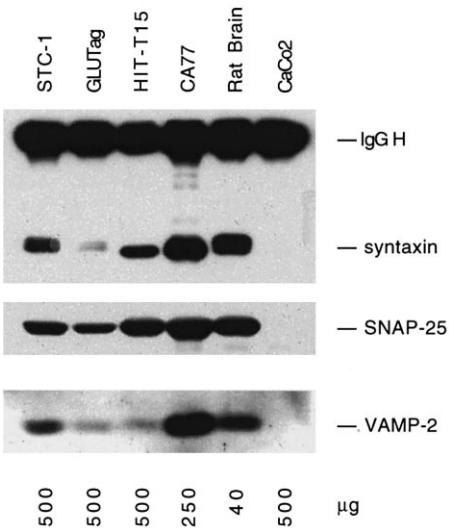

Fig. 3. Immunoprecipitation of SNAP-25-containing protein complexes from extracts of various endocrine cell lines. Cells were solubilized in Triton X-100 solubilization buffer for 15 min on ice. As indicated, different amounts of protein extracts were immunoprecipitated with the monoclonal anti-SNAP-25 antibody (SMI 81), and analyzed by Western blotting with monoclonal anti-syntaxin-1 (6D2), monoclonal anti-SNAP-25 (SMI 81) and polyclonal anti-VAMP-2 antibodies. The positions of SNAREs and of the high chain IgG (IgG H) are indicated (representative of 3 separate experiments).

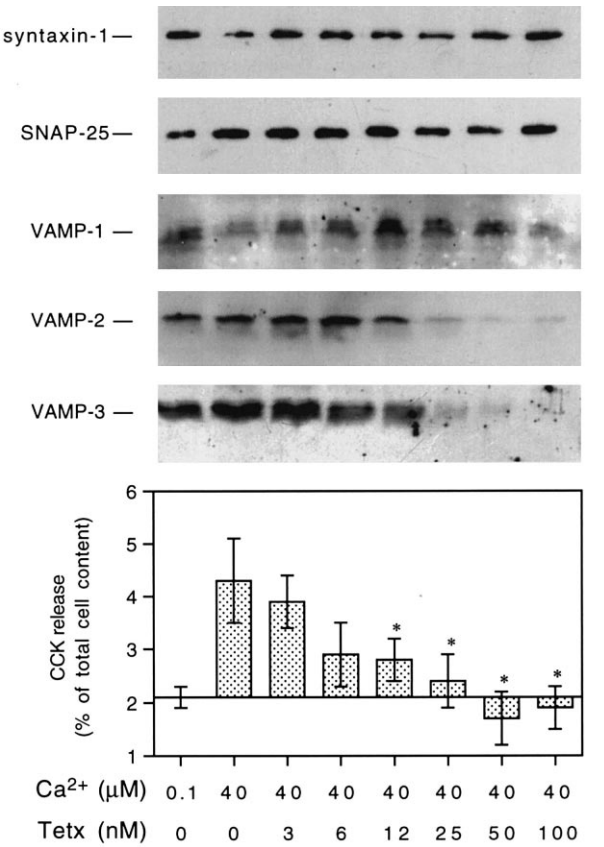

Fig. 4. Cleavage of VAMP-2 and of VAMP-3, and inhibition of CCK exocytosis, by light chain Tetx in SL-O-permeabilized STC-1 cells. Lower panel: Effect of Tetx treatment on Ca^{2+}-induced CCK release. STC-1 cells ($2.5\ 10^5$/tube) were treated with SL-O, then exposed to 40 µM Ca^{2+} in the presence of increasing concentrations of Tetx. Total CCK cell content and basal CCK release represented 1175 ± 147 and 25.3 ± 2.1 fmol/tube, respectively. *, $P < 0.05$ as compared with 40 µM Ca^{2+}-stimulated CCK release without toxin ($n = 3$). Upper panel: Corresponding representative immunoblot analysis of cell lysates for the presence of syntaxin-1, SNAP-25, VAMP-1, VAMP-2 and VAMP-3 to evaluate the specificity of Tetx cleavage.

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