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Botulinum neurotoxin light chains inhibit both Ca^{2+}-induced and GTP analogue-induced catecholamine release from permeabilised adrenal chromaffin cells

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Abstract Using digitonin-permeabilised bovine adrenal chromaffin cells, the effects of botulinum neurotoxin light chains on exocytosis triggered by Ca^{2+} or by GppNHp were examined. Botulinum neurotoxin D light chain, prepared as a His_6-tagged recombinant protein, cleaved VAMP and substantially inhibited catecholamine release due to Ca^{2+} and GppNHp. Botulinum neurotoxin C1 and E light chains produced partial inhibition of both Ca^{2+}- and GppNHp-induced catecholamine release. These results suggest that Ca^{2+}-dependent exocytosis and Ca^{2+}-independent exocytosis triggered by a non-hydrolysable GTP analogue occurs via a SNARE-dependent mechanism in chromaffin cells.

Key words: Exocytosis; Chromaffin cell; Neurotoxin; VAMP; Secretion

1. Introduction

The discovery that clostridial neurotoxins are proteases [1], and the characterisation of their specific substrates [2,3], has been a major step forward in the identification of proteins essential for synaptic neurotransmission. The synaptic vesicle protein known either as synaptobrevin or VAMP [4,5] is a substrate for tetanus toxin and for botulinum neurotoxins B, D, F and G [2,3]. SNAP-25 and syntaxin were originally identified as presynaptic membrane proteins [6,7]. SNAP-25 is the target for botulinum neurotoxins A and E and syntaxin for botulinum neurotoxin C1 [2]. VAMP, syntaxin and SNAP-25 are found in a stoichiometric complex in detergent extracts of brain membranes and were suggested to function as a synaptic vesicle docking complex [8,9] that could act as a receptor for α-SNAP and NSF, two proteins with functions in multiple vesicular transport pathways [10]. The ability of the neurotoxin substrates to bind α-SNAP led to their designation as SNAREs (soluble NSF-attachment protein (SNAP) receptors). A variety of evidence suggests that these various proteins are key components of the machinery involved in Ca^{2+}-triggered exocytosis in the synapse, though their exact functions remain a subject of debate [11-13].

Clostridial neurotoxins inhibit exocytosis in several endocrine and other secretory cell types. Inhibition of exocytosis in a non-neuronal cell by these neurotoxins was first shown for adrenal chromaffin cells [14] which have been an important model cell for the study of Ca^{2+}-regulated exocytosis [15,16]. Chromaffin cells express all three neurotoxin substrates [17,18] which can be isolated in a complex [18] and Ca^{2+}-dependent exocytosis in these cells is sensitive to neurotoxins that target VAMP (tetanus and botulinum B neurotoxins) [19-24] or SNAP-25 (botulinum A and E neurotoxins) [23,25-28]. Insulin-secreting cells are also sensitive to these two classes of neurotoxins [29-31] and studies on these cells have suggested that VAMP is essential for Ca^{2+}-induced secretion but not for insulin release activated by the non-hydrolysable GTP analogue, GTPγS, since this was insensitive to tetanus toxin and botulinum B neurotoxin [29]. These data on insulin-secreting cells, coupled with studies on constitutive vesicle traffic in epithelial cells in which basolateral but not apical exocytosis is inhibited by botulinum neurotoxins [32], appear to suggest that exocytosis could occur by either SNARE-dependent or SNARE-independent pathways. Exocytosis in chromaffin cells is triggered by Ca^{2+} [15,16] but exocytosis can also be activated in a Ca^{2+}-independent manner by GTP analogues in permeabilised chromaffin cells [33-36]. We have, therefore, used botulinum neurotoxins to investigate whether exocytosis activated by a non-hydrolysable GTP analogue in permeabilised chromaffin cells, occurs by a SNARE-dependent mechanism based on sensitivity to recombinant neurotoxin light chains. The results show that Ca^{2+}-independent catecholamine release from permeabilised chromaffin cells in response to a non-hydrolysable GTP analogue is sensitive to clostridial neurotoxins.

2. Materials and methods

Bovine adrenal chromaffin cells were dissociated from the adrenal medulla and maintained in culture for 3–6 days in 24-well trays as previously described [37]. Recombinant botulinum neurotoxins were expressed as His_6-tagged fusion proteins [38] from plasmids which were a generous gift from Dr Heiner Neimann (Tubingen, Germany). Proteins were purified from *Escherichia coli* M15 [pREP4] (Qiagen) after induction of expression with 0.5 mM isopropyl-D-thiogalactopyranoside for 2.5–5 h, cell lysis by ultrasonication and affinity chromatography on Ni-NTA-agarose. Proteins were eluted in 20 mM HEPES, 200 mM KCl, 2 mM β-mercaptoethanol, 0.5 mM ATP, 10% v/v glycerol pH 7.0 using a 50–100 mM imadazole gradient generated with a Pharmacia FPLC system and active fractions pooled and stored at −20 or −80°C.

