Many secretory cells utilize a GTP-dependent pathway, in addition to the well characterized Ca\(^{2+}\)-dependent pathway, to trigger exocytic secretion. However, little is currently known about the mechanism by which this may occur. Here we show the key signaling pathway that mediates GTP-dependent exocytosis. Incubation of permeabilized PC12 cells with soluble RalA GTPase, but not RhoA or Rab3A GTPases, strongly inhibited GTP-dependent exocytosis. A Ral-binding fragment from Sec5, a component of the exocyst complex, showed a similar inhibition. Point mutations in both RalA (RalA\(^{E38R}\)) and the Sec5 (Sec5\(^{T11A}\)) fragment, which abolish RalA-Sec5 interaction also abolished the inhibition of GTP-dependent exocytosis. Moreover, transfection with wild-type RalA, but not RalA\(^{E38R}\) enhanced GTP-dependent exocytosis. In contrast the RalA and the Sec5 fragment showed no inhibition of Ca\(^{2+}\)-dependent exocytosis, but cleavage of a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein by Botulinum neurotoxin blocked both GTP- and Ca\(^{2+}\)-dependent exocytosis. Our results indicate that the interaction between RalA and the exocyst complex (containing Sec5) is essential for GTP-dependent exocytosis. Furthermore, GTP- and Ca\(^{2+}\)-dependent exocytosis use different sensors and effectors for triggering exocytosis whereas their final fusion steps are both SNARE-dependent.

Sustained activation of one or more GTP-binding proteins by nonhydrolyzable GTP analogues (GppNHp\(^{1}\) and GTP-S) induces exocytosis secretion without elevating intracellular Ca\(^{2+}\). This has been observed in many different secretory cell types, including mast cells (1, 2), chromaffin cells (3, 4), melanotrophs (5), pituitary gonadotrophs (6), insulin-secreting \(\beta\) cells (7), and platelets (8, 9). Although this phenomenon is well established, the underlying mechanisms are poorly understood.

Many monomeric and trimeric GTPases (GTP/GDP-binding proteins) exist in mammalian cells. In particular, the Rho, Rab, and Ral families of low molecular weight proteins, which are monomeric GTPases, have been suggested as regulators of exocytosis. Rho family proteins have been proposed to regulate exocytosis in mast cells (10), because Rho GDP dissociation inhibitor (RhoGDI) (11) has been shown to inhibit GTP\(\gamma\)-S-induced exocytosis in mast cells (12). RhoGDI is known to inhibit GDP/GTP exchange in Rho family proteins including Rho, Cdc42, and Rac (11, 13, 14). The Rab family of proteins, particularly Rab3A, has long been a focus of exocytosis studies (15–17). Rab3A, a neuron-specific isoform localized on the secretory vesicles (18), is considered the mammalian orthologue of Sec4p GTPase in yeast (19). Sec4p is essential for exocytosis in yeast and the exocyst complex consisting of eight proteins (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70, Exo84) (20, 21) was identified as an effector for Sec4p (22). In mammalian cells including neurons, a similar protein complex designated as the mammalian exocyst complex (also called Sec6-Sec8 complex), was identified (23). However, the mammalian exocyst complex is not able to interact with Rab3A, but rather interacts with RalA and RalB (24–26), suggesting that Ral GTPase may regulate exocytosis. RalA and RalB physically bind to Sec3, a component of the exocyst complex, in a GTP-dependent manner (25–27). In addition, Ral is localized on dense granules in platelets (28) and synaptic vesicles in neurons (29), supporting a possible function of Ral in exocytosis in these cells.

In yeast, exocyst components are essential for secretion (20, 21). Functional analysis of exocyst proteins in higher organisms including Drosophila has only been recently initiated (30, 31). The mammalian exocyst is localized at the plasma membrane of nerve terminals in neurons (23). In differentiated PC12 cells, the exocyst was found in a punctate distribution at terminals of cell processes, or at or near sites of granule exocytosis (32). These results suggest the involvement of the mammalian exocyst in exocytic processes, although its exact function in exocytosis is not yet understood.

