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Synaptobrevin cleavage by the tetanus toxin light chain is linked to the inhibition of exocytosis in chromaffin cells

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Abstract Exocytosis of secretory granules by adrenal chromaffin cells is blocked by the tetanus toxin light chain in a zinc specific manner. Here we show that cellular synaptobrevin is almost completely degraded by the tetanus toxin light chain within 15 min. We used highly purified adrenal secretory granules to show that synaptobrevin, which can be cleaved by the tetanus toxin light chain, is localized in the vesicular membrane. Proteolysis of synaptobrevin in cells and in secretory granules is reversibly inhibited by the zinc chelating agent dipicolinic acid. Moreover, cleavage of synaptobrevin present in secretory granules by the tetanus toxin light chain is blocked by the zinc peptidase inhibitor captopril and by synaptobrevin derived peptides. Our data indicate that the tetanus toxin light chain acts as a zinc dependent protease that cleaves synaptobrevin of secretory granules, an essential component of the exocytosis machinery in adrenal chromaffin cells.

Key words: Adrenal medulla; Exocytosis; Secretory granule; Synaptobrevin; Tetanus toxin

1. Introduction

Tetanus and botulism are caused by clostridial neurotoxins which preferentially block transmitter release from glycinergic and cholinergic nerve terminals [1]. Recent observations indicate that these neurotoxins attack membrane proteins of synaptic vesicles or of the presynaptic plasmalemma which are likely components of the docking/fusion complex in nerve endings [2–10]. Earlier investigations have shown that clostridial neurotoxins also block exocytosis of secretory granules from adrenal chromaffin cells. Indeed the basic mechanisms of the neurotoxins' action have first been detected in adrenal chromaffin cells [11–18] and have subsequently been confirmed in neurons [19–23]. From these studies it is evident that the neurotoxins act intracellularly, that chain separation is a prerequisite for the inhibition of exocytosis by the light chains of the neurotoxins, and that they specifically block calcium triggered process(es) within the cascade of events leading to exocytosis. Recently, we identified the metal binding motif of the tetanus toxin light chain (TeTxL) as a key structure which mediates inhibition of exocytosis in adrenal chromaffin cells specifically when complexed with zinc [24]. Here we report that this action of the tetanus toxin light chain is due to the specific cleavage of synaptobrevin (SB) and identify synaptobrevin as an essential component of the exocytosis machinery of chromaffin cells.

2. Experimental

2.1. Materials

2.1.1. Toxins, peptides and chemicals. Tetanus toxin was isolated and characterized as previously described [25,26]. During toxin purification EDTA (4 mM) was present in the first column step (HIC). Tetanus toxin was separated into its heavy and light chains by isoelectric focusing in a sucrose gradient with ampholyte under reducing conditions in 2 mol/l urea [27]. Before use the purified tetanus toxin light chain (TeTxL) preparation was dialyzed against potassium glutamate medium (see below). Streptolysin O, used for controlled permeabilization of the chromaffin cells [28,29] was purified from group A streptococci (Richards strain) by SH-specific chromatography followed by HIC chromatography (Weller et al. unpublished).

The peptides QFET and ASQFETS were kindly prepared by J. Neckermann (Ulm, Germany) by solid phase synthesis using an automatic synthesizer (431A, Applied Biosystems, Foster City, CA, USA) employing F-moc chemistry. The peptides were detached with TFA from the resin, analyzed by HPLC on a Pep-S 5 μm column (Pharmacia, Freiburg, Germany) and characterized by Edman sequencing (model 473 A sequenzer, Applied Biosystems, Foster City, CA, USA). Peptides 1 (DALQAGASVFESSAAKLKRK) and 2 (DALQAGASQFETSAAKLKRK) were generous gifts from H. Niemann (Tübingen, Germany). Captopril (3-mercapto-2-methylpropionyl-L-proline) was from Bristol-Myers Squibb (München, Germany). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Cultivation, permeabilization and incubation of chromaffin cells with TeTxL. Bovine adrenal chromaffin cells were isolated and grown as described [24] except that the cells were plated at a higher density (7×10^5 cells/well). After two days in culture the cells were permeabilized (at 30°C for 2 min) with streptolysin O (63 hemolytic units/well) in potassium glutamate medium (150 mM potassium glutamate, 10 mM PIPES (pH 7.2), 5 mM EDTA, 0.5 mM EGTA) containing 2 mM Mg^{2+}-ATP, 7.67 mM magnesium acetate, 0.1% BSA and 1 mM dithiothreitol [24,29,30]. The medium was then replaced by the same medium containing TeTxL and/or the chemicals to be tested, followed by incubation for 25 min at 30°C. Finally, the cells of each well were lyzed with 100 μl SDS-sample buffer and proteins (determined with the BCA method, Pierce, Oud-Beijerland, Netherlands) were prepared for SDS-PAGE.

