Transient expression of botulinum neurotoxin C1 light chain differentially inhibits calcium and glucose induced insulin secretion in clonal β-cells

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Abstract We have investigated the effect of botulinum neurotoxin (BoNT) C1 light chain (LC) on insulin exocytosis from the clonal β-cell line HIT-T15. In streptolysin-O permeabilized cells, the β-cell impermeant BoNT C1 cleaved mainly syntaxin 1 and inhibited Ca^{2+} as well as GTPγS induced exocytosis. To study the effect of BoNTs in intact cells, we transiently coexpressed the BoNT LC together with a reporter gene for insulin release. BoNT C1 inhibited K^+ induced insulin secretion by 95% but reduced insulin release stimulated by glucose only by 25%. Thus a component of glucose stimulated insulin release is insensitive to BoNT C1.

Key words: Exocytosis; Ca^{2+}; Guanosine triphosphate; Glucose; Botulinum neurotoxin C1; Insulin

1. Introduction

Alterations in cytosolic levels of the second messenger Ca^{2+} control regulated secretion by exocytosis in neuronal and endocrine cells [1–3]. In the pancreatic β-cells glucose metabolism causes insulin secretion from large dense core vesicles (LDCV) by two mechanisms. First, exposure of β-cells to the sugar induces closure of ATP-sensitive K^+ (K_{ATP}) channels with subsequent membrane depolarization and voltage dependent Ca^{2+} influx [1]. The ensuing increase in cytosolic Ca^{2+} $[Ca^{2+}]_c$ evokes insulin release [1,3]. Second, glucose further enhances Ca^{2+} induced insulin secretion by a mechanism distinct from closure of K_{ATP} channels, alterations in $[Ca^{2+}]_c$ or activation of protein kinases A or C [4]. This second pathway, however, requires stimulatory levels of $[Ca^{2+}]_c$ [4,5] and is completely inhibited by Ca^{2+} channel blockers [4].

The mechanism of the K_{ATP} independent glucose effect on exocytosis has not yet been identified. Molecular dissection of the general exocytotic process in yeast, flies and mammals has prompted the SNARE hypothesis to summarize the detailed molecular events [6,7]. Exocytosis requires the assembly of the membrane proteins syntaxin, SNAP-25 and synaptobrevin/VAMP to a multi-subunit aggregate, the SNARE complex, which subsequently binds the cytosolic components NSF (NEM sensitive factor) and α/β-SNAP. The role of SNARE complex proteins in Ca^{2+} induced neurotransmitter release and neuroendocrine secretion has been confirmed by the correlation between their selective cleavage and the blockade of exocytosis by clostridial neurotoxins [8,9]. Tetanus toxin and botulinum neurotoxin (BoNT) B, D, F and G proteolyse specifically VAMP/cellubrevin, whereas BoNT A and E cleave SNAP-25. BoNT C1 acts on syntaxin (isoforms 1A, 1B and 2) and in addition on SNAP-25 [10–12].

In pancreatic β-cells several isoforms of the major components of the exocytotic machinery have been identified previously [13,17]. More importantly, their functional implication has been demonstrated for VAMP/synaptobrevin and SNAP-25 using the β-cell impermeant clostridial neurotoxins in streptolysin-O (SL-O) permeabilized cells [14,15,18].

The function of the SNARE protein syntaxin is less evident in β-cells. Employing anti-syntaxin antibodies or peptides corresponding to a helical domain of syntaxin 1 a role of syntaxin in Ca^{2+} induced insulin exocytosis was deduced [19,20]. As overexpression of syntaxin 1A in the clonal β-cell line βTC3 suppressed glucose stimulated exocytosis, syntaxin 1A was proposed as a negative regulator in insulin exocytosis [21]. Therefore we have investigated the effect of the β-cell impermeant BoNTs C1 and E on insulin exocytosis. Furthermore, we report a method which permits the use of the cell impermeant BoNTs in intact cells.

