Biochem. J. (1992) 283, 899–904 (Printed in Great Britain)

Amylase release from streptolysin O-permeabilized pancreatic acinar cells

Effects of Ca^{2+}, guanosine 5′-[γ-thio]triphosphate, cyclic AMP, tetanus toxin and botulinum A toxin

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The molecular requirements for amylase release and the intracellular effects of botulinum A toxin and tetanus toxin on amylase release were investigated using rat pancreatic acinar cells permeabilized with streptolysin O. Micromolar concentrations of free Ca^{2+} evoked amylase release from these cells. Maximal release was observed in the presence of 30 μM free Ca^{2+}. Ca^{2+}-stimulated, but not basal, amylase release was enhanced by guanosine 5′-[γ-thio]triphosphate (GTP[S]) (3–4 fold) or cyclic AMP (1.5–2 fold). Neither the two-chain forms of botulinum A toxin and tetanus toxin, under reducing conditions, nor the light chains of tetanus toxin, inhibited amylase release triggered by Ca^{2+}, or combinations of Ca^{2+} + GTP[S] or Ca^{2+} + cAMP. The lack of inhibition was not due to inactivation of botulinum A toxin or tetanus toxin by pancreatic acinar cell proteolytic enzymes, as toxins previously incubated with permeabilized pancreatic acinar cells inhibited Ca^{2+}-stimulated [^{3}H]noradrenaline release from streptolysin O-permeabilized adrenal chromaffin cells. These data imply that clostridial neurotoxins inhibit a Ca^{2+}-dependent mechanism which promotes exocytosis in neural and endocrine cells, but not in exocrine cells.

INTRODUCTION

Botulinum A toxin (Botx A) and tetanus toxin (Tetx) are examples of potent bacterial neurotoxins synthesized by Clostridia as single-chain proteins with a molecular mass of 150 kDa. The primary gene products, which have been sequenced at the DNA level (Eisel *et al.*, 1986; Binz *et al.*, 1990), are subsequently proteolytically cleaved, giving pharmacologically more active molecules (two-chain form) (Maisey *et al.*, 1988; Weller *et al.*, 1988). The two-chain form consists of a heavy (100 kDa) and a light (50 kDa) chain linked by a disulphide bond (DasGupta, 1989; Matsuda, 1989). Tetx and Botx A are reported to inhibit release of neurotransmitters from all neurons investigated to date (Bigalke *et al.*, 1981; Habermann & Dreyer 1986; Bergey *et al.*, 1987; Simpson, 1989).

Several lines of evidence suggest that clostridial neurotoxins target a selective intracellular mechanism. When directly injected into adrenal chromaffin cells, Tetx inhibits exocytosis of catecholamines, as demonstrated by increased membrane capacitance due to the incorporation of secretory vesicle membranes into the plasma membrane (Penner *et al.*, 1986). Clostridial neurotoxins can also be introduced into cells by permeabilization with digitonin or streptolysin O (SLO). Moreover, Botx A and Tetx potently inhibit catecholamine release from permeabilized PC12 cells (pheochromocytoma cells) (Ahnert-Hilger *et al.*, 1989*a*; McInnes & Dolly, 1990) and bovine adrenal chromaffin cells (Bittner & Holz 1988; Ahnert-Hilger *et al.*, 1989*c*; Stecher *et al.*, 1989*a*,*b*).

Furthermore, using SLO-permeabilized adrenal chromaffin cells, PC12 cells and mammalian neurosecretory nerve terminals, it has been demonstrated that inhibition of exocytosis by Tetx and Botx A is increased in the presence of a reducing agent which causes chain separation (Ahnert-Hilger *et al.*, 1989*a*; Stecher *et al.*, 1989*a*; Dayanithi *et al.*, 1990, 1992). Finally, the light chains of either Botx A or Tetx alone were found to inhibit Ca^{2+}-dependent release of catecholamines from adrenal chromaffin cells (Bittner *et al.*, 1989*a*,*b*; Stecher *et al.*, 1989*b*; Ahnert-Hilger *et al.*, 1989*c*), PC12 cells (McInnes & Dolly, 1990), and of vasopressin from nerve terminals (Dayanithi *et al.*, 1990, 1992).

