A tetanus toxin sensitive protein other than VAMP 2 is required for exocytosis in the pancreatic acinar cell

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Abstract The neurotoxin sensitivity of regulated exocytosis in the pancreatic acinar cell was investigated using streptolysin-O permeabilized pancreatic acini. Treatment of permeabilized acini with botulinum toxin B (BoNT/B) or botulinum toxin D (BoNT/D) had no detectable effect on Ca^{2+}-dependent amylase secretion but did result in the complete cleavage of VAMP 2. In comparison, tetanus toxin (TeTx) treatment both significantly inhibited Ca^{2+}-dependent amylase secretion and cleaved VAMP 2. These results indicate that regulated exocytosis in the pancreatic acinar cell requires a tetanus toxin sensitive protein(s) other than VAMP 2.

Key words: Pancreatic acinus; Botulinum neurotoxin; Tetanus toxin; Exocytosis; Secretion

1. Introduction

The SNARE (SNAP receptor) complex mediates exocytosis [1–3]. The complex consists of integral membrane proteins resident in both the vesicle membrane (v-SNAREs) and the plasma membrane (t-SNAREs), in addition to soluble cytosolic proteins including NSF (*N*-ethylmalemide sensitive factor) and α-SNAP (α-soluble NSF attachment protein) [1–3]. Numerous SNARE proteins have been identified, however, only a limited number of these appear to be required for regulated exocytosis. The predominant exocytotic v-SNARE is VAMP 2 (vesicle associated membrane protein) [1,2]. Exocytotic t-SNAREs include syntaxins 1, 2, 3 and 4, SNAP-25 (synaptosomal associated protein) and SNAP-23 [1–7].

The majority of the SNARE proteins are targets for clostridial neurotoxins, including tetanus toxin (TeTx) and the seven serotypes of botulinum toxin (BoNT/A–G) [8,9]. The toxins are heterodimers consisting of a heavy chain that mediates cell association and internalization, and a light chain that is a zinc metalloprotease with unique substrate specificity [8,9]. The light chain is only active after it is released from the heavy chain following internalization into the host cell. Cellular substrates for the toxins include VAMP 2 (TeTx, BoNT/B, BoNT/D), syntaxins 2, 3 and 4 (BoNT/C1) and SNAP-25 (BoNT/A and BoNT/E). Because of this unique characteristic the clostridial neurotoxins have been very effective tools for defining the roles of specific SNARE proteins in regulated exocytosis.

Several SNARE proteins have been identified in pancreatic acinar cells [10–12]. Of these, only VAMP 2 (zymogen granule), syntaxin 2 (apical plasma membrane) and syntaxin 3 (zymogen granule) are associated with organelles involved in regulated exocytosis and are thought to function in exocytosis [10,11]. The involvement of VAMP 2 in exocytosis in pancreatic acinar cells has been investigated using tetanus toxin [10]. Toxin treatment of streptolysin-O (SLO) permeabilized acini cleaved VAMP 2 and significantly inhibited Ca^{2+}-dependent secretion suggesting that VAMP 2 is required for exocytosis in the acinar cell [10]. Here we report that BoNT/B and BoNT/D cleaved VAMP 2 but did not influence Ca^{2+}-dependent amylase secretion from SLO permeabilized pancreatic acini. In comparison, TeTx both cleaved VAMP 2 and significantly inhibited amylase secretion. These findings indicate that a tetanus toxin sensitive protein other than VAMP 2 is required for regulated exocytosis in the pancreatic acinar cell.

2. Materials and methods

Collagenase (CLSPA) was purchased from Worthington Biochemicals, Freehold, NJ, USA. The neurotoxins were obtained from Calbiochem, La Jolla, CA, USA. All other biochemicals were acquired from Sigma Chemical Co., St. Louis, MO, USA. The anti-VAMP 2 monoclonal antibody (Cl 69.1) [13] and anti-cellubrevin antibody were generously donated by Dr. R. Jahn (Yale University) and Dr. P. DeCamilli (Yale University), respectively.

