Presence of a Complex Containing Vesicle-associated Membrane Protein 2 in Rat Parotid Acinar Cells and Its Disassembly upon Activation of cAMP-dependent Protein Kinase*

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Amylase release from parotid acinar cells is mainly induced by the accumulation of intracellular cAMP, presumably through the phosphorylation of substrates by cAMP-dependent protein kinase (PKA). However, the molecular mechanisms of this process are not clear. In a previous study (Fujita-Yoshigaki, J., Dohke, Y., Hara-Yokoyama, M., Kamata, Y., Kozaki, S., Furuyama, S., and Sugiya, H. (1996) J. Biol. Chem. 271, 13130–13134), we reported that vesicle-associated membrane protein 2 (VAMP2) is localized at the secretory granule membrane and is involved in cAMP-induced amylase secretion. To study the formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex containing VAMP2 in parotid acinar cells, we prepared rabbit polyclonal antibody against the peptide corresponding to Arg47-Asp64 of VAMP2 (anti-SER4256). The recognition site of anti-SER4256 overlaps the domain involved in binding target membrane SNAREs (t-SNARES). Then we examined the condition of VAMP2 by immunoprecipitation with anti-SER4256. VAMP2 was not included in the immunoprecipitate from solubilized granule membrane fraction under the control conditions, but incubation with cytosolic fraction and cAMP caused immunoprecipitation of VAMP2. The effect of cytosolic fraction and cAMP was reduced by addition of PKA inhibitor H89. Addition of both the catalytic subunit of PKA and the cytosolic fraction allowed immunoprecipitation of VAMP2, whereas the PKA catalytic subunit alone did not. These results suggest that (1) the t-SNARE binding region of VAMP2 is masked by some protein X and activation of PKA caused the dissociation of X from VAMP2; and (2) the effect of PKA is not direct phosphorylation of X, but works through phosphorylation of some other cytosolic protein.

In rat parotid acinar cells, stimulation of β-adrenergic receptors and the subsequent accumulation of cAMP induces the exocytosis of amylase (1). Although most regulated exocytosis systems are mediated by elevation of intracellular calcium ions, there are some exocytosis systems that are also regulated by intracellular cAMP. For example, glucagon-induced insulin release from pancreatic beta cells and luteinizing-hormone secretion from pituitary cells are mediated by cAMP (2, 3). In these systems, however, the amount of secretion induced by cAMP alone is less than that induced by calcium. In contrast, amylase secretion from parotid acinar cells is mainly regulated by intracellular cAMP without elevation of calcium. Therefore, this is an appropriate system in which to study the mechanism of cAMP-dependent exocytosis.

In calcium-dependent systems, several calcium-binding proteins such as synaptotagmin, synclillin, and calcium-dependent activator protein for secretion, were identified as candidates for the calcium sensor(s) that suppress exocytosis at low concentrations of cytosolic calcium ions and enhance by binding with calcium (4–7). In cAMP-regulated exocytic systems, activation of cAMP-dependent protein kinase (PKA)1 is thought to play an essential role in the process. During amylase secretion from parotid acinar cells, the activity of PKA was enhanced (1), and the catalytic subunit of PKA was shown to be sufficient to cause amylase secretion in permeabilized acinar cells (8). Therefore, it is likely that PKA phosphorylates a protein involved in exocytosis. Several proteins were reported to be phosphorylated upon β-adrenergic stimulation (9–11). However, it is not clear which phosphorylation is crucial for triggering cAMP-dependent exocytosis.

We previously reported (12) that vesicle-associated membrane protein 2 (VAMP2) is localized at the secretory granule membrane of rat parotid acinar cells and that it plays an important role in cAMP-dependent amylase secretion. However, target membrane SNAREs (t-SNAREs) such as syntaxin 1 or SNAP-25 were not detected in rat parotid acinar cells. In adipocytes, VAMP2 is also localized to vesicles that store the glucose transporter GLUT4, and functions cooperatively with syntaxin 4 in the translocation of GLUT4 to the cell surface (13, 14). Neutrophils and mast cells also express syntaxin 4 and VAMP2, and these are possibly involved in the exocytosis of secretory granules (15, 16). Aquaporin-2 is translocated from intracellular vesicles to the apical membrane upon production of cAMP induced by vasopressin in kidney collecting duct cells (17–19), in which VAMP2 and syntaxin 4 were detected at intracellular vesicles and the apical membrane, respectively (20, 21). Although many syntaxin isoforms were identified, only syntaxin 1 and 4 can associate with VAMP2 in vitro (22). The...
combination of VAMP2 and syntaxin4 is possibly a common pairing in regulated exocytosis and vesicular trafficking outside the neuron.