2. Materials and methods

2.1. Materials

Recombinant pore forming toxins were kindly provided by Dr U. Weller (Mainz, Germany) and recombinant clostridial neurotoxins were produced by standard procedures in *Escherichia coli*. pEGFP was purchased from Clontech (Heidelberg, Germany). mAb anti-VAMP, polyclonal anti-SNAP-25 and polyclonal anti-BoNT C1 light chain (LC) were generously provided by Dr. R. Jahn (MPI Göttingen, Germany), Dr. C. Montecucco (Padua, Italy) and Dr. S. Kozaki (Osaka, Japan), respectively. To construct peGFP/BoNT C1, the *Eco*RI/*Pst*I fragment of pQE/BoNT C1 LC was inserted into the same cleavage sites of peGFP-C2.

2.2. Cell culture, transient cotransfection, FACS and immunofluorescence/immunoblots

HIT-T15 cells were cultured, transiently cotransfected and purified by fluorescent activated cell sorting as published [17,22,23]. Western

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Abbreviations: α/β-SNAP, soluble NSF attachment proteins; BoNT, botulinum neurotoxin; BoNT C1, botulinum neurotoxin C1; BoNT E, botulinum neurotoxin E; eGFP/BoNT C1, enhanced GFP-botulinum neurotoxin C1 fusion protein; NSF, *N*-ethylmaleimide sensitive factor; phINS, plasmid encoding human insulin; SNARE, soluble NSF attachment receptor; VAMP, vesicle associated membrane protein

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blots and immunofluorescence were performed as described previously [17]. Primary antibodies were used at the following dilutions: anti-syntaxin (Sigma) 1:4000; mAb anti-SNAP-25 C-terminus (Sternberger Monoclonals, Baltimore, MD, USA) 1:2000; mAb anti-VAMP 1:10000; polyclonal anti-SNAP-25 N-terminus 1:1000 [12].

2.3. Cell permeabilization

Preparation of intracellular buffers and permeabilization procedures were as described [5,17,22,23]. Na_2ATP (5 mM) was present throughout the experiment. BoNTs were added to the permeabilized cells only during 9 min preincubation in intracellular buffer at 0.1 μM free Ca^{2+}. Subsequently, this solution was exchanged for intracellular buffer containing the indicated concentrations of free Ca^{2+} or 0.1 μM free Ca^{2+} and 100 μM GTPγS (Boehringer, Rotkreuz, Switzerland) in the absence of BoNTs [16,17,22].

2.4. Statistical analysis

Results are presented as mean ± S.D. from experiments performed independently on at least three different cell preparations. Statistical analysis was performed by Student's two-tailed *t*-test for unpaired data (2p) if not stated otherwise.

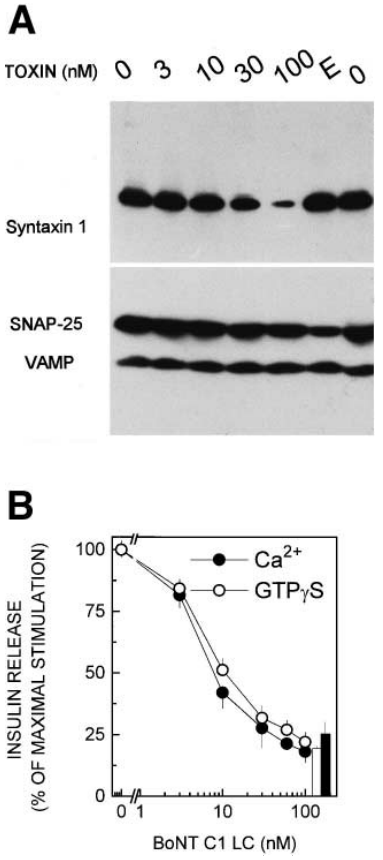

Fig. 1. Effect of BoNT C1 light chain on SNARE cleavage and insulin exocytosis in SL-O permeabilized HIT-T15 cells. A: HIT-T15 cells were permeabilized with SL-O and incubated for 9 min with the indicated concentrations of BoNT C1 or with 2 nM BoNT E. Subsequently cells were homogenized, separated by SDS/PAGE and immunoblots performed (1×10^5/lane) with antibodies recognising intact syntaxin 1, SNAP-25 C-terminus (SNAP-25) or VAMP. B: Effect of BoNTC 1 pretreatment in SL-O permeabilized cells on subsequent exocytosis stimulated by Ca^{2+} or GTPγS. The difference between 0.1 and 10 μM CA^{2+} (filled symbols) or 0.1 μM CA^{2+} in the absence or presence of 100 μM GTPγS (open symbols) in the controls was normalized to 100%. Bars: effect of 2 nM BoNT E on exocytosis stimulated by Ca^{2+} (filled bar) or GTPγS (open bar). $n = 6$–18 from four independent experiments.