2.1. Isolation of rat pancreatic acini

Rat pancreatic acini were prepared by collagenase digestion of pancreatic tissue slices according to the procedure we have previously described [14,15].

2.2. Secretion assay

10 ml of the final acinar suspension was centrifuged at $300 \times g$ for 5 min and the resulting acinar pellet resuspended in ice cold permeabilization buffer (139 mM K-glutamate, 20 mM PIPES, pH 6.6). This process was repeated three times to remove all traces of the Krebs Ringer buffer used to prepare the acini. Streptolysin-O was added to the acini (0.5 IU/ml) and the suspension incubated on ice for 5 min. This allows the SLO to bind to the acinar cell plasma membrane, efficient permeabilization of the acinar cells is obtained when the acini are warmed above room temperature. The excess unbound SLO was then removed from the acinar suspension by repeated sedimentation and resuspension in fresh permeabilization buffer. The acinar suspension was divided into two 5 ml aliquots to which were added 5 ml of priming buffer (139 mM K-glutamate, 20 mM PIPES pH 6.6, 4 mM ATP, 0.4 mM EGTA, 8 mM $MgCl_2$ and 2 mg/ml rat brain cytosol) with or without a designated concentration of neurotoxin. Immediately before use, BoNTs and TeTx were incubated with 5 mM dithio-

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threitol for 1 h at 22°C to reduce the interchain disulphide bonds and activate the toxins. The two acinar suspensions were incubated, with or without the neurotoxin, at 37°C. After 30 min the acinar suspensions were divided into 200 μl aliquots to which were added an equal volume of stimulation buffer (139 mM K-glutamate, 20 mM PIPES pH 6.6, 7.6 mM EGTA, 2 mM ATP, 4 mM $MgCl_2$, and sufficient $CaCl_2$ to give the desired concentration of free Ca^{2+}). The acini were then incubated for a further 30 min at 37°C. The aliquots of acini not used for the secretion studies were immediately pooled, spun down, lysed and prepared for Western blot analysis. The secretion incubations were stopped by placing the experimental tubes in an ice bath for 5 min after which the acini were pelleted by centrifugation in an Eppendorf microfuge (2000×*g* for 2 min), and the supernatant removed. The supernatant and the lysed acinar pellet were then assayed for amylase as described in [16]. The secretory response was calculated as the % of total cellular amylase secreted.

2.3. Western blot analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the procedure of Laemmli [17]. For Western blotting, acinar cell lysates (10 μg total protein per lane) were separated on 12% SDS–PAGE gels and electrophoretically transferred onto PVDF membranes, which were then incubated with anti-VAMP 2 primary antibodies. The bound primary antibodies were then detected using a horseradish peroxidase conjugated secondary antibody in conjunction with the ECL detection system (Amersham Pharmacia Biotech). Typically exposure times for the blots were 1–2 min.