If these SNARE proteins are involved in cAMP-dependent amylase secretion, the formation of the SNARE complex may be regulated by phosphorylation by PKA. SNARE proteins are thought to function in the recognition and docking between vesicles and target membrane by interacting with each other. It has been reported that the interaction of recombinant syntaxin4, and SNAP-23 was modulated by phosphorylation of syntaxin4 by exogenous PKA (23). However, Foster et al. (23) showed that VAMP2 binding to syntaxin4 was not affected by PKA phosphorylation of syntaxin4.

In this study, we tried to identify the t-SNAREs that interact with VAMP2 in parotid acinar cells and to clarify the regulatory mechanism of VAMP2. To study the condition of VAMP2 in rat parotid acinar cells, we prepared antibodies against VAMP2, and immunoprecipitated VAMP2 using the antibody. Whether VAMP2 is immunoprecipitated or not may depend on the proteins bound to VAMP2.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Collagenase and the catalytic subunit of cAMP-dependent protein kinase were purchased from Roche Molecular Biochemicals GmbH. Trypsin, trypsin inhibitor, and control rabbit IgG were purchased from Sigma. Protein A-Sepharose 4FF was from Amersham Pharmacia Biotech. Peptides corresponding to the amino acid sequence of Ser-Pro-Pro-Pro-Pro-Asp^44 of VAMP2 were synthesized and conjugated to keyhole limpet hemocyanin. Anti-SER4253 and anti-SER4256 were raised against each conjugated peptide and purified using peptide-coupled affinity columns (Fig. 2A). Affinity-purified anti-syntaxin4 antibody was a kind gift from Dr. Amira Klip (The Hospital for Sick Children, Toronto).

Preparation of Cytosolic and Total Membrane Fractions—Parotid glands were minced and dispersed by trypsin and collagenase. Dispersed acinar cells were homogenized with 320 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 20 mM Hepes-NaOH, pH 7.5. The nuclear fraction was removed from the homogenate by centrifugation at 750 g for 10 min, and the post-nuclear supernatant was centrifuged at 100,000 g for 60 min. The resultant supernatant and pellet were used as the cytosolic and total membrane fractions, respectively.

Preparation of Secretory Granule Membranes—Secretory granules of the rat parotid glands were isolated by Percoll gradient centrifugation as described previously (24), with some modification. Homogenization was carried out in 300 mM sucrose, 30 mM MgCl2, 1 mM DTT, 1 mM benzamidine, 0.4 mM PMSF, and 20 mM Hepes-NaOH, pH 7.5. After centrifugation, the Percoll suspension was fractionated from the bottom into 20 tubes. Secretory granules were recovered in the densest fraction. The specific activity of amylase in the final granule fraction was usually 4.5-fold higher than that in the homogenate. Purified whole granules were suspended in buffer A (1 mM MgCl2, 1 mM DTT, 1 mM PMSF, and 20 mM Hepes-NaOH, pH 7.5) containing 4% Triton X-100, and incubated for 45 min. Unsolubilized materials were removed by centrifugation (20,000 × g for 20 min). The supernatants were dialyzed against buffer B containing 1% Triton X-100 overnight. After centrifugation at 20,000 × g for 20 min, the supernatants were obtained as solubilized membrane fractions.

Solubilization of Total Membrane and Secretory Granule Membrane—Total membrane and secretory granule membrane fractions were suspended in solubilizing buffer B (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 20 mM Hepes-NaOH, pH 7.5) containing 4% Triton X-100, and incubated for 45 min. Unsolubilized materials were removed by centrifugation (20,000 × g for 20 min). The supernatants were dialyzed against buffer B containing 1% Triton X-100 overnight. After centrifugation at 20,000 × g for 20 min, the supernatants were obtained as solubilized membrane fractions.