For secretion experiments, chromaffin cells were permeabilised by incubation with 20 μM digitonin in permeabilisation buffer (139 mM potassium glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM ATP, 2 mM $MgCl_2$, pH6.5) for 10 min, incubated with or without purified botulinum neurotoxin light chains (in column elutions buffer or after dialysis against permeabilisation buffer) for 25 min in permeabilisation buffer and then challenged by addition of permeabilisation buffer with no added Ca^{2+} (0 Ca^{2+}, controls), with 10 μM Ca^{2+} or 100 μM GppNHp. Catecholamine released over a 20 min period was assayed fluorometrically and expressed as a percentage of total cellular catecholamine [37]. In some experiments, as indicated, ATP was omitted

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Abbreviations: GTPγS, guanosine 5'-[γ-thio]triphosphate; GppNHp, guanosine 5'-[βγ-imido]triphosphate

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from the final incubation step. All data shown are derived from a typical experiment and are expressed as mean ± S.E.M.

For the analysis of protein cleavage by botulinum neurotoxin D, cells were taken after a 10 min permeabilisation in digitonin and a 25 min incubation with 20 nM neurotoxin, solubilised in SDS dissociation buffer, separated on 12.5% SDS-polyacrylamide gels and immunoblots probed with antisera against VAMP (a gift from Dr Clifford Shone, CAMR, Porton Down, Salisbury), SNAP-25 [18] or syntaxin (HPC-1 monoclonal, Sigma Chemical Co.) as previously described [18].

3. Results and discussion

The protocol used here was to permeabilise chromaffin cells for 10 min using digitonin and then to incubate the cells over a further 25 min period, before stimulation with 10 μM Ca^{2+} or 100 μM of the non-hydrolysable GTP analogue guanosine 5′-[βγ-imido]triphosphate (GppNHp). As shown in Fig. 1, GppNHp stimulates catecholamine release above basal levels in the absence of added Ca^{2+} with 5 mM EGTA present. This Ca^{2+}-independent secretion triggered by GppNHp, as in previous work [33,34], is smaller than that triggered by 10 μM Ca^{2+} but was reproducibly observed. The release due to GppNHp was 2.5 ± 0.2% of total cellular catecholamine above basal from 17 cell batches. This extent of release could conceivably be accounted for by release of only docked secretory granules (corresponding to 1.5–3% of total granules per cell [15,39]). This is potentially of importance since it has been suggested that SNARE proteins of docked vesicles would not be accessible to neurotoxins [38]. It has been suggested that release of docked granules corresponds to that component of release that is MgATP-independent [39]. Release of catecholamine due to GppNHp was, however, reduced by about 50% in the absence of ATP, as is the case for Ca^{2+}-induced release (Fig. 1) indicating that the limited extent of GppNHp-induced release cannot be explained on the basis of specific release of docked, ATP-independent granules.

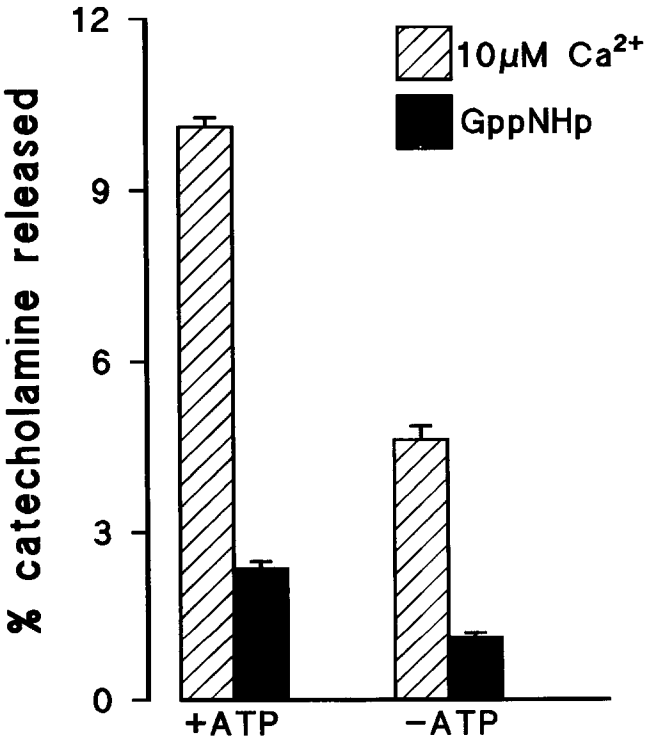

Fig. 1. Catecholamine release stimulated by Ca^{2+} or GppNHp and requirement for ATP. Chromaffin cells were permeabilised with digitonin for 10 min, incubated for a further 25 min and then challenged with 0 Ca^{2+}, 10 μM Ca^{2+} or 0 Ca^{2+} plus 100 μM GppNHp in the presence or absence of ATP ($n = 6$ in each case). Catecholamine release over a 20 min period was assayed and expressed as a percentage of total cellular catecholamine. The catecholamine released in response to 10 μM Ca^{2+} or GppNHp is shown as release above basal (0 Ca^{2+}) levels.