2.2.2. Isolation of chromaffin vesicle membranes and incubation with TeTxL. Chromaffin vesicles were isolated as described [31,32]. Briefly, bovine adrenal glands were perfused with 5 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM EDTA. The medullae were homogenized in (20% w/v) 20 mM MOPS (pH 7.0), 5 mM EDTA, 340 mM sucrose in a Teflon-to-glass homogenizer. From the postnuclear supernatant chromaffin vesicles were harvested by centrifugation for 20 min at 12,000 × *g*. They were placed on a discontinuous sucrose gradient consisting of 4 ml of 2.4 M and 2 ml of each 2.2, 2.0, 1.8 and 1.6 M sucrose in 20 mM MOPS (pH 7.0), 5 mM EDTA. Centrifugation was done for 1 h at 40,000 rpm in a 50.2 Ti rotor (Beckman). Absorbance at 280 nm after precipitation of the protein with 10% (w/v) TCA was measured to conveniently locate chromaffin vesicles in the gradient while arylsulfatase served to identify fractions containing lysosomes and other subcellular membranes [31,32]. The chromaffin vesicles were recovered from the gradient, diluted 1:4 with 20 mM MOPS (pH 7.0), 5 mM EDTA and centrifuged

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for 1 h at 100,000 × g. The purified chromaffin vesicles were osmotically lyzed by addition of a ten-fold excess of 20 mM MOPS (pH 7.0) with 5 mM EDTA. Chromaffin vesicle membranes were recovered by centrifugation for 1 h at 100,000 × g and washed twice in potassium glutamate medium (see above) containing 6 mM magnesium acetate. The final pellet was suspended in the same medium and stored at −20°C until use. Chromaffin vesicle membranes (100 μg) were solubilized with 0.5 mg of β-D-octylglucopyranoside and incubated in a volume of 50 μl for 30 min at 30°C in potassium glutamate medium (see above) containing 20 pmol TeTxL and/or the different chemicals to be tested. The reaction was stopped by cooling on ice, lipids were extracted by chloroform/methanol [33] and the samples were processed for SDS-PAGE.

2.2.3. Immunoblotting. The samples of chromaffin vesicle membranes or of chromaffin cells were separated by SDS-PAGE (12.5%) [34] and blotted onto nitrocellulose as described [35]. Binding of the monoclonal anti-synaptobrevin antibody (Cl. 10.1; diluted 1:2,000) [36] or of the monoclonal anti-synaptotagmin I antibody (Cl. 41.1; diluted 1:2,000) [37] was detected in samples of chromaffin cells by peroxidase labelled anti-mouse IgG antibodies (Dianova, Hamburg) and the enhanced chemiluminescence method (Amersham Buchler, Braunschweig). For proteins prepared from chromaffin vesicle membranes, biotinylated anti-mouse IgG (Dianova, Hamburg) and avidin labelled peroxidase (Vectastain, Burlingame, CA, USA) and detection by DAB was employed. For controls we used a polyclonal neuron specific enolase (NSE) antibody (Dako Diagnostika, Hamburg, Germany).

3. Results and discussion

Blockade of transmitter release from neurons by tetanus toxin is paralleled by the cleavage of synaptobrevin, a membrane protein of synaptic vesicles [2,3,6]. Exocytosis of hormone containing secretory granules is also inhibited by tetanus toxin [12,13,15,16,38]. More recently we established the role of zinc and the HELIH motif of the tetanus toxin light chain (TeTxL) for the inhibition of calcium induced catecholamine release from adrenal chromaffin cells [24]. Here we probed the functional significance of synaptobrevin for exocytosis of adrenal chromaffin cells using TeTxL as a tool.