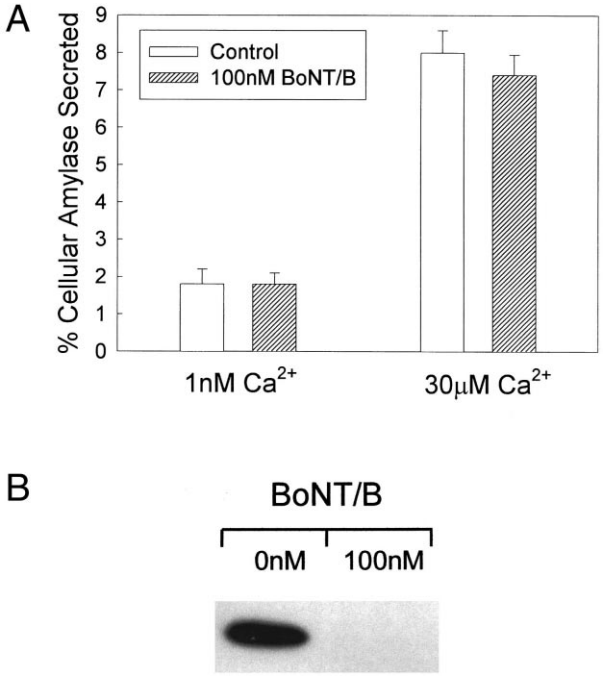

Fig. 1. Treating SLO permeabilized pancreatic acini with BoNT/B has no effect on Ca^{2+}-dependent amylase secretion but does result in the cleavage of VAMP 2. Pancreatic acini were permeabilized with SLO and then incubated with or without 100 nM BoNT/B for 30 min in the presence of 2 mM ATP and 1 mg/ml rat brain cytosol. A: Aliquots of untreated and toxin treated acini were removed and secretion stimulated by addition of Ca^{2+}. After 30 min the incubations were terminated and the amount of amylase secreted determined. The data are the means ± S.E.M. from three separate experiments conducted on acini isolated from three different rats. B: The remaining untreated and toxin treated acini were lysed and 10 μg (total protein) samples analyzed for VAMP 2 by Western blot analysis. The blot shown is representative of the Western blots obtained from the three experiments conducted.

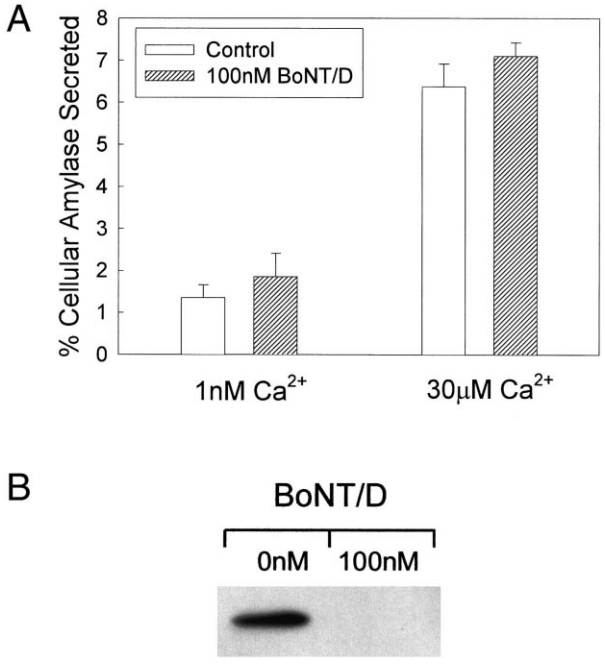

Fig. 2. Treating SLO permeabilized pancreatic acini with BoNT/D has no effect on Ca^{2+}-dependent amylase secretion but does result in the cleavage of VAMP 2. Pancreatic acini were permeabilized with SLO and then incubated with or without 100 nM BoNT/D for 30 min in the presence of 2 mM ATP and 1 mg/ml rat brain cytosol. A: Aliquots of untreated and toxin treated acini were removed and secretion stimulated by addition of Ca^{2+}. After 30 min the incubations were terminated and the amount of amylase secreted determined. The data are the means ± S.E.M. from three separate experiments conducted on acini isolated from three different rats. B: The remaining untreated and toxin treated acini were lysed and 10 μg (total protein) samples analyzed for VAMP 2 by Western blot analysis. The blot shown is representative of the three experiments conducted.

3. Results and discussion

To examine if VAMP 2 is required for regulated exocytosis in the pancreatic acinar cell, I determined the influence of BoNT/B (Fig. 1A) and BoNT/D (Fig. 2A) on Ca^{2+}-dependent amylase secretion from SLO permeabilized pancreatic acini. The majority of the cleavage sites for the neurotoxins in SNARE proteins, including VAMP 2, lie within domains that are involved in the protein–protein interactions that form and stabilize the SNARE complex [18]. This means that SNARE proteins present in SNARE complexes are resistant to toxin proteolysis [19,20]. The problem of toxin accessibility has been overcome by employing incubation conditions that actively promote the ATP-dependent priming of the exocytotic machine [21]. During priming there is an NSF-catalyzed rearrangement of the SNARE complex that renders the SNARE proteins susceptible to toxin proteolysis. Taking the above observation into consideration the permeabilized acini were incubated with BoNT/B and D in the presence of 2 mM ATP and 1 mg/ml rat brain cytosol, conditions known to promote the priming of exocytosis in the acinar cell (P. Padfield, unpublished observation). Neither BoNT (100 nM) had any detectable effect on Ca^{2+}-dependent amylase secretion from the SLO permeabilized acini.