Immunoprecipitation of VAMP2—Solubilized total membrane fraction (200 μg) and solubilized granule membrane fraction (6 μg) were suspended in reaction buffer (150 mM NaCl, 20 mM NaF, 10 mM MgCl2, 2 mM ATP, 10 μg/ml leupeptin, 10 μg/ml antipain, 1 mM PMSF, 1 mM DTT, 1 mM EGTA, 1% Triton X-100, and 20 mM Hepes-NaOH, pH 7.5) in the absence or presence of cytosolic fraction (50 μg), and/or 0.2 mM cAMP, a catalytic subunit of PKA (6 units), and then incubated at 30 °C for 10 min. Samples were added to protein A-Sepharose 4FF conjugated with normal rabbit IgG, and were incubated at 4 °C for 60 min. After centrifugation, the supernatants were recovered and incubated with anti-SER4256 conjugated with protein A-Sepharose 4FF at 4 °C for 2 h. Then protein A-Sepharose was collected by centrifugation and boiled in sample buffer for SDS-polyacrylamide gel electrophoresis.

Western Blotting—The proteins were separated on 12 and 15% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blotted with anti-syntaxin4 (1:1,000) and anti-SER4253 (1:10), respectively. Immunoreactivity was determined by means of the ECL chemiluminescence reaction (Amersham Pharmacia Biotech).

RESULTS

Syntaxin 4 Was Detected in the Total Membrane Fractions but not in the Granule Membrane Fraction of Rat Parotid Acinar Cells—In our previous study (12), neither syntaxin 4 nor SNAP-25, which interact with VAMP2 in neuronal cells, were detected in rat parotid acinar cells. A candidate for the t-SNARE target of VAMP2 is syntaxin4 because it binds to VAMP2 in vitro (22) and is reported to interact with VAMP2 in adipocytes (25). To examine the expression of syntaxin4 in parotid acinar cells, cytosolic and total membrane fractions of rat parotid acinar cells were prepared by centrifugation. Similar fractions were prepared from skeletal muscle to serve as positive controls for immunoblotting (22). Western blotting analysis of these samples showed that 35-kDa protein, which reacts with anti-syntaxin4 antibody, is detected in pellets of both parotid acinar cells and skeletal muscle (Fig. 1A). Next, the intracellular localization was analyzed. Cytosol, total membrane, and granule membrane fractions from parotid acinar cells were prepared and used for Western blotting analysis. Syntaxin 4 was detected in the total membrane fraction, but not in the granule membrane fractions where the majority of the VAMP2 was detected (Fig. 1B). The difference in localization suggests that syntaxin4 and VAMP2 function as classical t-SNAREs and vesicle SNAREs, respectively, in rat parotid acinar cells. This is consistent with the previously reported result that botulinum neurotoxin C1 did not inhibit CAMP-dependent amylase secretion (12), because syntaxin4 does not have a cleavage site for botulinum neurotoxin C1.

Immunoprecipitation of VAMP2 from Solubilized Membrane Fractions—The total membrane fractions were solubilized with Triton X-100, and the extracts were used for immunoprecipitation using anti-SER4256. The immunoprecipitates were subjected to Western blotting analysis using anti-SER4253. VAMP2 was not detected in the precipitates from untreated extracts (Fig. 2B, lane 1). Because VAMP2 was present in the solubilized total membrane fraction, it seems likely that the epitope was masked by another protein bound to VAMP2. We attempted to find conditions under which VAMP2 could be made accessible to the
We used purified catalytic subunit of PKA in the experiment. The effect of cytosolic fraction and cAMP is due to the activation in cAMP-dependent, but not in calcium-dependent amylase consistent with our previous finding that VAMP2 is involved.

Calcium ion in reactions 4 and 5 is buffered at 10 mM, eliminating free calcium ions, whereas the concentration of calcium ion neither allowed VAMP2 precipitation itself (Fig. 3A, lane 5), nor enhanced the effect of cAMP (Fig. 3B, lane 5). PKA inhibitor H89 decreased the ability of cytosol to allow immunoprecipitation of VAMP2 (Fig. 4A, lane 4). In contrast, incubation with both cytosolic fraction and PKA subunit efficiently induced precipitation similar in extent to cytosolic fraction and cAMP (Fig. 4A, lanes 3 and 5). It was confirmed that the effect of cytosolic fraction and cAMP is at least partly due to the activity of PKA. Because addition of cytosolic fraction was necessary, a cytosolic factor other than PKA is also necessary for this reaction.