Taken together, these studies suggest that clostridial neurotoxins block exocytosis at a common point in both neurons and endocrine cells. Accordingly, it was of interest to determine if exocytosis by exocrine cells is also sensitive to clostridial neurotoxins. To address this question, exocrine pancreatic acinar cells from rats were isolated and permeabilized with SLO. In the present study amylase release from SLO-permeabilized pancreatic acinar cells was investigated with respect to: (*a*) the ability of Ca^{2+} to stimulate amylase release, and (*b*) the effects of cyclic AMP (cAMP) and guanosine 5′-[γ-thio]triphosphate (GTP[S]) on Ca^{2+}-stimulated amylase release. Additionally, the influences of Botx A or of Tetx or its separated light and heavy chains on amylase release evoked by different stimuli was examined.

EXPERIMENTAL

Materials

Botx A, Tetx and the light and heavy chains of the latter were isolated and characterized as previously described (Weller *et al.*, 1989; Stecher *et al.*, 1989*b*), with modifications according to Sathyamoorthy & DasGupta (1985). SLO was kindly provided by S. Bhakdi, Institut für medizinische Mikrobiologie, Mainz, Germany. Special reagents were obtained from the following sources: cAMP, GTP[S] from Sigma (München, Germany); collagenase from Serva (Heidelberg, Germany); soybean trypsin inhibitor from Boehringer (Mannheim, Germany); Phadebas

Abbreviations used: Botx A, botulinum A toxin; cAMP, cyclic AMP; DTT, dithiothreitol; GTP[S], guanosine 5′-[γ-thio]triphosphate; h.u., haemolytic units; KG buffer, potassium glutamate buffer; NTA, nitrilotriacetic acid; SLO, streptolysin O; Tetx, tetanus toxin.

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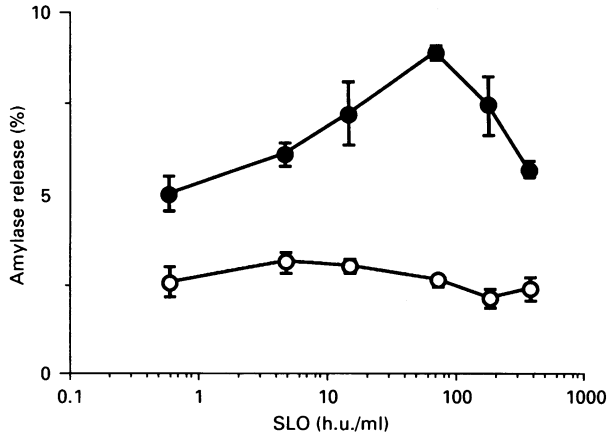

Fig. 1. Effects of different SLO concentrations on amylase release from pancreatic acinar cells

Pancreatic acinar cells were permeabilized with SLO. The permeabilization medium was discarded and replaced by KG buffer. After preincubation for 25 min at 25 °C, cells were centrifuged and challenged with KG buffer containing no Ca^{2+} (○) or 30 μM free Ca^{2+} (●) for 10 min at 25 °C. The amylase released into the supernatant and that remaining in the cells was determined as described (see the Experimental section). Values are means ± S.D. of three determinations.

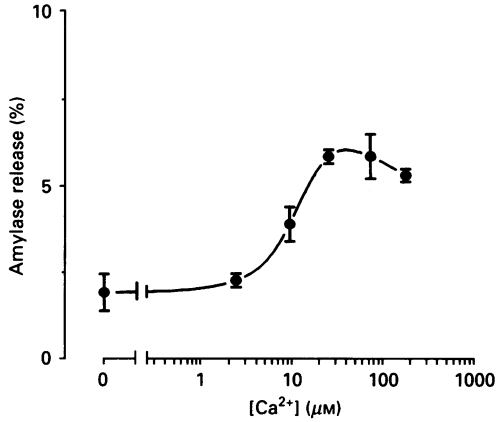

Fig. 2. Amylase release from SLO-permeabilized pancreatic acinar cells as a function of the free Ca^{2+} concentration

Cells were permeabilized with SLO (40 h.u./ml) in KG buffer. The permeabilization medium was replaced by KG buffer. After 25 min at 25 °C, the cells were centrifuged and challenged with KG buffer containing the free Ca^{2+} concentrations indicated. The amylase released within 10 min at 25 °C was measured (see the Experimental section) and is expressed as a percentage of total amylase content present in the cells prior to challenge with Ca^{2+}. Values are means ± S.D. of three determinations.

amylase assay kit from Pharmacia (Feiburg, Germany). All other chemicals were of analytical grade.