As controls to check that toxin treatment did result in the proteolysis of VAMP 2, aliquots of untreated and toxin

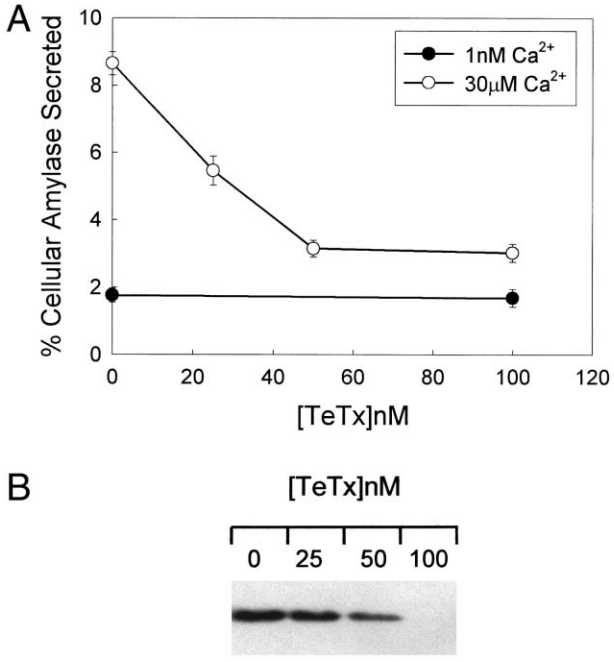

Fig. 3. Treating SLO permeabilized pancreatic acini with TeTx inhibits Ca^{2+}-dependent amylase secretion and results in the cleavage of VAMP 2. Pancreatic acini were permeabilized with SLO and then incubated with increasing concentrations of TeTx for 30 min in the presence of 2 mM ATP and 1 mg/ml rat brain cytosol. A: Aliquots of treated acini were removed and amylase secretion stimulated by addition of Ca^{2+}. After 30 min the incubations were terminated and the amount of amylase secreted determined. The data are the means ± S.E.M. from three separate experiments conducted on acini isolated from three different rats. B: The remaining acini were lysed and 10 μg (total protein) samples analyzed for VAMP 2 by Western blot analysis. The blot shown is representative of the three experiments conducted.

treated acini were removed, lysed and analyzed by Western blot for VAMP 2 (Figs. 1B and 2B). No VAMP 2 band was observed in the samples obtained from the toxin treated acini suggesting that BoNT treatment had cleaved all the VAMP 2 present in the acini. To eliminate the possibility that a small residual pool of VAMP 2 which was below the threshold of detection using standard blotting conditions (see Section 2) was present in the toxin treated acini, I increased sample loading (up to 50 μg protein per lane) and/or extended the exposure times of the blots (up to 60 min). Even using these modified conditions no VAMP 2 band was observed in the gel lanes loaded with samples prepared from the BoNT toxin treated acini (data not shown). Thus it must be assumed that BoNT treatment did result in the complete elimination of VAMP 2 from the permeabilized acini. This observation plus the fact that neither BoNTs have any detectable influence on Ca^{2+}-dependent amylase secretion indicates that VAMP 2 is not required for regulated exocytosis in the pancreatic acinar cell.