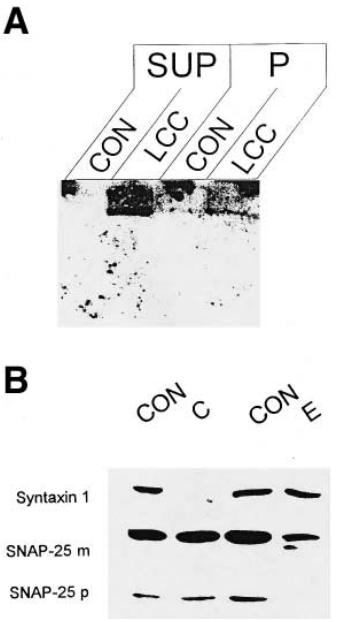

Fig. 2. BoNT C1 LC expression and SNARE protein cleavage by transiently expressed BoNT C1 LC in intact cells. A: BoNT C1 LC expression. Cells cotransfected with phINS and pcDNA3 (CON) or with phINS and pCMV/BoNT C1 LC (LCC) were homogenized, separated into supernatant (SUP) and particulate fraction (P) by centrifugation (14000×*g*, 30 min) and immunoblots performed with a polyclonal antibody against BoNT C1 LC. B: Cells were transfected with peGFP/BoNT C1 or cotransfected with peGFP and pCMV/BoNT E LC and sorted by FACS. Aliquots of sorted cells (0.5×10^5/lane) were subjected to SDS-PAGE followed by immunoblot. CON, control, C, peGFP/BoNT C1 transfected positive cells; E, pCMV/BoNT E LC positive cells. Syntaxin, mAb anti-syntaxin; SNAP-25 m, monoclonal anti-SNAP-25 antibody; SNAP-25 p, polyclonal anti-SNAP-25 C-terminus antibody. The blot shown is representative of three independent experiments.

3. Results and discussion

3.1. Effect of BoNTs C1 and E in permeabilized cells

We first examined the acute effect of BoNT C1 on SNARE protein cleavage. In contrast to neurons and chromaffin cells, β-cells are impermeant to clostridial neurotoxins. Therefore, HIT-T15 cells were permeabilized by SL-O [16,17,22,23]. Under these conditions, BoNT C1 LC cleaved syntaxin 1 in a dose dependent manner (see Fig. 1A). SNAP-25 immunoreactivity was detected with a polyclonal C-terminally directed antibody, which only recognizes the uncleaved protein [12]. A decrease in SNAP-25 levels occurred only at BoNT C1 concentrations above 10 nM. In contrast, 2 nM BoNT E markedly reduced SNAP-25 immunoreactivity. As expected, none of the toxins altered the amount of the SNARE VAMP/synaptobrevin. Pretreatment of SL-O permeabilized cells by BoNT C1 LC did not influence the basal release of insulin at 0.1 μM Ca^{2+} (8.7 ± 1.3 ng insulin/well in the absence of BoNT C1 LC versus 7.8 ± 1.6 ng in the presence of 100 nM BoNT C1 LC, $n = 9$–14). Exposing permeabilized cells to 10 μM Ca^{2+}, the maximal stimulatory Ca^{2+} concentration [3,17,22,23], or 100 μM GTPγS [16] increased insulin release 4–7- and 2–3-fold, respectively. For a better comparison between the two stimuli, the release over basal evoked by 10 μM Ca^{2+} or by GTPγs in the absence of BoNT C1 LC was normalized to 100%. Exocytosis evoked by Ca^{2+} or GTPγS was inhibited to a similar extent by BoNT C1 (Fig. 1B). Half-