Synaptobrevin can readily be detected in immunoblots of adrenal chromaffin cells. Within 15 min of incubation of permeabilized chromaffin cells with TeTxL the synaptobrevin band of 18 kDa was greatly diminished and it was barely detectable after 30 min (Fig. 1, lower panel). In contrast, TeTxL treatment does not affect the electrophoretic mobility of other components of chromaffin cells such as synaptotagmin (ST) (Fig. 1, upper panel) or neuron specific enolase (NSE) (Fig. 2, upper panel). Moreover, we did not notice a change in the pattern of protein bands in Coomassie and silver stained gels of chromaffin cells (not shown). Although this does not completely rule out that there may be TeTxL targets other than synaptobrevin [39,40], it is particularly intriguing that the specific degradation of synaptobrevin observed here and inhibition of exocytosis reported earlier [15,16,24] occured under identical conditions.

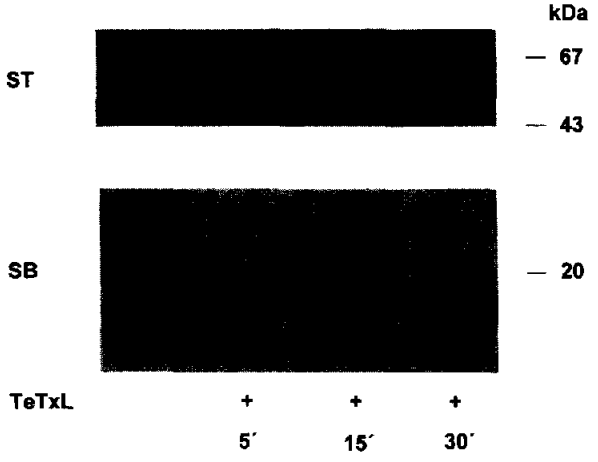

Fig. 1. Synaptobrevin cleavage within chromaffin cells by the tetanus toxin light chain as a function of time. After permeabilization the cells were incubated for 5, 15 or 30 min with 100 nM TeTxL at 30°C. The reaction was stopped by addition of SDS-sample buffer, followed by immunoblotting and synaptobrevin detection by the enhanced chemiluminescence method. The lower panel shows almost complete disappearance of synaptobrevin (SB) with time of incubation. The upper panel displays the same blot after immunodetection of synaptotagmin (ST), demonstrating the same amounts of samples in all lanes.

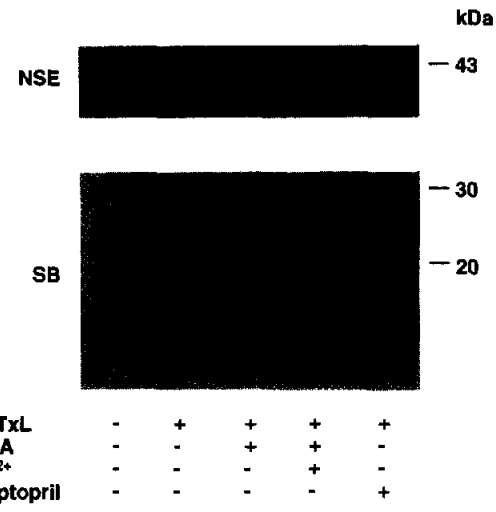

Fig. 2. Synaptobrevin cleavage within chromaffin cells by the tetanus toxin light chain depends on zinc. As observed in immunoblots (lower panel), incubation of permeabilized chromaffin cells with 100 nM TeTxL results in a decrease of the synaptobrevin (SB) immunoreactive band. Addition of 100 μM dipicolinic acid (DPA) or of 1 mM captopril abolished this effect. The activity of TeTxL is restored after addition of 100 μM Zn^{2+}. The upper panel shows the control with anti-NSE.

