β-Adrenergic-induced cytosolic redistribution of Rap1 in rat parotid acini: role in secretion

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D’Silva, Nisha J., Kerry L. J.acobson, Sabrina M. Ott, and Eileen L. Watson. β-Adrenergic-induced cytosolic redistribution of Rap1 in rat parotid acini: role in secretion. Am. J. Physiol. 274 (Cell Physiol. 43): C1667–C1673, 1998.—Rap1 has recently been identified on the secretory granule membrane and plasma membrane of rat parotid acinar cells (N. J. D’Silva, D. Dij ulio, C. B. Belton, K. L. J.acobson, and E. L. Watson. J. Histochem. Cytochem. 45: 965–973, 1997). In the present study, we examined the cellular redistribution of Rap1 following treatment of acini with isoproterenol (ISO), the β-adrenergic agonist, and determined the relationship between translocation and amylase release. In the presence of ISO, Rap1 translocated to the cytosol in a concentration- and time-dependent manner; this effect was not mimicked by the muscarinic agonist, carbachol. Translocation was maximal at 1 μM ISO and paralleled amylase release immediately after ISO stimulation. Rap1 translocation and amylase release were blocked by the β-adrenergic antagonist, propranolol, whereas okadaic acid, a downstream secretory inhibitor, significantly blocked amylase release but did not inhibit Rap1 redistribution. Results suggest that the translocation of Rap1 is causally related to secretion and that the role of Rap1 in secretion is at a site proximal to the exocytotic event.

Small GTP-binding protein; translocation; salivary gland; exocrine secretion; amylase release

In the rat parotid gland, protein secretion occurs via the regulated, constitutive, or paragranular pathways (39). In regulated secretion, secretory granules fuse with the plasma membrane and release secretory granule contents in response to cell stimulation. The actual fusion event (exocytosis) involves the fusion of the outer surface of the secretory granule with the inner surface of the plasma membrane and release of secretory granule contents. On the basis of results published by Gomperts (14), showing that guanosine 5′-O-(3-thiotriphosphate) can stimulate secretion when upstream signaling pathways are blocked, it has been suggested that downstream secretory events may be regulated by a GTP-binding protein, G_E. However, it is not clear whether G_E is a heterotrimeric or monomeric (small) GTP-binding protein (Smg) or represents more than one GTP-binding protein. The identification of Smgs on secretory granules of exocrine (9) and other secretory cells (7, 26) supports a role for these proteins in exocytosis.

Several Smgs have been implicated in exocytosis. For example, Rab3 proteins have been associated with regulated exocytosis in neuronal and neuroendocrine cells (7, 11, 12). Another Smg, Rap1, has also been linked to secretion in human neutrophils (26). Rap proteins are members of the Ras subfamily of the Ras superfamily of GTP-binding proteins (4). These Rap proteins have 50% homology to Ras oncproteins and are found in nearly all tissues but differ from Ras in cellular and subcellular localization (4, 24, 26). Rap proteins are divided into two subgroups, Rap1 and Rap2, each of which has two subtypes: A and B (4). Evidence in yeast suggests that the secretory role of Rap1 is at the exocytotic step (27). In neutrophils, the identification of Rap1 primarily on the specific granules suggested that it plays a crucial role in phagocytosis, an exocytotic event that involves degranulation of the specific and azurophilic granules (26). This hypothesis was supported by studies that showed that, on stimulation of the Ca2⁺-dependent pathway, Rap1A translocated from the secretory granules to the plasma membrane with cytochrome b, a component of the nicotinamide adenine dinucleotide phosphate oxidase system (26, 31). The recent identification of Rap1 on secretory granules of rat parotid acinar cells (9) also suggests that it plays a role in secretion in a manner analogous to that postulated for Rap1A in neutrophils (26) and Rap1B in platelets (10, 28).