Next I determined how treating permeabilized acini with TeTx influenced Ca^{2+}-dependent amylase secretion (Fig. 3A) and VAMP 2 concentration (Fig. 3B). Previous studies have demonstrated that TeTx both significantly inhibits amylase secretion and cleaves VAMP 2 in SLO permeabilized acini. 50 to 100 nM TeTx produced a maximal, 75–80%, inhibition of Ca^{2+}-dependent amylase secretion. This level of inhibition is two to three times higher than previously observed in SLO permeabilized acini [10]. This disparity probably reflects the difference in incubation conditions and times used. In particular, in the present study ATP and cytosol were included in the toxin incubation buffer to promote the priming of exocytosis and so optimize SNARE availability. Most recently, the involvement of VAMP 2 in exocytosis in the acinar cell was explored using an in vitro assay that monitors the fusion of isolated zymogen granules with purified acinar cell plasma membranes [4]. In this study, TeTx cleaved VAMP 2 but only produced a small reduction in granule/plasma membrane fusion. However, how accurately the in vitro fusion assay reconstitutes Ca^{2+}-dependent exocytosis is unclear. In addition to inhibiting amylase secretion, 100 nM TeTx also cleaved all the VAMP 2 present in the acini. In the light of these findings and the results of the BoNT studies, it appears that a TeTx sensitive protein other than VAMP 2 is required for Ca^{2+}-dependent exocytosis in the pancreatic acinar cell. Given that TeTx and BoNT/B cleave the same peptide bond (Gln–Phe) it might appear surprising that BoNT/B did not have the same effect as TeTx and inhibit secretion. Detailed examination of the enzymological properties of the two toxins indicates that TeTx and BoNT/B interact differently with their substrates [22], therefore it is likely that under a specific set of conditions there is a unique set of substrates for each toxin.

Over the past 2 to 3 years numerous SNARE proteins, both v-SNAREs and t-SNAREs, have been identified. The toxin sensitivity of these proteins does vary, for example a TeTx insensitive VAMP isoform has recently been identified in Caco2 cells [23]. Thus it is possible, if not probable, that VAMP 2 is not the sole TeTx sensitive protein expressed in the pancreatic acinar cell. A BoNT/B insensitive protein immunologically related to VAMP 2 has been identified on the zymogen granule membrane [24], however, this protein has not been isolated or further characterized. It is therefore impossible to say whether this protein is the BoNT/B and BoNT/D insensitive, but TeTx sensitive, factor required for exocytosis in the acinar cell. In addition, a small quantity of cellubrevin (VAMP 3) is present in the granule membrane [25,26]. In neuroendocrine cells cellubrevin is cleaved by TeTx, BoNT/B and BoNT/D, however, under the conditions used in this study it is possible that the cellubrevin present in the acinar cells is resistant to one or more of the toxins. We therefore examined the influence of the three bacterial neurotoxins on cellubrevin concentration in SLO permeabilized acini (Fig. 4). All three neurotoxins (100 nM) cleaved the entire pool cellubrevin present in the permeabilized acini indicating that cellubrevin, like VAMP 2, does not appear to be required for exocytosis in the acinar cell. It has been suggested that

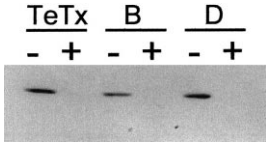

Fig. 4. Treating SLO permeabilized pancreatic acini with TeTx, BoNT/B or BoNT/D results in the cleavage of cellubrevin. Pancreatic acini were permeabilized with SLO and then incubated with or without 100 nM TeTx, BoNT/B or BoNT/D for 30 min in the presence of 2 mM ATP and 1 mg/ml rat brain cytosol. The untreated and toxin treated acini were lysed and 30 μg (total protein) samples analyzed for cellubrevin by Western blot analysis. The blot shown is representative of the three experiments conducted.

TeTx inhibits neurotransmitter release by stimulation of transglutaminase [27,28]. Although there is no evidence that transglutaminase has any significant influence on exocytosis in exocrine cells, this study does highlight the possibility that TeTx inhibits exocytosis in the acinar cell by influencing a protein other than a VAMP isoform.

In summary, this study demonstrates that Ca^{2+}-dependent exocytosis in SLO permeabilized acini is sensitive to TeTx but not BoNT/B and D. In contrast, all three toxins cleaved VAMP 2. The difference in toxin sensitivities indicates that a TeTx sensitive protein other than VAMP 2 is required for exocytosis in the acinar cell. Currently studies are underway to identify the TeTx sensitive protein.