Zinc can be removed from the catalytic site of TeTxL by dipicolinic acid (DPA) [41], a chelating agent with high affinity for zinc [42]. Concomitantly the inhibiton of secretory granule exocytosis by TetxL is abolished [24,41]. We found that synaptobrevin cleavage within chromaffin cells is impaired by dipicolinic acid (Fig. 2). Moreover, addition of zinc restored the activity of TeTxL (Fig. 2). Together these data suggest a causal link between zinc dependent cleavage of synaptobrevin by TeTxL and inhibition of exocytosis. The zinc peptidase inhibitor captopril and synaptobrevin derived peptides abolish the effect of TeTxL on neurotransmitter release and cleavage of synaptobrevin in isolated synaptic vesicles [2,3]. Moreover the inhibition of vasopressin release from neurohypophysial nerve terminals by TeTxL is blocked by these substances [41]. However, neither synaptobrevin derived peptides nor captopril block the effect of TeTxL on catecholamine release by chromaffin cells [24]. One could argue that breakdown of peptides and of the peptide derivative captopril by endogenous enzymes of chromaffin cells or absence of synaptobrevin from chromaffin vesicle membranes [43,44] could account for these observations.

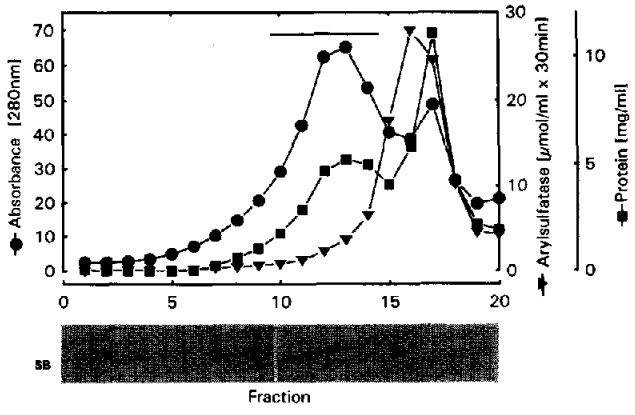

Fig. 3. Sucrose density gradient fractionation of adrenal chromaffin vesicles. Chromaffin vesicles obtained by differential centrifugation were further purified on a discontinuous sucrose gradient (see section 2). The fractions were collected from the bottom of the gradient. Absorbance at 280 nm of the TCA supernatants, protein and the lysosomal enzyme arylsulfatase were measured. Chromaffin vesicles, marked by a bar, exhibit the highest density while lysosomes and other contaminating organelles such as mitochondria [31,32] are found at lower sucrose densities. Synaptobrevin (immunoblot at bottom) was only found in chromaffin vesicle fractions.

In order to clarify these issues, we first isolated adrenal chromaffin vesicles by differential centrifugation and further purified them in hyperosmotic sucrose gradients (see section 2). This procedure yields highly purified chromaffin vesicles with negligible contamination by lysosomes and other subcellular organelles [31,32]. As revealed by immunoblotting, the chromaffin vesicle fractions of the gradient clearly do contain synaptobrevin (see Fig. 3). We then recovered chromaffin vesicles from the sucrose gradients, lyzed them hypotonically (see section 2) and used their membranes as a substrate for the tetanus