Isolation of exocrine pancreatic acinar cells

Pancreatic acinar cells were prepared as described previously (Amsterdam & Jamieson, 1972) with modifications (Streb & Schulz, 1983). Briefly, pancreatic tissue from two male Wistar rats (200 g) was chopped with scissors and digested for 15 min in an isolation medium (pH 7.4) containing 145 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-$MgCl_2$, 2 mM-$CaCl_2$, 10 mM-Hepes, 15 mM-glucose, 0.2% (w/v) BSA, 0.01% (v/v) soybean trypsin inhibitor and 150 units of collagenase/ml. Cells were continuously shaken and gassed with 100% O_2 in a 37 °C water bath, subsequently washed in fresh isolation medium (supplemented with 1 mM-EDTA while omitting collagenase and $CaCl_2$) and then incubated again in collagenase (210 units/ml) for 50–60 min. The cells were then mechanically dissociated by sequential passages through polypropylene pipettes of increasing tip diameter. Cell suspensions were centrifuged for 5 min at 85 *g*; the cell pellet was resuspended in fresh isolation medium, filtered through medical gauze, centrifuged again (85 *g*, 5 min) in isolation medium containing 8% (w/v) BSA and finally washed (three times) in fresh isolation medium.

Amylase release from permeabilized exocrine pancreatic acinar cells

Cell suspensions were washed twice with Locke buffer (pH 7.2) containing 140 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 0.5 mM-ascorbic acid, 15 mM-Pipes, 11 mM-glucose, and once with Ca^{2+}-free buffer (pH 7.2) containing 150 mM-potassium glutamate (KG), 10 mM-Pipes, 0.5 mM-EGTA and 5 mM-nitrilotriacetic acid (NTA). After centrifugation for 3 min at 40 *g*, cells were resuspended in 2 ml of KG buffer (pH 7.2) containing 150 mM-potassium glutamate, 10 mM-Pipes, 0.5 mM-EGTA, 5 mM-NTA, 2 mM-Mg^{2+}/ATP, 1 mM free Mg^{2+} and 0.1% (w/v) BSA. Permeabilization was achieved by incubating samples of pancreatic acinar cells with SLO in KG buffer containing 1 mM-dithiothreitol (DTT) for 5 min on ice, followed by centrifugation (1000 *g*, 30 s). The activity of SLO, determined using rabbit erythrocytes, is expressed in haemolytic units per ml (h.u./ml) (Lind *et al.*, 1987; Ahnert-Hilger *et al.*, 1989*b*). H.u. are indicated in the Figure legends. Permeabilization of the cells was controlled by determining Azur A dye exclusion, as previously described (Föhr *et al.*, 1989; Ahnert-Hilger *et al.*, 1989*b*).

The supernatant was removed and the cells were incubated with KG buffer containing DTT, supplemented or not with clostridial neurotoxins or their constituent chains. Following incubation for 25 min at 25 °C (water bath), cells were centrifuged for 30 s at 5000 *g*. Amylase release was stimulated by resuspending the cells in KG buffer supplemented with Ca^{2+}, Ca^{2+}+GTP[S] or Ca^{2+}+cAMP for 10 min at 25 °C. Subsequently, cell suspensions were centrifuged again and amylase content was determined in the supernatant and in the cell pellets after lysis with 0.2% (v/v) Triton X-100 using a commercially available assay (Phadebas amylase assay; Pharmacia). The amount of released amylase is expressed as a percentage of the total amylase content present in the cells before stimulation.

The free Ca^{2+} concentrations in the stimulation media were calculated using a computer program and monitored with a Ca^{2+}-selective minielectrode (Föhr *et al.*, 1992). The neutral carrier incorporated into a polyvinyl chloride membrane was kindly provided by W. Simon (ETH Zürich, Switzerland).

Culture of chromaffin cells

Chromaffin cells from bovine adrenal medullas were isolated by collagenase digestion as described previously (Livett, 1984), with modifications as outlined in Stecher *et al.* (1989*a*).