In this study, we sought to identify a key GTPase and its effector(s) that mediate GTP-dependent exocytosis using neuroendocrine PC12 cells. Permeabilized PC12 cells have proven to be a powerful model for the molecular dissection of exocytosis (33–41). It was previously shown that permeabilized, cytosol-depleted PC12 cells are capable of secretion in response to nonhydrolyzable GTP analogues (42, 43). Here we show that RalA-exocyst is the key sensor-effector for GTP-dependent exocytosis in PC12 cells. Because PC12 cells show robust Ca\(^{2+}\)-dependent secretion, we also sought to explain the relationship between GTP-dependent exocytosis and Ca\(^{2+}\)-dependent exocytosis. We suggest that GTP-dependent exocytosis and Ca\(^{2+}\)-dependent exocytosis use different sensors and effectors for triggering exocytosis.

**EXPERIMENTAL PROCEDURES**

**PC12 Secretion Assay**—The secretion assay followed the protocols of previously published work (33, 36, 37, 43). Briefly PC12 cells were labeled with \(^{3}H\)norepinephrine (NE, PerkinElmer Life Sciences or...
Amersham Biosciences) in the presence of 0.5 mM ascorbic acid. After washes, the cells were harvested in KGlub buffer (20 mM HEPES, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, and 2 mM EGTA) with 0.1% bovine serum albumin (BSA), permeabilized with a ball homogenizer (33, 36) or with freeze-and-thaw (37, 43), and incubated for 1–3 h on ice in the presence of 10 mM EDTA to extract the cytotoxic proteins. (Similar results were obtained from both methods of permeabilization, as previously shown, Ref. 43.) Permeabilized PC12 cells were washed three times with KGlub buffer containing 0.1% BSA. GTP-dependent NE secretion assays were done in KGlub buffer with 0.08% BSA containing various concentrations of GppNHp (trisodium salt, Sigma) (typically 100 μM), unless otherwise indicated) and recombinant proteins for 20–35 min at 30 °C. Ca2+–dependent secretion assays were done in KGlub buffer containing 0.1% BSA, 2 mM MgATP, 1 mM MgCl2, 1.72 mM CaCl2 in KGlub buffer, which contains 2 mM EDTA, produced 1–3 μM [Ca2+]i (36). Secretion was terminated by chilling to 0 °C, and samples were centrifuged at 4 °C for 5 min. Supernatants were removed, and the pellets were solubilized in 1% Triton X-100 for liquid scintillation counting. In the *Botulinum neurotoxin type E (BoNT/E–LC* experiments, permeabilized PC12 cells were washed once with KGlub buffer with 0.1% BSA, incubated with 100 mM BovNT–LC for 8 min at 30 °C (36) and then washed twice with KGlub buffer with 0.1% BSA before inducing secretion. Concentrations of free Mg2+ in the presence of EDTA were calculated based on Ref. 44, using a program written by Dr. Reinhard Jahn (Calbiochem). 

**Immunocytochemistry and Confocal Laser-scanning Microscopy**—PC12 cells transfected with pCMV9E101myc–RalA<sup>WT</sup> or pCMV9E10myc–Ral<sup>Δ315</sup> were grown on polylysine-coated glass coverslips, washed loaded with either GDP, GTPγS, or GppNHp, fixed for 1 h at room temperature in PBS containing 0.3% bovine serum albumin. Primary antibodies against Mye (mouse 9E10 ascites diluted 1:1000, Covance) and secretogranin II (rabbit polyclonal antisemum diluted 1:1000, GED Biosciences) were diluted in blocking buffer and applied to the permeabilized cells for 1 h at room temperature. Following three washes in blocking buffer, goat fluorescein-conjugated anti mouse (diluted 1:1000) and goat rhodamine red-x-conjugated anti-rabbit antibodies (diluted 1:5000) (Jackson ImmunoResearch Laboratories) were diluted in blocking buffer and applied for 1 h at room temperature. Samples were washed three times in blocking buffer and mounted in FluorSave<sup>TM</sup> reagent (Calbiochem). Immunofluorescence staining was monitored with a Zeiss laser confocal scanning microscope (LSM 410) with a water immersion objective lens (×63).