In general, Smgs regulate vesicular transport by cycling between membrane-bound and soluble forms, depending on the GTP- vs. GDP-bound state or modifications such as phosphorylation or isoprenylation (13, 15, 20). Translocation of a Smg in association with secretion has been used as evidence for its involvement in the latter (26), with the underlying assumption that proteins regulate secretion by shuttling between donor and acceptor compartments (39). For example, in nerve terminals, Rab3A and Rab3C dissociate from synaptic vesicles on Ca2⁺-dependent exocytosis (11, 12). This dissociation occurs during or after the exocytotic event and is dependent on the latter.

In the present study, we explored the secretory function of Rap1 by examining the translocation of Rap1 in rat parotid acini in response to isoproterenol (ISO) and its relationship to amylase release. Results show that ISO causes the redistribution of Rap1 from the particulate to the cytosolic fraction in a concentration- and time-dependent manner and that this event is coincident with amylase release. Data also show that both ISO-induced translocation and amylase release are blocked by the β-adrenergic receptor antagonist, propranolol, whereas okadaic acid, a downstream inhibitor of secretion, inhibits amylase release without an effect on translocation. Results suggest that Rap1 is involved in secretion but at a site that is proximal to the exocytotic event. Furthermore, data suggest that CAMP-mediated phosphorylation is important for the translocation of Rap1.
METHODS

Preparation of rat parotid acini. Rat parotid glands were isolated from male Sprague-Dawley rats (100–120 g), and parotid acini were prepared as described previously (9).

Preparation of rat parotid crude membrane fraction and cytosol. Rat parotid acini were homogenized with a glass Teflon pestle tube with a 0.012- to 0.014-in. clearance (Kon- tes, Vineland, NJ), and 250 g supernatant and pellet fractions were obtained as described by Robinovitch et al. (33). The cytosol was prepared from the 250 g supernatant, which was centrifuged at 12,000 g for 10 min and then followed by a centrifugation at 100,000 g for 1 h. The resultant supernatant was collected as the cytosolic fraction, and the pellet was discarded. For preparation of the crude membrane fraction, rat parotid acini were homogenized and centrifuged at 12,000 g for 10 min. The resultant pellet, which selected for organelles of high buoyant density including secretory granules, was designated the crude membrane fraction. Proteins were quantified using the Folin method of Hartree (16) with BSA as a standard.

Translocation studies. For these studies, cell aliquots were treated with an agonist in the presence or absence of an antagonist. Control samples were treated with distilled water and/or the appropriate vehicle of suspension for the antagonist. In all experiments, the cytosolic fraction was prepared from control and stimulated cells.

Amylase release. After treatment of parotid acini with the appropriate pharmacological agent, cell aliquots were retrieved for amylase determinations according to the method of Bernfeld (3). One-milliliter samples were centrifuged at 14,000 rpm for 30 s in a microcentrifuge. Amylase determinations were made for both the supernatant and pellet fractions. Amylase released into the supernatant was expressed as percent of total amylase, as described by Ito et al. (18). Data are expressed as means ± SE.

Immunochemiluminescent analysis. The cytosolic fractions were resolved by SDS-12% PAGE and transferred to 0.2-µm polyvinylidene difluoride (PVDF) (Novex, Inc, San Diego, CA) or 0.1-µm Immobilon PVDF filters (Millipore) filters. For most of the studies, Novex PVDF filters were used. However, Immobilon filters, because of their smaller pore size, were utilized in the latter studies. The filters were blotted with rabbit anti-Rap1 affinity-purified polyclonal antibody, and antibody binding was detected with the enhanced chemiluminescence Western blotting detection system (Amersham, Arlington Heights, IL) using affinity-purified horseradish peroxidase-linked donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as a secondary antibody.

Data analysis. Densitometric analysis of the chemilumograms was done using Molecular Dynamics' ImageQuant software. Several chemilumograms at different exposure durations were obtained for each experiment. The optical density obtained for the Rap1 signal on every chemilumogram was plotted against time. Only data that were in the linear range of the film were accepted for further analysis. The average optical density for the linear exposures was calculated, and data from treated samples were expressed as percent of the corresponding control. Statistical analysis was done by a one-tailed Student’s t-test. A one-tailed Student’s t-test was used because a cytotoxic increase in Rap1 was predicted, based on results from platelets (10). Data are presented as means ± SE unless otherwise indicated.