[^{3}H]Noradrenaline release from permeabilized adrenal chromaffin cells

Bovine adrenal chromaffin cells cultured for 3–6 days were preloaded with [^{3}H]noradrenaline and permeabilized as described previously (Stecher *et al.*, 1989*a*; Ahnert-Hilger *et al.*, 1989*b*, 1992). Briefly, the cells were permeabilized with SLO (240 h.u./ml) in KG buffer supplemented with 1 mM-DTT, for 2 min at 37 °C. The buffer was replaced with fresh buffer containing either no toxins or the neurotoxin preparation to be tested. After incubation for 25 min at 37 °C, the supernatant was removed

and the cells were stimulated for 10 min with KG buffer containing 30 μM free Ca^{2+}.

[^{3}H]Noradrenaline release was monitored by measuring the radioactivity present in the supernatants and in the cells after lysis with 0.2% (w/v) SDS. The amount of released [^{3}H]-noradrenaline is expressed as a percentage of the total radioactivity present in the cells before addition of Ca^{2+}.

RESULTS

Effects of SLO on amylase release from pancreatic acinar cells in the absence or presence of Ca^{2+}

After permeabilization with various concentrations of SLO (see the Experimental section), amylase release from freshly isolated rat pancreatic acinar cells was determined in the presence or absence of Ca^{2+}. SLO was used at concentrations ranging from 0.7 to 400 h.u./ml (Fig. 1). Ca^{2+}-dependent amylase release (with 30 μM-Ca^{2+}) was increased in a dose-dependent manner, reaching a maximum at 75 h.u. of SLO/ml (Fig. 1, closed symbols). The fraction of cells permeabilized under these conditions, as judged by Azur A staining, was $\geqslant$ 95%. At lower or higher concentrations of SLO, a smaller secretory response was observed. Determination of permeability by Azur A staining indicated that this response was due to either insufficient or too extensive permeabilization. Basal release in the absence of Ca^{2+} remained low with increasing SLO concentrations (Fig. 1, open symbols). The SLO concentration used in all subsequent experiments was that which facilitated the greatest difference between basal and Ca^{2+}-stimulated amylase release.

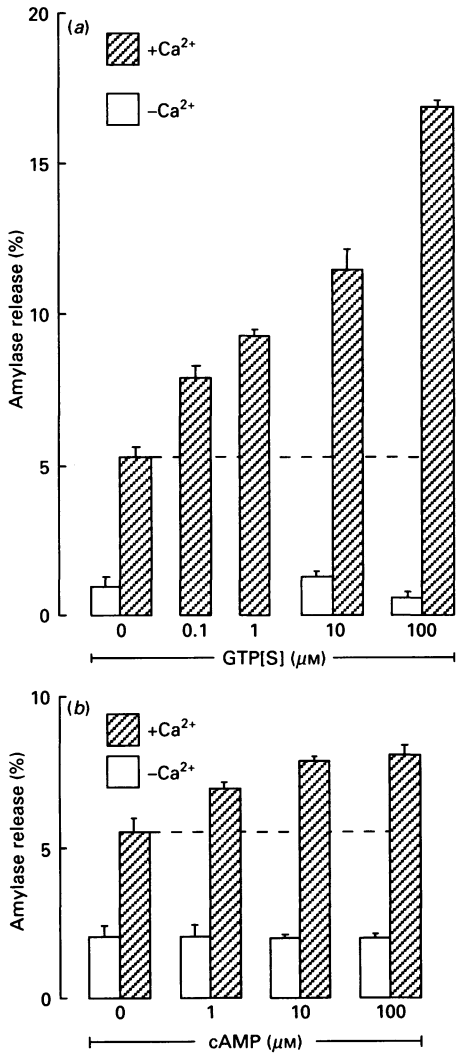

Fig. 3. Effects of GTP[S] and cAMP on Ca^{2+}-stimulated amylase release from SLO-permeabilized pancreatic acinar cells

Cells were permeabilized with SLO (40 h.u./ml) and subsequently incubated in KG buffer for 25 min at 25 °C. Cells were then incubated for 10 min at 25 °C with KG buffer containing the indicated concentrations of GTP[S] (Fig. 3*a*) or cAMP (Fig. 3*b*) in the presence or absence of 30 μM free Ca^{2+}. Values are means ± S.D. of three determinations.