**RESULTS**

**Pharmacological Characterization of GppNHp-induced Exocytosis**—We first confirmed a previous finding that GppNHp together with Mg2+ induces NE secretion from permeabilized PC12 cells (43). As previously reported (43), the dose–response curve was bell-shaped, and with high concentrations of Mg-GppNHp, NE release was suppressed (Fig. 1A). To clarify the relative role of Mg2+ for this suppression, we tested GppNHp-induced NE release in the absence of Mg2+. Low concentrations (0.1–10 μM) of GppNHp and Mg-GppNHp induced similar levels of exocytosis. However, at high concentrations (100 μM to 1 mM), GppNHp stimulated increased levels of exocytosis relative to Mg-GppNHp (Fig. 1A). This result suggests that suppression at high doses is primarily because of high concentrations of Mg2+. To further validate our findings we also examined the effects of Mg2+ concentrations on GppNHp-induced exocytosis by varying the concentration of free Mg2+ ([Mg2+]free) independent of GppNHp by using a Mg2+/EDTA buffer. When Mg2+ was chelated by 1.8 mM EDTA (0 μM [Mg2+]free), GppNHp-induced secretion was completely abolished, suggesting an essential role for Mg2+ in GTP-dependent exocytosis. In contrast 0.7 μM [Mg2+]free rescued GppNHp-induced exocytosis (Fig. 1B). However, at 6–45 μM [Mg2+]free, GppNHp-induced exocytosis was less efficient than at 0.7 μM [Mg2+]free (Fig. 1B). Thus, we conclude that while submicromolar to micromolar [Mg2+]free is required for GppNHp-induced exocytosis, more than 10 μM [Mg2+]free inhibits GppNHp-induced exocytosis.

The complex action of Mg2+ on GppNHp-induced exocytosis is reminiscent of the interaction between Mg2+ and GTPases. Thus, we hypothesized that GppNHp-induced exocytosis is mediated by activated GTPases and that the inhibition of exocytosis by high concentrations of Mg2+ is due to the stabilizing effects of Mg2+ on the nucleotide-bound form of GTPases (48, 49). After permeabilization and washes, GTPases in PC12 cells are mostly in a GDP-bound form and Mg2+ prevents GppNHp from binding to the target GTPase by drastically decreasing the off-rate of GDP (48, 49). Therefore, we predicted that GppNHp-induced exocytosis would be inhibited by increased concentrations of GDP. Indeed, GDP inhibited GppNHp (100 μM)-induced exocytosis in a concentration-dependent manner (Fig. 1B).
squares), 6 μM [Mg\(^{2+}\)]\(_{\text{free}}\) (open diamonds), and 45 μM [Mg\(^{2+}\)]\(_{\text{free}}\) (closed diamonds). Error bars indicate S.E. (n = 5). C, PC12 cells stimulated by 100 μM GppNHp in the presence of indicated concentrations of GDP. In each experiment the average of GppNHp-induced NE release without GDP was set to 100%, and the average of background NE release without GppNHp was set to 0%. Error bar indicates S.E. (n = 11).

**Inhibition of GTP-dependent Exocytosis by Soluble RalA, but Not RhoA or Rab3A**—We sought to identify the GTPase involved in GppNHp-induced exocytosis. Three small GTPases were considered as possible candidates: RhoA, Rab3A, and RalA. We hypothesized that if one of these GTPase proteins was responsible for GTP-dependent exocytosis, it would inhibit exocytosis by functioning as a dominant negative protein. This is a realistic possibility because the recombinant proteins generated in E. coli lack prenylation for proper membrane localization. On incubation of permeabilized PC12 cells with soluble recombinant proteins, we found that the strong inhibition was mediated by recombinant RalA, but not by RhoA or Rab3A (Fig. 2B). This effect was not caused by variable purities among GST fusion proteins as determined by SDS-PAGE and Coomassie Blue staining (Fig. 2A). Strong inhibition by RalA was not caused by competition for binding of GppNHp to native GTPases (buffering effect) because the concentration of recombinant proteins used in Fig. 2B was ~1 μM, 100 times lower than the concentrations of GppNHp (100 μM). A dose-response curve indicated that the inhibition by RalA became evident at concentrations of 0.1 μM (Fig. 2C). Furthermore, higher concentrations of GppNHp (e.g., 1 mM) did not reverse the inhibition by RalA (Fig. 2D), thus further arguing against a potential buffering of available GppNHp. Therefore, we reasoned that recombinant RalA inhibits exocytosis as a dominant negative by binding to the target of endogenous GTPase(s).