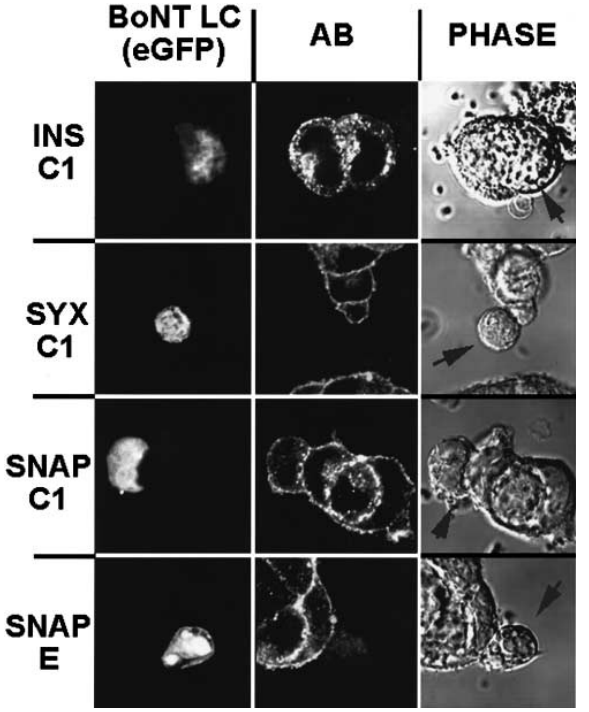

Fig. 3. Syntaxin, SNAP-25 and insulin immunoreactivity in HIT-T15 cells transiently expressing BoNT C1 LC or BoNT E LC. Cells transiently transfected with peGFP/BoNT C1 (C1) or with peGFP and pCMV/BoNTE LC (E) were fixed, permeabilized and stained with anti-insulin (INS, 1:200), anti-syntaxin (SYX, 1:100) or polyclonal anti-SNAP-25 C-terminus (SNAP, 1:100) antibodies. Antibodies used and type of transiently expressed toxins are given on the left. Left panel: eGFP fluorescence; middle panel: antibody binding as revealed by rhodamine coupled second antibodies; right panel: phase contrast with arrows indicating eGFP expressing cells.

maximal inhibition was apparent at 10 nM BoNT C1 LC, a toxin concentration which did not reduce SNAP-25 levels (see Fig. 1A). Ca^{2+} or GTPγS induced exocytosis was also abolished by BoNT E LC (Fig. 1B). These data indicate that both pathways leading to exocytosis require the SNARE proteins syntaxin and SNAP-25 in HIT-T15 cells.

Although SL-O permeabilized cells offer a useful model to study exocytosis [23,24], the final step in the secretory pathway, permeabilized cells preclude a number of interesting studies. We therefore transiently expressed clostridial neurotoxin light chains in clonal β-cells thereby providing a defined intracellular source as demonstrated previously for tetanus toxin [25,26]. Using a polyclonal antibody against BoNT C1 LC we found specific expression of the toxin in homogenates and the soluble fraction of transiently transfected cells, but not in the membrane pellet or in untransfected cells (Fig. 2A).

To study BoNT induced cleavage of SNAREs in HIT-T15 cells, we transiently expressed green fluorescent protein (eGFP) fused in frame with BoNT C1 LC (peGFP/BoNT C1) or eGFP and BoNT E. The cells expressing BoNTs were purified by fluorescent activated cell sorting and the SNARE proteins syntaxin and SNAP-25 were examined by immunoblotting (see Fig. 2). As expected, we observed a large reduction in syntaxin immunoreactivity in homogenates from sorted cells expressing BoNT C1 LC. In contrast, we could not detect cleavage in SNAP-25 using a monoclonal C-terminal antibody, which still recognizes the truncated form [15]. Likewise, a polyclonal C-terminal antibody, which detects only intact SNAP-25 [12], did not reveal a major change between homogenates from control cells or those expressing BoNT C1 LC. For comparison we immunoblotted homogenates from purified cells expressing BoNT E. In this case, a reduction or cleavage of SNAP-25 was clearly visible.

We confirmed these observations by immunofluorescence of cotransfected cells. The amount of insulin and the distribution of secretory granules were not altered by the expression of peGFP/BoNT C1 LC (Fig. 3). Again, the expression of BoNT C1 LC resulted in a loss of syntaxin 1 immunoreactivity, whereas a change in SNAP-25 immunoreactivity was not detected (Fig. 3). In contrast, the coexpression of BoNT E LC and eGFP led to a complete loss of SNAP-25 immunoreactivity in GFP positive cells (Fig. 3).