RESULTS

In the rat parotid gland, significant amylase release has been shown to occur on stimulation of the β-adrenergic receptor acting via the cAMP-protein kinase A (PKA) pathway (32). Hence, in initial experiments, the translocation of Rap1 to the cytosol following stimulation of parotid acini with ISO was determined. Rap1 was found to translocate to the cytosol when rat parotid acinar cells were stimulated with 1 µM ISO for 20 min (Fig. 1A), a time frame in which a significant amount of amylase release occurs in both glands and dispersed acini (Fig. 1A) (18, 25). The increase in cytosolic Rap1 was 189 ± 44% (n = 4) of the corresponding control (Fig. 1A) and corresponds to a decrease in Rap1 in the membrane fraction (Fig. 1B). In
contrast to the results obtained with ISO, there was no significant change in cytosolic Rap1 in cells treated for 20 min with the muscarinic receptor agonist, carbachol (50 μM) (Fig. 1A). Increasing the Ca²⁺ concentration in the incubation buffer from 0.2 to 1.28 mM had no effect on Rap1 translocation to the cytosol (data not shown).

In other studies, a concentration response to ISO at 20 min showed that maximal translocation to the cytosol was observed in rat parotid acinar cells stimulated with 1 μM ISO (Fig. 2). Occasionally (e.g., Fig. 2), in ISO-stimulated cells, cytosolic Rap1 resolved as a doublet and both signals increased in intensity. This doublet was thought to represent phosphorylated or unphosphorylated forms of Rap1 (20) or be due to the presence (24 kDa) or absence (22 kDa) of a reducing agent during Rap1 solubilization (6, 8, 30). The doublet was likely a function of incomplete reduction because the Rap1 antibody used in these studies did not predictably detect the low-molecular-mass Rap1 signal in cytosol samples solubilized in the absence of a reducing agent (data not shown). Translocation of Rap1 to the cytosol paralleled amylase release; maximal amylase secretion was also observed at a concentration of 1 μM ISO (Fig. 2) (1, 2).

On the basis of these findings, further studies were conducted to determine the time course of ISO-induced translocation of Rap1. The first time point taken was at 30 s because this was the earliest time at which samples could be collected. As shown in Fig. 3, translocation of Rap1 to the cytosol was time dependent and occurred as early as 30 s after stimulation with ISO. Translocation appeared to be maximal at 20 min and decreased slightly thereafter, consistent with the shutting or recycling phenomenon of Smgs, described by Wagner and Williams (40). Amylase release, on the other hand, increased up to 40 min, the last time period monitored. It was also noted that there was a slight increase in the density of the Rap1 signal in control samples with time, which is likely due to basal secretion previously noted by Spearman et al. (34).

To further explore the involvement of Rap1 in amylase secretion, the redistribution of Rap1 was investigated when amylase release was blocked with propranolol, a β-adrenergic antagonist that competitively binds the β-adrenergic receptor, thereby inhibiting secretion by acting upstream in the secretory cascade. Rat parotid acini were preincubated with 10 μM propranolol or distilled water for 2 min followed by a 15-min incubation with 1 μM ISO. At 15 min, sufficient amylase was released, and the cytosolic redistribution of Rap1 was readily detectable (see Fig. 3). As shown in Fig. 4, ISO-induced translocation of Rap1 to the cytosol was 167 ± 24% (n = 3) of the control and was competitively inhibited by 10 μM propranolol. ISO-induced amylase secretion, which was 159% greater than control, was also completely inhibited in the presence of 10 μM propranolol.