**A Ral Binding Fragment from Sec5, but Not RhoGDI, Inhibits GTP-dependent Exocytosis**—The exocyst has been recently identified as an effector complex for Ral (24–26). Ral physically binds to Sec5, one of the eight proteins comprising the exocyst complex, in a GTP-dependent manner. We hypothesized that if the exocyst functions as the effector for Ral in GTP-dependent exocytosis, a fragment of Sec5 should inhibit exocytosis by preventing endogenous Ral from binding to the exocyst complex. Indeed we found that this Sec5 fragment inhibited GTP-dependent exocytosis in a concentration-dependent manner (Fig. 3, A and B). We also examined the effects of RhoGDI (11), which inhibits signaling pathways mediated by Rho family proteins, including Rho, Rac, and Cdc42 (11, 13, 14), on exocytosis. If Rho family proteins are critical for GTP-dependent exocytosis, it is expected that RhoGDI will inhibit the exocytosis. In our preparation, we found no inhibition by RhoGDI (Fig. 3A). Thus, the RalA-exocyst complex, which contains Sec5, and not the Rho signaling pathway, is important for GTP-dependent exocytosis. However, maximal inhibition by soluble RalA and the Sec5 fragment was ~70% of GTP-dependent exocytosis. Thus our results do not exclude the possibility that another GTPase may mediate GppNHp-induced exocytosis.
We found that wild-type RalA bound to the exocyst in a GTP-dependent manner, whereas binding of RalAE38R to the exocyst was at background levels in the presence or absence of GppNHp or GDP (Fig. 4B). Thus, the mutated residue in RalAE38R is sufficient to abolish the interaction between these two recombinant proteins, as measured by isothermal titration calorimetry (27). We expressed both mutant RalAE38R and wild-type RalA as GST fusion proteins and showed that the purity of these two fusion proteins was similar (Fig. 4A). Then, we examined recombinant RalA binding to the exocyst complex in brain homogenates using a GST pull-down assay in which the presence of the exocyst was examined with antibodies against Sec5 and Sec8, two major components of the exocyst complex. We found that wild-type RalA bound to the exocyst in a GTP-dependent manner, whereas binding of RalAE38R to the exocyst was at background levels in the presence of 6.25 μM recombinant GST, GST-Sec5 (residues 2–120), and GST-RhoGDI. Error bars indicate S.E. (n = 6). GST-Sec5 significantly inhibited GTP-dependent exocytosis compared with GST alone; t_{0.05} = 26.2, p < 0.0001, indicated by *.

**FIG. 3.** The Sec5 fragment (residues 2–120) inhibits GTP-dependent exocytosis. A, permeabilized PC12 cells were stimulated with 100 μM GppNHp in the absence or presence of 6.25 μM recombinant GST, GST-Sec5 (residues 2–120), and GST-RhoGDI. Error bars indicate S.E. (n = 6). GST-Sec5T11A inhibited GTP-dependent exocytosis compared with GST alone; t_{0.05} = 26.2, p < 0.0001, indicated by *.

**FIG. 4.** The specificity of the inhibition by RalA and the Sec5 fragment as demonstrated by site-directed mutagenesis. A, analysis of purified GST fusion Sec5T11A mutant, RalA-WT, and RalAE38R mutant (~15–25 μg) by SDS-PAGE and Coomassie Blue staining. B, pull-down experiments from brain homogenate using recombinant GST-RalA proteins loaded with GDP or GppNHp. C, pull-down experiments from brain homogenate loaded with GDFβ5 or GppNHp using recombinant GST-Sec5 proteins. D, permeabilized PC12 cells stimulated with GppNHp in the absence or presence of 0.5 μM GST-RalA (wild-type, mutant) or 3.75 μM GST-Sec5-(2–120) (wild-type, mutant). Error bar indicates S.E. (n = 8).