There is no obvious explanation for the difference in BoNT C1 produced SNARE cleavage between neurons and chromaffin cells on the one hand [10–12] and endocrine β-cells on the other. The two recently identified SNAP-25 isoforms SNAP-23 and syndet [27,28] have a broad tissue distribution and may be resistant to BoNT C1. However, the anti-SNAP-25 antibodies used here did not cross-react with GST-syndet or GST-SNAP-23 (data not shown), excluding the possibility that such a cross-reactivity could have masked SNAP-25 cleavage. It is of interest that experiments using BoNT C1 in cultured dorsal root ganglia also did not reveal SNAP-25 cleavage [29]. This may suggest a high degree of cell specificity in the action of BoNT C1 on SNAP-25. Alternatively, the amount of BoNT C1 LC expressed was rather small and perhaps only sufficient to cleave syntaxin. The latter is in agreement with our results in permeabilized cells indicating that syntaxin is more sensitive to BoNT C1 LC than SNAP-25.

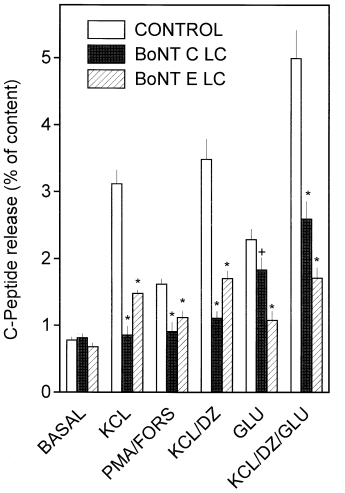

Fig. 4. Effect of transient expression of BoNT C1 LC on human insulin C peptide secretion from intact cells. Cells were cotransfected with phINS and either pcDNA3 (open bars), pCMV/BoNT C1 LC (filled bars) or pCMV/BoNT E LC (hatched bars) as given in Section 2 and 48 h later exposed for 15 min to KRB without glucose (BASAL), 48 mM KCl (KCL), 100 nM of the phorbol ester PMA together with 10 μM forskolin and 0.5 mM IBMX (PMA/FORS), 5 mM glucose (GLU), 30 mM KCl/250 μM diazoxide (KCL/DZ), or 30 mM KCl/250 μM diazoxide/5 mM glucose (KCL/DZ/GLU). $n=6$–12; $^{+}1P<0.05$; $^{*}2P<0.05$ as compared to the absence of toxins.

3.2. Transient expression of BoNT C1 or E and insulin secretion from intact cells

Having established the expression of BoNT C1 and E LC, their effect on insulin release was studied. For this purpose, cells were cotransfected with pCMV/BoNT C or E and a plasmid encoding human insulin (phINS) as reporter gene for exocytosis in the hamster insulin secreting cell line HIT-T15. This permits the study of hormone release exclusively from cotransfected cells [17,22,23,30]. First, we tested whether expression of BoNT C1 LC inhibits exocytosis similar to the acute effects of recombinant toxin in SL-O permeabilized cells described above. To create small pores preventing the leakage of expressed BoNT C1 LC or BoNT E LC, cotransfected cells were permeabilized with α-toxin from *Staphylococcus aureus* [5,16,24]. Under this condition, GTPγS or 10 μM Ca^{2+} increased hormone release in control cells (cotransfected with phINS and pcDNA3) by 845 ± 33% and 1686 ± 81%, respectively ($n = 6$ for each condition). Cotransfection with phINS and pCMV/BoNT C1 LC resulted in 91.3 ± 5.5% inhibition of GTPγS induced exocytosis and 95.1 ± 2.4% inhibition of Ca^{2+} evoked exocytosis ($n = 6$ for each condition). A comparable inhibition of exocytosis by both stimuli was observed after coexpression of human insulin and BoNT E LC. For both toxins human insulin C-peptide content and C-peptide release at 0.1 μM Ca^{2+} remained unchanged (data not shown).

