Vesicle-associated Membrane Protein 2 Is Essential for cAMP-regulated Exocytosis in Rat Parotid Acinar Cells

THE INHIBITION OF cAMP-DEPENDENT AMYLASE RELEASE BY BOTULINUM NEUROTOXIN B*

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Amylase exocytosis of the parotid gland is mediated by intracellular cAMP. To investigate whether cAMP-dependent secretion has a mechanism similar to that of regulated neuroexocytosis, we examined the expression of synaptosome-associated proteins. In rat parotid acinar cells, we found 25 (p25) and 18 kDa (p18) proteins reacted with antibodies against Rab3A and vesicle-associated membrane protein 2 (VAMP-2), respectively. On the other hand, syntaxin 1 and SNAP-25, which interact with VAMP-2 at synapses, were undetectable. Rab3A-like p25 and VAMP-2-like p18 were also expressed in other exocrine acinar cells. The latter was localized at secretory granule membranes, and the former was detected in secretory granule and cytosolic fractions. The antibody against VAMP-2 used in this study did not react with cellubrevin, and p18 was cleaved with botulinum neurotoxin B. Thus, we identified p18 as VAMP-2.

Botulinum neurotoxin B inhibited the cAMP-induced amylase release from streptolysin O-permeabilized acinar cells. Therefore, VAMP-2 is required for cAMP-regulated amylase release in rat parotid acinar cells. This is the first report that VAMP-2 is involved in regulated exocytosis that is independent of Ca2+.

Some regulated exocytosis systems are mediated by intracellular cAMP without an elevation of cytosolic Ca2+. For example, the insulin release of pancreatic β-cells is induced mainly by glucose and also by hormones such as glucagon (1). Glucose-induced insulin release is mediated by an increase in cytosolic Ca2+, and the secretion induced by glucagon is mediated via a cAMP-dependent pathway without an increase in cytosolic Ca2+ (2). In sheep pituitary cells, gonadotropin-releasing hormone increases intracellular free Ca2+ and cAMP levels, both of which induce luteinizing-hormone secretion (3). Nevertheless, the maximal effect of cAMP is small compared with stimulation by Ca2+ (1, 3). Thus, Ca2+ is a dominant mediator of exocytosis in these systems. In contrast, amylase secretion of the parotid gland is mainly mediated by intracellular cAMP (4). Therefore, the parotid gland is convenient system with which to study the mechanism of cAMP-dependent exocytosis.

In the parotid gland, the stimulation of β-adrenergic recep-

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Materials—Streptolysin O and Hanks’ balanced salt solution were purchased from Life Technologies, Inc. Trypsin and soybean trypsin inhibitor were from Sigma. Collagenase and hyaluronidase were from Boehringer Mannheim. Botulinum neurotoxins were prepared principally according to the method as described previously (25). Mouse monoclonal antibodies against synaptotagmin I (mAb 1D12) (26, 27) and syntaxin1 (mAb 10H5) (28), and rabbit polyclonal antibodies against SNAP-25 (29), Rab3A, and VAMP-2 (synaptobrevin II) in rat parotid acinar cells. Moreover, we examined the effect of neurotoxins, which inhibit neurotransmitter release from neuronal cells (17, 22–24), upon amylase release from rat parotid acinar cells.

EXPERIMENTAL PROCEDURES

The abbreviations used are: VAMP, vesicle-associated membrane protein; BNT, botulinum neurotoxin; SLO, streptolysin O; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; v-SNARE, vesicle docked SNARE; t-SNARE, target membrane SNARE; GTPγS, guanosine 5′-O-3-thiotriphosphate.
Fig. 1. Immunoblot analysis of synaptosome-associated proteins in rat parotid acinar cells. The supernatants and pellets from PC12 and rat parotid acinar cells were fractionated electrophoretically on SDS-polyacrylamide gel electrophoresis gels, blotted, and probed with mouse monoclonal anti-synaptotagmin I (A), anti-synaptophysin (B), anti-syntaxin 1 (C), rabbit polyclonal anti-SNAP-25 (D), anti-Rab3A (E), and anti-VAMP-2 (F). Immunoreactive bands were visualized using an ECL kit (Amersham Corp.). The migration of molecular mass standards is shown on the left-hand side of each panel.

SECRETORY GRANULE MEMBRANES—From dispersed acinar cells, we prepared fractions of the subcellular fractions. The membranes were homogenized in ice-cold homogenizing buffer, composed of 250 mM sucrose, 20 mM Hepes-NaOH (pH 7.2), containing 1 mM EGTA, 0.1 mM MgCl2, and 0.5 mM dithiothreitol in the absence of 10 μM EDTA, respectively. The 100,000 g pellet from rat parotid acinar cells was incubated with activated or inactivated BNT-B at 37°C for 60 min. Thereafter, the samples were boiled with buffer containing SDS and used for Western blotting analysis using anti-VAMP-2 antibody.