To determine whether this cytosolic factor is a protein or not, we treated cytosolic fraction with trypsin. Treatment with trypsin significantly decreased the ability of cytosol to allow immunoprecipitation (Fig. 4B, lane 3). Cytosolic fraction protected by trypsin inhibitor before incubation with trypsin retained the activity (Fig. 4B, lane 4). Therefore, the activity is attributable to some protein.

Another issue is the substrate of PKA. To determine whether the substrate is cytosolic factor(s) or granular membrane protein(s), we performed the phosphorylation reaction separately and mixed cytosolic and solubilized granule membrane fractions. After incubation of the cytosolic fraction with PKA at 30 °C for 10 min, H89 (final concentration, 10 μM) and the solubilized granule membrane fraction were added to the mixture and incubated at 30 °C for an additional 10 min. VAMP2 was precipitated under these conditions as efficiently (Fig. 4B, lane 5) as seen with the cytosolic and solubilized granule membrane fractions incubated with PKA in the same reaction (Fig. 4B, lane 2). Next, the solubilized granule membrane fraction was incubated with PKA at 30 °C for 10 min, following which H89 and the cytosolic fraction were added and incubated for an additional 10 min. In this case, the efficiency of precipitation was decreased (Fig. 4B, lane 6). Therefore, phosphorylation of some protein in the cytosolic fraction, but not in the granule membrane fraction, is sufficient for the immunoprecipitation of VAMP2.

However, the immunoprecipitation occurring due to the phosphorylation of the solubilized granule membrane fraction is not negligible (Fig. 4B, lane 6). This result may indicate that the inhibitory activity of H89 was incomplete. H89 did not completely inhibit the immunoprecipitation caused by incubation with cytosolic and solubilized granule membrane fractions and cAMP either (Fig. 3A, lane 5). Another possible explanation is that a small amount of the protein phosphorylated by PKA is also present in the granule membrane fraction. In that case, another cytosol-specific protein would also be required.
because incubation of the solubilized granule membrane and catalytic subunit of PKA without the cytosolic fraction induced no immunoprecipitation (Fig. 4A, lane 4). DISCUSSION

We demonstrated here that the putative t-SNARE binding region of VAMP2 is exposed in a cAMP-dependent manner, which causes immunoprecipitation of VAMP2 with antibody that binds to Arg^{27}-Asp^{64} of VAMP2 (anti-SER4256). The ability of anti-SER4256 to immunoprecipitate VAMP2 probably reflects the state of VAMP2 and its binding protein in the solubilized granule membrane fractions. When anti-SER4256 cannot immunoprecipitate VAMP2, it suggests that the epitope on VAMP2 is masked by some unidentified protein(s). Disruption of this binding allows VAMP2 to be immunoprecipitated. The epitope of the antibody is included within the region that is necessary for binding to t-SNAREs (27). Therefore, through the exposure of this region, VAMP2 gains the ability to interact with t-SNAREs. In our previous study, we have shown that VAMP2 plays an essential role in cAMP-dependent amylase secretion (12). However, it has not been clarified how cAMP regulates the function of VAMP2. The main function of cAMP is thought to be to activate PKA, but it is unknown what protein mediates between the activation of PKA and exocytosis. The exposure of the t-SNARE binding region of VAMP2 also took place through the activation of PKA. This is the first report of a biochemical function of PKA in cAMP-mediated exocytosis.

A postulated model of this regulatory mechanism is shown in Fig. 5. VAMP2 is possibly masked by some protein X under the control conditions. Upon stimulation, activated PKA phosphorylates some cytosolic protein Y, which subsequently removes X from VAMP2 directly or indirectly. Consequently, VAMP2 interacts with t-SNAREs. Although the t-SNARE proteins involved in amylase secretion have not been identified, syntaxin4 and SNAP-23 are promising candidates because of their VAMP2 binding activity (22, 28). The disassembly of the complex containing VAMP2 is probably the first step of cAMP-dependent exocytosis of amylase.

What proteins are X and Y? One candidate for X is t-SNARE.
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