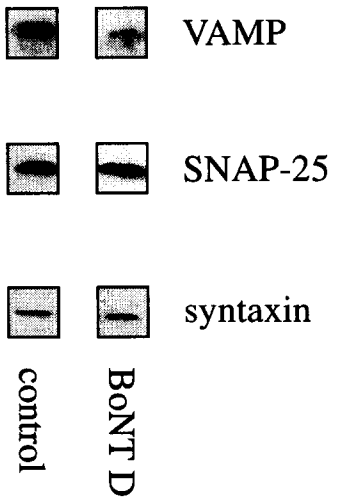

Fig. 2. Cleavage of VAMP in permeabilised chromaffin cells by botulinum neurotoxin D light chain. After digitonin-permeabilisation, chromaffin cells were treated with or without 20 nM His_6-tagged light chain of botulinum neurotoxin D (BoNT D) for 25 min. The cells were analysed by SDS-polyacryamide gel electrophoresis and immunoblotting with antisera against VAMP, SNAP-25 or syntaxin as indicated. VAMP was cleaved by 62% by botulinum neurotoxin D in this experiment.

In order to examine the sensitivity of Ca^{2+}- and GppNHp-induced release to clostridial neurotoxins, we introduced the active light chains into the permeabilised cells in the incubation preceding stimulation. The neurotoxin light chains were expressed and purified as His_6-tagged recombinant proteins in *E. coli* which would avoid any potential problems due to contamination of the neurotoxins with other types of clostridial toxins. The recombinant light chain of botulinum D neurotoxin (20 nM) was found to cleave VAMP leading to a loss of around 60% of VAMP detected by immunoblotting and had no effect on levels of syntaxin on SNAP-25, after incubation with permeabilised cells (Fig. 2), consistent with data on neuronal preparations showing that this neurotoxin specifically cleaves VAMP [40,41]. Treatment of permeabilised cells with botulinum D neurotoxin light chain led to a substantial inhibition of both Ca^{2+}- and GppNHp-induced catecholamine release (Fig. 3). This neurotoxin light chain, at 20 nM, produced a greater than 50% inhibition in all 11 experiments with Ca^{2+}-induced release and a mean inhibition of 74.1 ± 9.2% in 7 separate experiments with GppNHp as the stimulus. As shown in Fig. 3, at higher neurotoxin D light chain concentrations release due to GppNHp was abolished. These results suggest, therefore, that VAMP is involved not only in Ca^{2+}-induced release in chromaffin cells but also in release induced by GppNHp. Direct analysis of membrane capacitance has directly shown that GppNHp induces exocytosis [36] and it is likely that the same final steps in the exocytotic pathway are activated by Ca^{2+} or by GppNHp. Previous work on insulin-secreting cells had shown that secretion

induced by GTPγS was insensitive to tetanus and botulinum B neurotoxins despite extensive cleavage of VAMP, and inhibition of Ca^{2+}-induced secretion, leading to the suggestion that VAMP is essential for the Ca^{2+}-induced secretion but not that induced by non-hydrolysable GTP analogues [29]. The present data from chromaffin cells show, in contrast, that both stimuli are neurotoxin D-sensitive and therefore act via a VAMP-dependent pathway.

The requirement for the other two neurotoxin sensitive substrates for secretion due to Ca^{2+} or GppNHp in chromaffin cells was examined using recombinant light chains of the botulinum C1 neurotoxin, which was shown to preferentially cleave syntaxin [42,43] and E neurotoxin which cleaves SNAP-25 [44,45]. The maximum concentration of these light chains that could be used was limited by their instability following dialysis. Both neurotoxins produced partial inhibition of Ca^{2+}-induced release. A similar partial inhibition was also seen for GppNHp-induced release (Fig. 4) of 59.3 ± 9.4% (n=6 experiments) for botulinum neurotoxin C1 and 58.8 ± 14.0% (n=4 experiments) for neurotoxin E compound to control values.