**Point Mutations in RalA (RalAE38R) and the Sec5 (Sec5T11A) Fragment, Which Abolish RalA-Sec5 Interaction, also Abolish the Inhibition of GTP-dependent Exocytosis**—We next examined the specificity of the inhibition by soluble RalA protein and the Sec5 fragment. A recent structural study of the RalA-Sec5 interaction identified potentially important residues for the interaction (27). Point mutants for these residues (RalAE38R, Sec5T11A) abolished the interaction between these two recombinant proteins, as measured by isothermal titration calorimetry (27). We expressed both mutant RalAE38R and wild-type RalA as GST fusion proteins and showed that the purity of these two fusion proteins was similar (Fig. 4A). Then, we examined recombinant RalA binding to the exocyst complex in brain homogenates using a GST pull-down assay in which the presence of the exocyst was examined with antibodies against Sec5 and Sec8, two major components of the exocyst complex. We found that wild-type RalA bound to the exocyst in a GTP-dependent manner, whereas binding of RalAE38R to the exocyst was at background levels in the presence or absence of GppNHp or GDP (Fig. 4B). Thus, the mutated residue in RalAE38R is sufficient to abolish the interactions with the native exocyst. Similarly, we expressed a mutant Sec5 fragment (Sec5T11A) (residues 2–120) as a GST fusion protein (Fig. 4A) and observed that this mutation abolished the GTP-dependent interaction between Sec5 and native RalA in the brain (Fig. 4C, upper panel). As a control we demonstrated that neither the wild-type nor the mutant Sec5 fragment bound Rab3A in brain homogenate (Fig. 4C, lower panel). Then we introduced these mutant proteins into permeabilized PC12 cells and examined whether they would mimic the inhibitory effects of recombinant wild-type RalA and the Sec5 fragment. These point mutations almost completely abolished the inhibition (Fig. 4D). Thus, the inhibition by recombinant wild-type RalA and the Sec5 fragment appears to be caused by their interactions with the native exocyst complex and RalA, respectively. Therefore, we conclude that the GTP-dependent interaction between RalA and the exocyst is essential for GTP-dependent exocytosis.

**Transfection with Wild-type RalA, but Not with RalAE38R, Enhances GTP-dependent Exocytosis**—To obtain independent evidence that RalA may function as a GTP sensor in GTP-dependent exocytosis, we conducted a co-transfection assay in which hGH functions as a marker of transfection. PC12 cells secrete transfected hGH when stimulated by KCl depolarization (46, 47). We found that similar to GppNHp-induced NE release, GppNHp triggers hGH secretion from permeabilized PC12 cells in a concentration-dependent manner. To test if RalA acts as a sensor for the GTP analogue in exocytosis, we co-transfected PC12 cells with hGH and wild-type RalA (RalA-WT), mutant RalA (RalAE38R) or a control plasmid. RalA-WT, and RalAE38R were expressed as Myc fusion proteins to facilitate their detection in PC12 cells (see below). Transfection with Myc-RalA-WT caused a major shift in the GppNHp response curve to lower GppNHp concentrations, making the cells responsive to 0.1 μM GppNHp. In addition, Myc-RalA-WT enhanced maximal secretion in response to GppNHp (Fig. 5A). Thus, transfected Myc-RalA-WT sensitized GppNHp-induced hGH secretion, indicating that RalA functions as a sensor for the GTP analogue in GTP-dependent exocytosis. In contrast, Myc-RalAE38R did not effect GppNHp-induced hGH secretion.

We verified that the lack of effect by RalAE38R was not caused by the mislocalization of this protein, because transfected Myc-RalA-WT and Myc-RalAE38R were both largely colocalized with Secretogranin II (SgII), a marker protein of secretory granules in PC12 cells (Fig. 5B). The localization of transfected Myc-RalAs on secretory granules is consistent with a previous finding of native Ral on dense granules in platelets (28). Therefore, we conclude that the RalA-exocyst interaction is essential for transfected RalA to act as a sensor for the GTP analogue in GTP-dependent exocytosis.