Table 1. Ca^{2+}-stimulated amylase release from SLO-permeabilized pancreatic acinar cells is not affected by Botx A or Tetx

Freshly isolated pancreatic acinar cells were permeabilized with SLO as described in the legend to Fig. 3. Subsequently they were incubated for 25 min at 25 °C with KG buffer, or the two-chain form, the light chain (LC) or the heavy chain (HC) of Tetx (*a*), or the two-chain form of Botx A (*b*) at the concentrations as indicated. The buffer was replaced by fresh KG buffer either with or without 30 μM free Ca^{2+}. The amylase released after 10 min was determined (see the Experimental section). Values are means ± S.D. of three determinations.

Preincubation conditions	Amylase released (%)	
	$-Ca^{2+}$	$+Ca^{2+}$
(*a*)		
KG buffer	1.84 ± 0.30	8.01 ± 0.48
Tetx (1 μM)	2.48 ± 1.04	7.99 ± 1.65
Tetx LC (100 nM)	2.34 ± 0.14	7.79 ± 0.33
Tetx HC (100 nM)	–	7.80 ± 0.50
(*b*)		
KG buffer	1.49 ± 0.15	6.50 ± 0.29
Botx A (50 nM)	1.60 ± 0.30	6.55 ± 0.61

Ca^{2+}-dependence of amylase release from SLO-permeabilized pancreatic acinar cells

Fig. 2 shows that amylase release from SLO-permeabilized pancreatic acinar cells could be triggered by increasing the free Ca^{2+} concentration in the medium. The maximum amount of amylase released corresponded to 6–8% of the total cellular content. This response was obtained by stimulation with 30 μM free Ca^{2+}. The EC_{50} of Ca^{2+} for amylase release was approximately 8 μM free Ca^{2+} (Fig. 2).

Enhancement of Ca^{2+}-stimulated amylase release from SLO-permeabilized pancreatic acinar cells by GTP[S] and cAMP

Figs. 3(*a*) and 3(*b*) show the effect of GTP[S], a poorly hydrolysable GTP analogue, and of cAMP, on amylase release from SLO-permeabilized pancreatic acinar cells. GTP[S] between 0.1 and 100 μM markedly enhanced amylase release triggered by 30 μM free Ca^{2+} (Fig. 3*a*). Ca^{2+}-stimulated amylase release was increased more than 3-fold in the presence of 100 μM-GTP[S] (Fig. 3*a*). However, GTP[S] in the absence of Ca^{2+} did not evoke amylase release (Fig. 3*a*).

cAMP also increased Ca^{2+}-induced amylase release, but this substance was less efficacious than GTP[S], since 100 μM-cAMP increased Ca^{2+}-stimulated amylase release by only 45% (Fig. 3*b*). Again, cAMP in the absence of Ca^{2+} did not elicit amylase release (Fig. 3*b*).

Table 2. Tetanus toxin has no effect on Ca^{2+} + GTP[S] or Ca^{2+} + cAMP-stimulated amylase release from SLO-permeabilized pancreatic acinar cells

The procedure followed that described in Table 1. Cells were either incubated with KG buffer or exposed to amounts and forms of Tetx as indicated. Stimulation of amylase release was initiated by incubating the cells with 10 μM-GTP[S], 10 μM-GTP[S] + 30 μM free Ca^{2+}, 10 μM-cAMP or 10 μM-cAMP + 30 μM free Ca^{2+}. Values are means ± S.D. of three determinations. Abbreviation: LC, light chain.

Preincubation conditions	Amylase released (%) by:			
	GTP[S]	Ca^{2+} + GTP[S]	cAMP	Ca^{2+} + cAMP
KG buffer	3.40 ± 0.75	15.84 ± 0.95	2.31 ± 0.44	10.03 ± 0.98
Tetx (1 μM)	–	16.29 ± 1.58	–	10.17 ± 0.23
Tetx LC (1 μM)	–	14.64 ± 0.47	–	9.95 ± 1.16

Table 3. Tetx and Botx A incubated with pancreatic acinar cells retain their inhibitory activities on [^{3}H]noradrenaline release from adrenal chromaffin cells

Adrenal chromaffin cells, preloaded with [^{3}H]noradrenaline and permeabilized with 240 h.u. of SLO/ml, were incubated for 25 min at 37 °C with KG buffer, the two-chain form or the light chain (LC) of Tetx, or the two-chain form of Botx A. The toxins had first been included in the incubation medium recovered from SLO-permeabilized pancreatic acinar cells. This recovered medium (after dilution) was added to permeabilized adrenal chromaffin cells. Subsequently, the cells were challenged for 10 min with KG buffer containing either no Ca^{2+} or 30 μM free Ca^{2+}. The released [^{3}H]-noradrenaline was determined as described (see the Experimental section) and is given as a percentage of total [^{3}H]noradrenaline present in the cells before stimulation. Release in the absence of Ca^{2+} (approx. 4.5%) has been subtracted. Values are means ± S.D. of three determinations.