As far as is currently known, botulinum neurotoxin D cleaves only VAMP 1 and VAMP 2 and the ubiquitous homologue cellubrevin [2]. We have shown here that recombinant His_6-tagged botulinum neurotoxin D light chain cleaves VAMP but not syntaxin or SNAP-25 in permeabilised chromaffin cells and substantially inhibits both Ca^{2+}-induced and GppNHp-induced catecholamine release suggesting that one or more VAMPs are essential in both cases. This differs from the situation in insulin-secreting cells where it has been suggested that VAMPs are not required for GTPγS-induced secretion [29]. While botulinum neurotoxin E is believed to be specific for SNAP-25, neurotoxin C1 has recently been shown to cleave both syntaxin and SNAP-25 in chromaffin cells [46]. Nevertheless, the data from the three neurotoxin light chain used here are consistent with a role for the SNARE proteins in GppNHp-induced secretion in chromaffin cells.

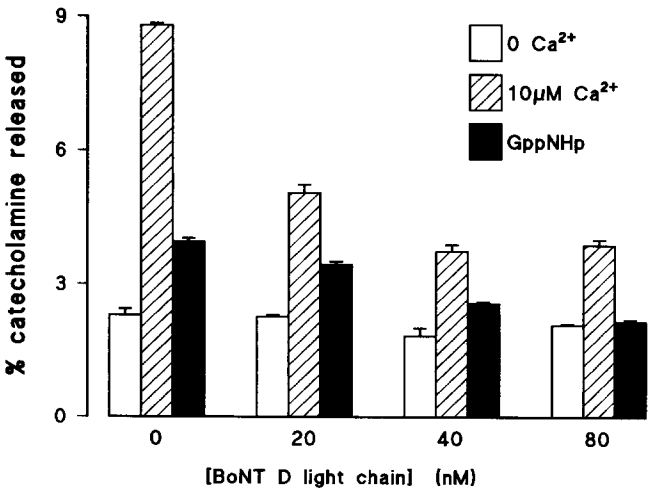

Fig. 3. Effect of botulinum neurotoxin D light chain on catecholamine release due to Ca^{2+} or GppNHp. After digitonin-permeabilisation for 10 min, cells were incubated for 25 min with the indicated concentration of His_6-tagged botulinum neurotoxin D light chain (BoNT D). The cells were then challenged with 0 Ca^{2+}, 10 μM Ca^{2+} or 0 Ca^{2+} plus 100 μM GppNHp and catecholamine release over a 20 min period assayed and expressed as a percentage of total cellular catecholamine (n=4).

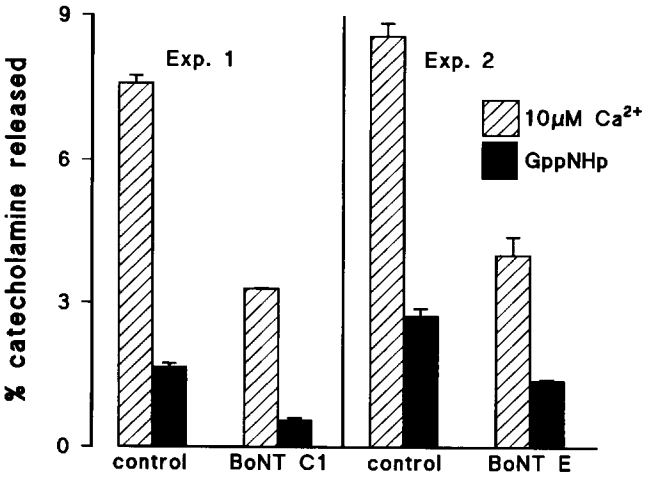

Fig. 4. Effect of botulinum neurotoxin C1 and E light chains on catecholamine release due to Ca^{2+} or GppNHp. After digitonin-permeabilisation for 10 min, cells were incubated for 25 min with no additions, with 60 nM His_6-tagged botulinum neurotoxin C1 light chain (BoNT C1) or with 10 nM His_6-tagged botulinum neurotoxin E light chain (BoNT E). The cells were then challenged with 0 Ca^{2+}, 10 μM Ca^{2+} or 0 Ca^{2+} plus 100 μM GppNHp and catecholamine release over a 20 min period assayed and expressed as a percentage of total cellular catecholamine (n = 4 in each case). The catecholamine release in response to 10 μM Ca^{2+} or GppNHp is shown as release above basal (0 Ca^{2+}) levels.

Various GTP analogues have multiple effects on secretion from permeabilised chromaffin cells [47] which includes stimulation of release from permeabilised cells [33-35] and exocytosis in patch-clamped cells [36] by GppNHp at very low free Ca^{2+} concentrations. Like Ca^{2+}-induced secretion, Ca^{2+}-independent catecholamine release stimulated by GppNHp is partially MgATP-dependent, is substantially inhibited by prior treatment with botulinum neurotoxin D and is partially inhibited by neurotoxins C1 and E. These results suggest that both activators of exocytosis activate a common mechanism leading to exocytosis that requires the SNARE proteins, VAMP, syntaxin and SNAP-25 and would argue against the presence of a SNARE-independent pathway for exocytosis in adrenal chromaffin cells.

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