Because results were consistent with a role for Rap1 in secretion, the next step was to investigate a possible site of action, i.e., downstream vs. upstream, in the secretory cascade. We rationalized that the best approach for determining whether Rap1 is directly involved in exocytosis would be to try to dissociate the translocation of Rap1 to the cytosol from the exocytotic event. Rat parotid acini were treated with 1 μM ISO in the presence or absence of okadaic acid, a phosphatase inhibitor that also inhibits secretion (35, 41). Because okadaic acid was found to inhibit cAMP-induced amylase release in rat parotid acini (35), Wagner et al. (41) suggested that it acts downstream in the secretory pathway, i.e., distal to cAMP-PKA. Okadaic acid at 10 and 1 μM significantly inhibited (70 and 50%, respectively) amylase release from ISO-treated rat parotid acini (Fig. 5) (35) and was, therefore, used as a tool to inhibit secretion. It was predicted that, if Rap1 directly mediates exocytosis, then its translocation would be blocked when secretion is inhibited downstream in the secretory pathway, i.e., proximal to the exocytotic event. This was based on findings that the translocation of a Smg from the secretory vesicle during or immediately following the exocytotic event is supportive of its direct role in exocytosis (11, 12). For these studies, rat parotid acini were treated with 1 μM ISO for 15 min in the presence of 10 or 1 μM okadaic acid or ethanol (control). Studies were done at both concentrations of okadaic acid to test whether effects on translocation would also be concentration dependent. Densitometric quantification of the immunoblot data (Fig. 5) showed that Rap1 translocation was not inhibited by either 10 or 1 μM okadaic acid, thereby indicating that Rap1 translocates to the cytosol even when secretion is inhibited. The
increase in cytosolic density of Rap1, expressed as percent of control, was 179 ± 11% with 1 µM ISO, 172 ± 3% with 10 µM okadaic acid + ISO, and 168 ± 10% with 1 µM okadaic acid + ISO.

Because 0.5–1 µM okadaic acid may fragment subcellular organelles after a 30-min incubation (42), cell viability and plasma membrane intactness were assessed by microscopic examination of trypan blue exclusion at the end of the ISO incubation period; >95% of the acini examined excluded the dye.

**DISCUSSION**

The parotid gland is an exocrine gland with two major signaling pathways. The inositol 1,4,5-triphosphate-Ca\(^{2+}\) pathway primarily mediates fluid and electrolyte release, whereas proteins destined for secretion are released primarily via the \(\beta\)-adrenergic-cAMP pathway (32). The localization and high levels of Rap1 on the secretory granule membrane suggested that it has a role in protein secretion in the parotid gland (9). The major findings of this study are that Rap1 is redistributed to the cytosol in rat parotid acini stimulated with the \(\beta\)-adrenergic agonist, ISO, and that Rap1 appears not to have a direct involvement in the exocytotic process. However, because secretion represents a series of sequential steps, Rap1 may play a direct role in secretion at a step proximal to the exocytotic site. We used translocation as an indicator of the role of Rap1 in secretion because Smgs have been shown to translocate in association with the latter (12, 26, 28). Our results confirm and extend the findings for a role for Rap1 in secretion in hematopoietic cells to the parotid gland. A close parallel was established between ISO-induced translocation of Rap1 and amylase release from both rat parotid tissue slices and dispersed acini, reported here and previously (18, 25).

To further explore the relationship between ISO-induced translocation and amylase release (exocytosis), we sought to separate the two events. We investigated this by inhibiting secretion with propranolol and okadaic acid (35), proximal and distal secretory inhibitors, respectively. Studies with propranolol clearly showed that translocation is coincident with amylase release. However, studies with okadaic acid showed that translocation occurred even when amylase release was sig-
significantly blocked (70%). It was unlikely that the ~30% of secretion that was not inhibited was correlated with Rap1 translocation. As shown in Fig. 2, amylase release and translocation are closely correlated (r = 0.953; critical value is 0.874 at the 1% level of significance), i.e., translocation does not appear to be an all or none phenomenon. Thus results suggest that Rap1 has a role in secretion proximal to the exocytotic event but distal to cAMP-PKA (35, 41). Its function may precede or be parallel to the okadaic acid-sensitive step. Yamashina (43) characterized the effects of okadaic acid in the rat parotid gland and recently reported that 0.5–1 µM okadaic acid disrupted the lamellated structure of Golgi, replacing it with vesicles within 10 min of exposure. This disruption was rapidly reversed on withdrawal of okadaic acid. In the studies shown here, Golgi disruption would not have affected interpretation of the translocation data because particulate material was removed during isolation of the cytosol fraction.