Preincubation conditions	Ca^{2+}-stimulated [^{3}H]noradrenaline release (%)
KG buffer	6.39 ± 0.25
Tetx (100 nM)	0.75 ± 0.20
Tetx LC (160 nM)	1.03 ± 0.50
Botx A (30 nM)	1.99 ± 0.50

Amylase release from SLO-permeabilized pancreatic acinar cells is not affected by clostridial neurotoxins

SLO-permeabilized pancreatic acinar cells were incubated with Tetx or Botx A for 25 min (see the Experimental section), in view of the observation that this period sufficed for these toxins to exert maximal inhibition of Ca^{2+}-dependent [^{3}H]noradrenaline release by adrenal chromaffin cells (Stecher *et al.*, 1989*a*,*b*; Ahnert-Hilger *et al.*, 1989*c*). Following preincubation with either toxin, the pancreatic acinar cells were exposed to Ca^{2+} alone or in combination with either GTP[S] or cAMP. Ca^{2+}-induced amylase release from SLO-permeabilized pancreatic acinar cells was not affected by the two-chain form of Tetx, its separate light and heavy chains (Table 1*a*), or the two-chain form of Botx A (Table 1*b*). Specifically, these cells, when incubated with the forms and concentrations of Tetx and Botx A described above, released the same amounts of amylase in response to 30 μM free Ca^{2+} as did the controls (preincubated with KG buffer). Moreover, basal release of amylase (in the absence of Ca^{2+}) also was unaffected by these toxins (Table 1). Likewise, amylase release triggered by 30 μM free Ca^{2+} plus 10 μM-GTP[S] or 30 μM free Ca^{2+} plus 10 μM-cAMP (Table 2) was not blocked by either Tetx or its light chain. Similar results were obtained with the two-chain form of Botx A (not shown).

Lack of inhibition of amylase release is not due to inactivation of clostridial neurotoxins

Besides amylase, proteolytic enzymes such as trypsin and chymotrypsin, which could impair the toxic activities of Tetx and Botx A, are released from pancreatic acinar cells. Therefore both neurotoxins included in media recovered following incubations with SLO-permeabilized pancreatic acinar cells were tested for their ability to inhibit Ca^{2+}-stimulated [^{3}H]noradrenaline release from SLO-permeabilized adrenal chromaffin cells. The recovered Botx A, Tetx and Tetx light chains, even in concentrations lower than those used in pancreatic acinar cells, were still very potent inhibitors of Ca^{2+}-stimulated [^{3}H]noradrenaline release from SLO-permeabilized adrenal chromaffin cells (Table 3). Compared with controls (preincubated with KG buffer), the inhibition of [^{3}H]noradrenaline release with the toxin concentrations used was 88% and 70% for the two-chain forms of Tetx and Botx A respectively, and 84% for the Tetx light chains (Table 3). The results are similar to data obtained following exposure of SLO-permeabilized adrenal chromaffin cells to 'fresh' toxins (Ahnert-Hilger *et al.*, 1989*c*; Stecher *et al.*, 1989*a*,*b*).

DISCUSSION

Previous reports disagree regarding the requirements for amylase release by permeabilized pancreatic acinar cells (Kimura *et al.*, 1986; Edwardson *et al.*, 1990; Kitagawa *et al.*, 1990). Accordingly, it was important to establish the requirements for amylase release from SLO-permeabilized pancreatic acinar cells before evaluating the effects of clostridial neurotoxins.