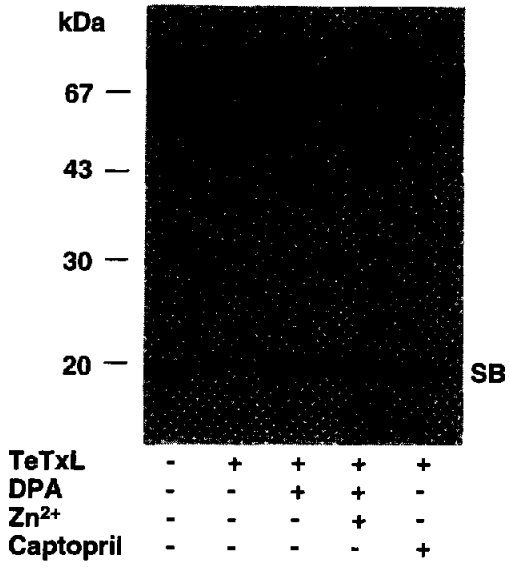

Fig. 4. Cleavage of synaptobrevin in chromaffin vesicle membranes by the tetanus toxin light chain depends on zinc. Chromaffin vesicle membranes (100 μg) were incubated for 30 min at 30°C with or without 20 pmol tetanus toxin light chain (TeTxL). Immunoblots revealed an almost complete disappearance of the synaptobrevin (SB) immunoreactive band. Addition of 400 μmolar dipicolinic acid (DPA) abolished the effect of TeTxL. Reactivation of TeTxL occurred with 400 μmolar zinc. Captopril (4 mM) prevented the cleavage of synaptobrevin by TeTxL.

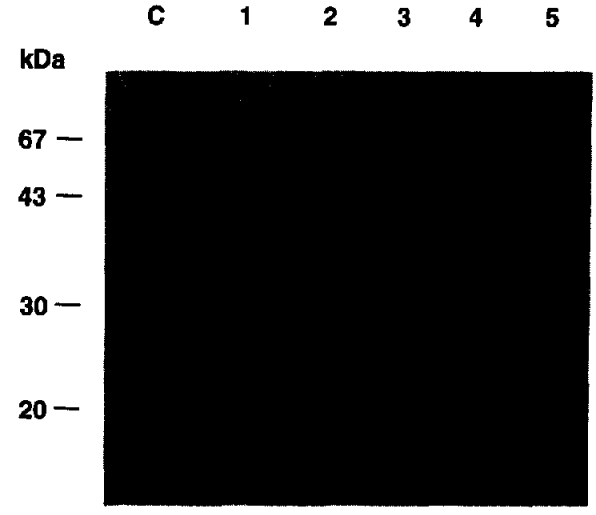

Fig. 5. Effect of synaptobrevin derived peptides on the cleavage of synaptobrevin in chromaffin vesicle membranes by the tetanus toxin light chain. Incubation of chromaffin vesicle membranes (100 μg) with 20 pmol TeTxL caused an almost complete disappearance of synaptobrevin analyzed in immunoblots (C, only chromaffin vesicle membrans; 1, with TeTxL). Peptides (400 μM) of different length (2, QFET; 3, ASQFETS; 4, peptide 1; 5, peptide 2) corresponding in sequence to the cleavage site of synaptobrevins (see section 2) abolished the effect of TeTxL on synaptobrevin degradation.

toxin light chain (TeTxL). We first determined whether synaptobrevin of chromaffin vesicle membranes is a substrate for TeTxL and whether the activity of the neurotoxin is blocked by DPA. This chelating agent has been shown to remove zinc from TeTxL [41], to abolish the inhibition of catecholamine release by TeTxL [24] and to block cleavage of synaptobrevin in chromaffin cells (see above). We found that TeTxL, within 30 min of incubation at 30°C, almost completely removed immunoreactive synaptobrevin from chromaffin vesicle membranes (Fig. 4). This effect was suppressed by DPA and was restored by zinc (Fig. 4). Furthermore, we found that the cleavage of synaptobrevin by TeTxL was blocked by captopril, an inhibitor of zinc peptidases (Fig. 4). Finally, peptides spanning the site of synaptobrevins cleaved by TeTxL in synaptic vesicles [2,3] were tested for an effect on synaptobrevin cleavage in chromaffin vesicle membranes. Both of the shorter peptides used (QFET and ASQFETS) and longer peptides (20-mers, see section 2) corresponding in sequence to synaptobrevin 1 and peptide 2 (a synaptobrevin 2 analogue) inhibited the cleavage of synaptobrevin in chromaffin vesicle membranes (Fig. 5).

Taken together our data indicate that TeTxL acts selectively as a zinc-dependent protease on synaptobrevin present in adrenal chromaffin vesicle membranes. Thus, it can be concluded that secretory granules (this study) and synaptic vesicles [2,3,6], the storage organelles of neurotransmitters, neuropeptides and hormones, share the target of the tetanus toxin. This target, synaptobrevin, together with other membrane proteins and soluble proteins is a central component of a complex, which is critical for exocytosis [9,10].

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