Amylase Release from Streptolysin O-permeabilized Acinar Cells—The incubation medium was composed of 100 mM KCl, 19.2 mM NaCl, 25 mM HEPES-NaOH (pH 7.2), 1 mM EGTA, 0.1 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride, dispersed by eight strokes in a glass-glass homogenizer. The homogenate was centrifuged at 150,000 g for 10 min. The pellet was resuspended in the homogenizing buffer containing 40% Percoll and centrifuged at 20,000 g for 20 min. Secretory granules were collected from the bottom of the tube, diluted with the homogenizing buffer, and centrifuged at 1,500 × g for 10 min. The resuspended in the homogenizing buffer containing 40% Percoll and centrifuged at 20,000 g for 20 min. Secretory granules were collected from the bottom of the tube, diluted with the homogenizing buffer, and centrifuged at 1,500 × g for 10 min. The resuspended in the homogenizing buffer containing 40% Percoll and centrifuged at 20,000 g for 20 min.

Amylase activity was determined with the ECL chemiluminescence reaction (Amersham Corp.).

RESULTS

Rat Parotid Acinar Cells Contain Vesicle-associated Membrane Protein-2-like p18 and Rab3A-like p25—The expression of the synaptosome-associated proteins in rat parotid acinar cells was compared to that in the catecholamine-secreting cell line, PC12. All proteins tested in this study were detected in PC12 cells: synaptotagmin I (65 kDa), synaptophysin (38 kDa), syntaxin 1 (35 kDa), SNAP-25 (25 kDa), Rab3A (25 kDa), and VAMP-2 (18 kDa). Rab3A was detected in both the supernatant and pellet fractions, at almost a 1:1 ratio (Fig. 1E), and other proteins were observed in the pellet fraction (Fig. 1, A–D and
The anti-syntaxin 1 antibody recognized two bands at a molecular mass of about 35 kDa that may correspond to syntaxins 1A and 1B (Fig. 1). In rat parotid acinar cells, anti-Rab3A and anti-VAMP-2 antibodies detected a protein band in the supernatant (Fig. 1E) and pellet fractions (Fig. 1F), respectively. The electrophoretic mobility of the protein bands detected with antibodies against Rab3A and VAMP-2 in rat parotid acinar cells was the same as those in PC12 cells (25 and 18 kDa, respectively). Synaptotagmin I, synaptophysin, syntaxin 1, and SNAP-25 were not detected in the rat parotid acinar cells under these conditions (Fig. 1A–D).

Rab3A-like p25 and VAMP-2-like p18 Are Expressed Commonly in Exocrine Glands—The 100,000 g supernatant and pellet fractions of exocrine gland acinar cells were prepared from various exocrine glands, including the parotid, submandibular lacrimal gland, and pancreas. Immunoblot analysis was performed using antibodies against Rab3A and VAMP-2. A Rab3A-like p25 was expressed in the submandibular and lacrimal acinar cells, but not in the pancreas (Fig. 2, top panel). A 25-kDa protein has been detected in the pancreas using the antibody against Rab3 (37). Because the antibody used in this study does not react with other Rab3 homologues, the Rab3 in the pancreas may be another isoform(s) such as Rab3B, C, or D.

The antibody against VAMP-2 reacted with an 18-kDa protein expressed by all the exocrine glands examined (Fig. 2, bottom panel). These results suggest that Rab3 and VAMP-2 have common roles in various exocrine glands. On the other hand, syntaxin 1, synaptotagmin I, synaptophysin, and SNAP-25 were not detected in the exocrine gland acinar cells under the same conditions (data not shown).

The Protein p18 Is Located at the Secretory Granule Membrane, and p25 Is in the Cytosol and Granule Membrane Fractions—To investigate the localization of Rab3A-like p25 and VAMP-2-like p18, we prepared nuclear-, mitochondrial-, microsomal-, and secretory granule membrane-rich and cytosol fractions of rat parotid acinar cells as described under "Experimental Procedures." Immunoblotting revealed that Rab3A was mainly localized in the cytosol fraction, and very little was detected in the granule membrane fraction (Fig. 3, top panel). About 99% of the Rab3A was detected in the cytosol, and less than 1% was localized at granule membranes. Rab3A was not detected in the other membrane fractions. In contrast, VAMP-2 is specifically localized at the granule membranes in rat parotid acinar cells (Fig. 3, bottom panel).

Botulinum Neurotoxin B Cleaved the p18—Neurotoxins are zinc endopeptidases that cleave synaptosome-associated proteins such as syntaxin 1 or SNAP-25. One of them, BNT-B, specifically cleaves VAMP-2. To ascertain whether or not p18 is VAMP-2, we cleaved the protein with BNT-B. Activated BNT-B was mixed with the 100,000 g pellet fraction of rat parotid acinar cells, and cleavage of the p18 in the pellet fraction was examined. The p18 band disappeared, and a novel 9-kDa band appeared (Fig. 3, lane 2). Thus, BNT-B cleaved p18. The 9-kDa proteins band may be the N-terminal fragment of the p18. This proteolytic activity was diminished in the presence of 10 mM EDTA because of the chelation of zinc ions (Fig. 3, lane 3). BNT-B also cleaves the VAMP homologue, cellubrevin (38). However, the antibody used in this experiment does not react with cellubrevin. Therefore, p18 was identified as VAMP-2.