Current evidence implies that cytosolic Ca^{2+} is a prerequisite promoter of exocytosis by pancreatic acinar cells (cf. Williams *et al.*, 1989; Schulz, 1989). This is further emphasized by our present findings that 30 μM-Ca^{2+} elicited maximal amylase release from SLO-permeabilized pancreatic acinar cells. The exocytotic release of amylase from pancreatic acinar cells permeabilized with SLO, preincubated with test substances for 25 min and stimulated with Ca^{2+} for 10 min accounts for about 6–8% of total cellular amylase. This value is comparable with values of 5.0% and 4.8% obtained on stimulation for 10 min with the agonists carbachol (10 μM) and ceruletid (0.3 nM) respectively in non-permeabilized cells prepared in the same way (T. P. Kemmer, C. Längle & P. Halfertheiner, unpublished work).

The characteristics of the Ca^{2+}-dependent effects on amylase release revealed in the present study differ from those reported from other laboratories where electric discharge (Knight & Koh, 1984), saponin and digiton (Kimura *et al.*, 1986) or SLO (Edwardson *et al.*, 1990; Kitagawa *et al.*, 1990) were used for

permeabilization of pancreatic acinar cells. Thus maximal amylase release was reported following exposure of cells to 10 μM-Ca^{2+}, 1 mM-Ca^{2+} or 13 μM-Ca^{2+} respectively. The reasons for the observed differences in Ca^{2+} sensitivities in these studies are not obvious, but could relate to use of different permeabilization techniques and experimental protocols.

A role for cAMP as an intracellular messenger in amylase release was deduced from earlier data. In fact, cellular cAMP levels are augmented in intact pancreatic acinar cells in response to different secretagogues and concomitant with amylase release (cf. Williams *et al.*, 1989; Schulz, 1989). In this regard, Kimura *et al.* (1986) reported that 2 mM-cAMP or cAMP analogues were necessary to augment amylase release in the presence of Ca^{2+} from digitonin- or saponin-permeabilized rat pancreatic acinar cells. In contrast, cAMP had no effect on amylase release from electrically permeabilized acinar cells (Knight & Koh, 1984). We found that cAMP, at concentrations close to physiological values, acted as a positive modulator of Ca^{2+}-stimulated amylase release from SLO-permeabilized pancreatic acinar cells without affecting basal release.

We further examined the effect of GTP[S], a poorly hydrolysable GTP analogue, on amylase release. Effects of GTP[S] on exocytosis have been demonstrated in a variety of secretory cells, but these effects of GTP[S] are not consistent in these different cells. For example, activation of exocytosis by GTP[S] has been demonstrated in permeabilized neutrophils (Barrowman *et al.*, 1986), platelets (Haslam & Davidson, 1984), mast cells (Howell & Gomperts, 1987; Cockcroft *et al.*, 1987), a rat insulinoma cell line (Vallar *et al.*, 1987) and adrenal chromaffin cells (Bader *et al.*, 1989). Furthermore, inhibitory effects of GTP[S] on exocytosis have been reported in PC12 cells (Ahnert-Hilger *et al.*, 1987) and adrenal chromaffin cells (Knight & Baker, 1985).

Using the patch–clamp technique it has been reported that GTP[S] affects exocytosis from pancreatic acinar cells (Maruyama, 1988). Specifically, application of GTP[S] (50–100 μM) through the patch pipette potentiated the acetylcholine-evoked response, as measured by the enhancement of cell membrane capacitance. However, the exact amount of GTP[S] reaching the cell interior with this technique is not known. On the other hand, our protocol allows determination of dose–response relationships by the direct application of GTP[S] in permeabilized pancreatic acinar cells. Using this methodology we found that GTP[S] (between 0.1 and 100 μM) greatly enhanced the Ca^{2+}-stimulated amylase release from SLO-permeabilized pancreatic acinar cells, whereas Ca^{2+}-independent amylase release was unaffected.