Next, we stimulated insulin secretion in intact HIT-T15 cells by activation of different pathways. Exposure of intact cells to KCl leads to membrane depolarization and voltage dependent Ca^{2+} influx triggering secretion in primary β- and clonal HIT-T15 cells [3]. As shown in Fig. 4, KCl induced insulin release was completely inhibited in intact HIT-T15 cells by expression of BoNT C or E LC. A similar effect was observed after stimulation of insulin release by combined activation of the protein kinases A and C through forskolin/IBMX and the phorbol ester PMA. In contrast, BoNT C1 LC diminished by only 25% insulin release induced by glucose, whereas BoNT E LC expression resulted in complete inhibition (Fig. 4). Glucose evokes insulin secretion not only through closure of K_{ATP} channels followed by membrane depolarization and voltage dependent Ca^{2+} influx, but also through a pathway independent of K_{ATP} channels. The effect of the sugar on K_{ATP} channels and $[Ca^{2+}]_c$ in primary β-cells can be circumvented by the use of diazoxide to keep K_{ATP} channels open and KCl to induce membrane depolarization resulting in voltage dependent Ca^{2+} influx. Under these conditions of raised $[Ca^{2+}]_c$, glucose still produces a further increase in insulin release [4]. This involves a pathway distinct from further changes in $[Ca^{2+}]_c$ or activation of protein kinases A and C. To validate the resistance of glucose mediated secretion to BoNT C1 LC, we used a similar protocol. As shown in Fig. 4, the concomitant presence of glucose further enhanced the insulin release stimulated by KCl/diazoxide in HIT-T15 cells. Whereas stimulation by KCl/diazoxide alone was abolished by BoNT C1 LC, a lesser degree of inhibition by BoNT C1 LC was observed when KCl/diazoxide was added in the presence of glucose.

These findings allow two main conclusions. First, functional syntaxin is required for Ca^{2+} evoked insulin release from LDCVs. The previously reported inhibitory role of overexpressed syntaxin 1A [21] may reflect scavenging of interacting SNARE proteins as also observed for VAMP [30]. Results based on overexpression can therefore not be taken per se as evidence of an inhibitory role of syntaxin. Indeed, in HIT-T15 cells large overexpression of syntaxin 1A produced similar inhibition (unpublished observations).

Second, an alternative pathway to exocytosis must exist, as a component of glucose induced insulin release is insensitive to BoNT C1. Increased levels of $[Ca^{2+}]_c$ are necessary to observe the K_{ATP} independent effect of glucose [4,5,31]. The Ca^{2+} requirement could reflect presensitization of exocytosis necessary to observe K_{ATP} independent glucose effects. Alternatively, the well-documented requirement of Ca^{2+} influx for glucose induced insulin secretion [1,3,31] may reflect an additional action of Ca^{2+} at sites distinct from exocytosis itself. Indeed, glucose has to be metabolized to elicit its secretory response [32] which involves several Ca^{2+} sensitive mitochondrial enzymes [5,33–35]. As transient expression of BoNT LCs did not alter the content or basal release of the reporter gene (human insulin C-peptide), effects on the biosynthetic pathway prior to exocytosis can be excluded. Interestingly an alternative pathway to exocytosis has also been suggested to be present in insulin secreting RINm5F cells, in which BoNT B and F cleave synaptobrevin/VAMP but do not inhibit depolarization induced exocytosis [36].

Our observations do not negate a role of Ca^{2+} regulated and BoNT C1 sensitive components of the exocytotic machinery in the initial period of glucose stimulated insulin secretion. The partial inhibition of glucose induced exocytosis by BoNT C1 may be caused by the toxin effect on this initial phase of insulin release. This point could not be directly addressed in this study due to the limited time resolution inherent in the biochemical assays and the levels of reporter gene expression. It is conceivable that more subtle events such as rapid hormone release, colocalization of secretory granules with Ca^{2+} influx sites [37] or pulsatility of secretion [38] are altered by the action of BoNT C1.

The present data demonstrate the usefulness of transiently expressed BoNT C1 to study the role of SNARE proteins in toxin impermeant intact cells. This approach is likely to be applicable to most of the clostridial neurotoxins. Future work should determine whether glucose induced exocytosis involves BoNT C1 resistant isoforms of syntaxin [39,40] or proceeds by a mechanism independent of syntaxin.

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