Amylase Release from SLO-permeabilized Cells Was Inhibited by BNT-B—The BNT-B-induced cleavage of synaptic vesicle VAMP-2 is considered to be the molecular basis of the BNT-B-induced neuroexocytotic blockade. Thus, we examined the effects of BNT-B on amylase secretion from SLO-permeabilized parotid acinar cells. In rat parotid acinar cells, cAMP stimulated amylase secretion (Fig. 5, lane 2). A prior incubation with BNT-B did not affect the amount of basal amylase secretion in the absence of cAMP (Fig. 5, compare lanes 1 and 3), but reduced the amount of amylase released by stimulation with cAMP down to 48% of the level in the absence of BNT-B (Fig. 5, lanes 2 and 4). Under these conditions, BNT-B cleaved only about 50% of VAMP-2 in the permeabilized cells (data not shown), corresponding to an inhibition efficiency of 48%. These results showed that VAMP-2 is involved in the cAMP-dependent pathway. In contrast, Ca\(^{2+}\)-stimulated amylase secretion was not affected by BNT-B (data not shown).

Other neurotoxins, BNT-A and BNT-C1, reportedly cleave SNAP-25 and syntaxin 1, respectively (39). In rat parotid acinar cells, VAMP-2 was detected, but SNAP-25 and syntaxin were not. We then investigated the effects of these neurotoxins on the cAMP-dependent amylase secretion. Although BNT-B inhibited the cAMP-dependent secretion, neither BNT-A nor BNT-C1 affected the basal or signal-dependent secretion (Fig. 5B, lanes 5–8). This result supports the notion that SNAP-25 and syntaxin 1 were not detected in the rat parotid acinar cells.

**DISCUSSION**

This is the first report that VAMP-2 is involved in the regulated exocytosis that is independent of Ca\(^{2+}\) elevation. In other systems for example, that of neuronal cells and chromafin, VAMP-2 is considered to play an important role in Ca\(^{2+}\).
VAMP-2 Is Essential for cAMP-dependent Exocytosis

Among synaptosome-associated proteins, we also found Rab3A in rat parotid acinar cells. Rab3A was mainly detected in the cytosol fraction. The amount of the protein localized at secretory granule membranes was less than 1%. In neuronal cells, Rab3A is mainly located at synaptic vesicles (40, 41), and in this study, about half of the total Rab3A was found in the pellet fraction of PC12 cells. Rab3A-like GTP-binding protein is also located on the cytosolic face of zymogen granules in pancreatic exocrine cells (37). It is possible that cytosol-located Rab3A translocates to granule membranes in a signal-dependen-ent manner. In a preliminary experiment, however, we did not find such large scale translocation in rat parotid acinar cells (data not shown). We have not yet determined the involvement of Rab3A in amylase secretion in the parotid gland.

In neuronal cells, the hypothesis is proposed that synaptic vesicles dock to a target membrane through the interaction of vesicular and target membrane proteins referred to as SNAP receptors (SNAREs) (11, 12). VAMP-2 is a vesicle-locked SNARE (v-SNARE), and syntaxin1 and SNAP-25 are target membrane SNAREs (t-SNAREs). In rat parotid acinar cells, t-SNAREs were not detected, although the v-SNARE was detected at the secretory granules. Moreover, the proteases that cleave the t-SNAREs did not inhibit the amylase secretion from rat parotid acinar cells. It is suggested that VAMP-2 interacts with proteins other than syntaxin 1 and SNAP-25 in parotid acinar cells. These unidentified t-SNAREs may be subject to cAMP-dependent protein kinase in rat parotid acinar cells.

Identification of a putative target protein of VAMP-2 in the parotid gland will be useful for investigating cAMP-dependent membrane traffic in other systems.

The next issue is to determine the target of VAMP-2 in cAMP-dependent exocytosis. The putative target can be required only for the cAMP-dependent pathway or it is common to Ca2+-dependent and cAMP-dependent systems. Either notion is possible. A VAMP-binding protein, VAP-33, has been identified in the Aplysia central nervous system, and an antibody against it inhibited synaptic transmission (42). VAP-33 locates at plasma membranes, and may play an essential role in membrane fusion by interacting with VAMP-2. Its amino acid sequence has no homology with those of syntaxin and SNAP-25 (42). Therefore, a protein with a function homologous to that of VAP-33 may also function independently of syntaxin and SNAP-25 in the mammalian parotid gland.

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