Our data are consistent with data from other laboratories (Edwardson *et al.*, 1990; Kitagawa *et al.*, 1990) also using SLO-permeabilized pancreatic acinar cells, but it should be noted that the stimulatory effects of GTP[S] we observed were much greater. Specifically, our experiments demonstrated that 100 μM-GTP[S] augmented Ca^{2+}-stimulated amylase release 3–4 fold; in contrast, an enhancement of Ca^{2+}-stimulated amylase release of only 1.5-fold following the same concentration of GTP[S] was reported by Edwardson *et al.* (1990) and Kitagawa *et al.* (1990). The enhanced secretory responses observed in our study are probably due to differences in experimental protocols. Most important, it should be noted that Edwardson *et al.* (1990) and Kitagawa *et al.* (1990) added simultaneously SLO, the substances to be tested for effects on secretion (e.g. GTP[S]) and Ca^{2+}-containing media; in our protocol permeabilization, preincubation and stimulation were carried out successively, which allowed exact control of the cell interior throughout the experiment. In addition, buffer and temperature conditions were different in their protocol. Furthermore, in the protocol used by Kitagawa *et al.* (1990), a rapid loss of secretory ability was evident by 10 min. With our protocol, cells retain their secretory responsiveness to Ca^{2+}, cAMP and GTP[S] for more than 35 min, an important prerequisite for the analysis of the action of Tetx and Botx A, which require in endocrine and nerve cells 10 min or more (Stecher *et al.*, 1989*a*,*b*; Ahnert-Hilger *et al.*, 1989*c*).

The location of the GTP[S]-sensitive components within the permeabilized cells is not yet clear. Heterotrimeric GTP-binding proteins, involved in signal transduction at the plasma membrane which leads to inositol 1,4,5-trisphosphate production and increases in intracellular Ca^{2+} concentrations (Berridge, 1987), are certainly not active in permeabilized cells. Moreover, the Ca^{2+} chelators present in the incubation media imply that any Ca^{2+} fluctuations are unlikely. However, heterotrimeric as well as low-molecular-mass GTP-binding proteins, associated with secretory vesicles of nerve cells (Matsuoka & Dolly, 1990; Fischer v. Mollard *et al.*, 1990), chromaffin cells (Toutant *et al.*, 1987; Burgoyne & Morgan, 1989; Doucet *et al.*, 1989; Darchen *et al.*, 1990) and pancreatic acinar cells (Lambert *et al.*, 1990), could also be effective in amylase release from permeabilized pancreatic acinar cells.

The characteristics of amylase release from SLO-permeabilized pancreatic acinar cells provided a baseline for examination of the effects of clostridial neurotoxins. It should be recalled that permeabilization with SLO permits the access of large molecules to the cytoplasm. The data reveal that amylase release evoked by Ca^{2+}, Ca^{2+} + cAMP or Ca^{2+} + GTP[S] was not affected by Botx A, Tetx or Tetx light chains. It is notable in this context that the concentrations of Botx A and Tetx which failed to inhibit amylase release were 10–150-fold greater than concentrations which exerted a strong inhibition of [^{3}H]noradrenaline release from SLO-permeabilized adrenal chromaffin cells (Ahnert-Hilger *et al.*, 1989*a*,*c*; Stecher *et al.*, 1989*a*,*b*). The absence of toxic effects was not due to inactivation of Botx A and Tetx by enzymes released from pancreatic acinar cells because the toxins, following incubation with pancreatic acinar cells, still potently inhibited [^{3}H]noradrenaline release from SLO-permeabilized adrenal chromaffin cells.

Adrenal chromaffin cells, like other endocrine cells, derive from the neural crest, and therefore have the same ontogenetic origin as neuronal cells (Le Douarin & Fontaine-Pérus, 1990). Cells of the exocrine pancreas are of endodermal origin and thus lack this developmental relationship to neuronal cells.

Neurons, chromaffin cells and pancreatic acinar cells share the property of regulated release of cellular products by exocytosis. In contrast to neurons and chromaffin cells, pancreatic acinar cells are insensitive to a broad range of concentrations of intracellularly applied Tetx and Botx A. Given the present data, it is reasonable to infer that pancreatic acinar cells lack the intracellular target for Botx A and Tetx. Moreover, sensitivity of neurons and endocrine cells to clostridial neurotoxins provides another common marker for neural and endocrine cells and emphasizes their close ontogenetic relationship, in that they both express cytological markers such as neuron-specific enolase, synaptic vesicle membrane antigens and chromogranins/secretogranins. These markers are not detectable in exocrine cells.

We thank Mrs. C. Längle and Mrs. H. Bogenschütz for their help in isolating pancreatic acinar cells, Mrs. U. Fröhlich for excellent technical assistance and Mr. H. Traurig (Department of Anatomy and Neurobiology, University of Kentucky) for his constructive comments and his help in the preparation of this manuscript. This study was supported by the Deutsche Forschungsgemeinschaft (GR 681).

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Received 23 July 1991/18 November 1991; accepted 6 December 1991