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References

[1] Gerst, J.E. (1999) Cell. Mol. Life Sci. 55, 707–734.
[2] Gaisano, H.Y. (2000) Pancreas 20, 217–226.
[3] Mochida, S. (2000) Neurosci. Res. 36, 175–182.
[4] Hansen, N.J., Antonin, W. and Edwardson, J.M. (1999) J. Biol. Chem. 274, 22871–22876.
[5] Paumet, F., Le Mao, J., Martin, S., Galli, T., David, B., Blank, U. and Roa, M. (2000) J. Immunol. 164, 5850–5857.
[6] Kawanishi, M., Tamori, Y., Okazawa, H., Araki, S., Shinoda, H. and Kasuga, M. (2000) J. Biol. Chem. 275, 8240–8247.
[7] Chen, D., Bernstein, A.M., Lemons, P.P. and Whiteheart, S.W. (2000) Blood 95, 921–929.
[8] Pellizzari, R., Rossetto, O., Schiavo, G. and Montecucco, C. (1999) Philos. Trans. Roy. Soc. Lond. 354, 259–266.
[9] Schiavo, G., Matteoli, M. and Montecucco, C. (2000) Physiol. Rev. 80, 717–766.
[10] Gaisano, H.Y., Sheu, L., Foskett, J.K. and Trimble, W.S. (1994) J. Biol. Chem. 269, 17062–17066.
[11] Gaisano, H.Y., Ghai, M., Malkus, P.N., Sheu, L., Bouquillon, A., Bennett, M.K. and Trimble, W.S. (1996) Mol. Biol. Cell 7, 2019–2027.
[12] Gaisano, H.Y., Sheu, L., Wong, P.P., Klip, A. and Trimble, W.S. (1997) FEBS Lett. 414, 298–302.
[13] Edelman, L., Hanson, P.I., Chapman, E.R. and Jahn, R. (1995) EMBO J. 14, 224–231.
[14] Padfield, P.J. and Panesar, N. (1997) Am. J. Physiol. 273, G655–G660.
[15] Padfield, P.J. and Panesar, N. (1998) Biochem. J. 330, 329–334.
[16] Bernfeld, P. (1955) Methods Enzymol. 1, 149–158.
[17] Leammli, U.K. (1970) Nature 227, 680–685.
[18] Niemann, H., Blasi, J. and Jahn, R. (1994) Trends Cell Biol. 4, 179–185.
[19] Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Sudhof, T.C. and Niemann, H. (1994) EMBO J. 13, 5051–5061.
[20] Pellegrini, L.L., O'Connor, V., Lottspeich, F. and Betz, H. (1995) EMBO J. 14, 4705–4713.
[21] Banerjee, A., Kowalchyk, J.A., DasGupta, B.R. and Martin, T.F.J. (1996) J. Biol. Chem. 271, 20227–20230.
[22] Foran, P., Shone, C.C. and Dolly, J.O. (1994) Biochemistry 33, 15365–15374.
[23] Galli, T., Zahraoui, A., Vaidyanathan, V.V., Raposo, G., Tian, J.M., Karin, M., Niemann, H. and Louvard, D. (1998) Mol. Biol. Cell. 9, 1437–1448.
[24] Braun, J.E., Fritz, B.A., Wong, S.M. and Lowe, A.W. (1994) J. Biol. Chem. 269, 5328–5335.
[25] Sengupta, D., Gumkowski, F.D., Tang, L.H., Chilcote, T.J. and Jamieson, J.D. (1996) Eur. J. Cell Biol. 70, 306–314.
[26] Gaisano, H.Y., Sheu, L., Grondin, G., Ghai, M., Bouquillon, A., Lowe, A., Beaudoin, A. and Trimble, W.S. (1996) Gastroenterology 111, 1661–1669.
[27] Facchiano, F., Valtorta, F., Benfenati, F. and Luini, A. (1993) Trends Biochem. Sci. 18, 327–329.
[28] Aston, A.C. and Dolly, J.O. (1997) J. Neurochem. 68, 649–658.