The mechanism by which ISO induces Rap1 translocation was not established. However, because ISO acts via the cAMP pathway, a likely possibility is that cAMP-mediated phosphorylation is involved, as has been found in human platelets (28). A direct role for Ca2+ in mediating Rap1 translocation was ruled out by demonstrating that carbachol, at a concentration (50 µM) that significantly increases free intracellular Ca2+ levels and is optimum for amylase release from rat parotid acini (38), failed to affect the cytosolic translocation of Rap1. However, as suggested previously (6), Rap1 itself may play a role in Ca2+ regulation.

Given that the present data suggest a role for Rap1 upstream in the secretory pathway, an important question relates to the potential targets for Rap1. In platelets, studies suggest that Rap1 plays a role in the regulation of phospholipase C (PLC) and metabolism of inositol phospholipids (21, 22). In these cells, thrombin is a potent agonist that acts via the PLC-γ1-inositol 1,4,5-triphosphate-Ca2+ pathway to mediate the release of α-granule contents (21). Torti and Lapetina (37) suggested that during platelet activation Rap1, which binds Ras GTPase-activating protein (RasGAP), recruits the RasGAP-PLCγ1 complex to the plasma membrane, thereby leading to PLCγ1-induced hydrolysis of membrane lipids and release of Ca2+, a signaling cascade that culminates in secretion. Activation of the cAMP pathway, on the other hand, inhibits secretion in thrombin-stimulated platelets (21) and human erythrocytes (22), which have characteristics similar to platelets. Phosphorylation of Rap1B via cAMP appears to uncouple the thrombin receptor from PLC by causing the translocation of Rap1 away from the membrane to the cytosol (37), thereby preventing the formation of the Rap1B-RasGAP-PLCγ1 complex at the plasma membrane. In addition, Rap1 has also been shown to regulate Ca2+ fluxes in platelets (6). In the presence of GTP, phosphorylated Rap1 increases Ca2+ uptake and intracellular Ca2+ concentration. The role of Rap1 in Ca2+ regulation is further supported by data showing a correlation between the expression of Rap1B and the 97-kDa sarco(endo)plasmic reticulum Ca2+-ATPase in platelets, hematopoietic cells, and some cancer lines (24). As suggested by Magnier et al. (24), regulation of Ca2+-ATPases may not be limited to platelets but might be a more general process representative of other cell types.

Rap1 has also been found to be associated with cytoskeletal assembly in human platelets (10). In the rat parotid gland, the presence of a cell web in proximity to the apical plasma membrane (17), where exocytosis occurs, suggests that cytoskeletal reorganization (29) and the accompanying amylase release in response to cell stimulation play a significant role in secretion, either by facilitating the movement of secretory granules or actually regulating the exocytotic event or both.

In summary, the studies shown here are significant in that a relationship between Rap1 and secretion, hitherto unexplored in exocrine glands, is established in the parotid gland. The unequivocal localization of Rap1 on the rat parotid secretory granule (9) and its time- and concentration-dependent translocation to the cytosol in ISO-stimulated cells in correlation with amylase release, as well as inhibition of this redistribution by propranolol and okadaic acid, suggest that this
protein has a role in secretion that is distal to PKA stimulation and proximal to the exocytotic event. This secretory role may be via cytoskeletal interactions or via Ca\(^{2+}\) regulation and cross talk between the cAMP- and inositol 1,4,5-trisphosphate-Ca\(^{2+}\)-dependent pathways.

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