**GDPS, RalA, and the Sec5 Fragment Show No Inhibition of Ca^{2+}-dependent Exocytosis**—We also examined the relationship between GTP-dependent and Ca^{2+}-dependent exocytosis. In PC12 cells, both GppNHp and Ca^{2+} triggers NE release from
large dense core vesicles, although Ca^{2+}/H^{11001} with MgATP and cytosolic factors can induce a more robust exocytosis response (33, 36, 37, 38, 43). If the same pathway utilized in GTP-dependent exocytosis contributes to Ca^{2+}/H^{11001}-dependent exocytosis, it would be expected that GDP, Ral and the Sec5 fragment, all strong inhibitors of GTP-dependent exocytosis, will at least partially inhibit Ca^{2+}/H^{11001}-dependent exocytosis. However, we found that none of these agents inhibited Ca^{2+}-dependent exocytosis (Fig. 6, A and B). Thus we conclude that independent pathways mediate GTP-dependent and Ca^{2+}-dependent exocytosis. These results also emphasize the specificity of the inhi-
bition of GTP-dependent exocytosis by recombinant RaIA and Sec5 fragments.

Cleavage of Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptor (SNARE) Inhibits both GTP and Ca\(^{2+}\)-Dependent Exocytosis—The SNARE complex, consisting of SNAP-25, syntaxin, and synaptobrevin (also called VAMP) (50), is essential for Ca\(^{2+}\)-dependent exocytosis (reviewed in Ref. 51). We examined the role of the SNAREs in GTP-dependent exocytosis by utilizing BoNT/E-LC, which specifically cleaves SNAP-25. BoNT/E-LC almost completely blocked both Ca\(^{2+}\)- and GppNHp-induced exocytosis (Fig. 7), which is in agreement with previous results that used native SNARE-clearing toxins including BoNT/E (42). Thus, SNARE function is essential for both Ca\(^{2+}\)- and GTP-dependent exocytosis.

**DISCUSSION**

In this study, we have discovered the long sought after signaling pathway that mediates GTP-dependent exocytosis. In various mammalian cell types, sustained activation of one or more GTPases by nonhydrolyzable GTP induces exocytotic secretion (1–9). Therefore, the discovery of a mediating GTPase for this process is important for the cell biology of secretion. We showed that the interaction between RaIA GTPase and the exocyst is critical for GTP-dependent exocytosis. The involvement of other GTPases (Rab3A and Rho) in GTP-dependent exocytosis has been previously suggested (12, 15). The experiments using the Rab3A fragment (effector loop peptide) in mast cells (15) are now being questioned because the fragment induces Ca\(^{2+}\) transients in mast cells, raising the possibility that its mode of action is nonspecific (52). In our preparation, we did not observe inhibitory effects by recombinant Rab3A, RhoA, or RhoGDI on GTP-dependent exocytosis. Because RhoGDI inhibits GTP\(S\)-induced exocytosis in mast cells (12), further experiments are required to investigate whether the RaIA-exocyst interaction is critical for GTP-dependent exocytosis in other secretory cells such as mast cells. In addition, we do not exclude involvement of other GTPase signaling pathways in GTP-dependent exocytosis in PC12 cells, because the maximal inhibition exerted by soluble RaIA and the Sec5 fragment is ~70%.

In contrast to GTP-dependent exocytosis, we found no role for Ral-exocyst interaction in Ca\(^{2+}\)-dependent exocytosis. Based on this finding, we suggest that Ca\(^{2+}\)-dependent and GTP-dependent exocytosis utilize independent pathways to trigger exocytosis. However, this finding appears to contradict the results of Moskalenko et al. (25), which suggested that overexpression of RaIA inhibited high K\(^+\)-induced, Ca\(^{2+}\)-dependent exocytosis. Although their work suggests that RaIA is a negative regulator of Ca\(^{2+}\)-dependent exocytosis, it is important, however, to note that overexpression of other GTPases, including Rab3 (53) and Rab11b (54), exhibited even stronger inhibition of high K\(^+\)-induced exocytosis. Thus, we suggest that the observed inhibition of high K\(^+\)-induced exocytosis by several GTPases may lack specificity.

The exocyst complex was first characterized in yeast (20, 21). In yeast, exocyst components are essential for secretion and the entire complex functions as an effector for Sec4 GTPase. Little is currently known of the function of the exocyst complex in higher organisms. In *Drosophila*, mutations of Sec5, a central component of the exocyst complex, resulted in embryonic lethality and general defects in membrane trafficking (31). Interestingly, however, Ca\(^{2+}\)-dependent neurotransmitter release from synaptic vesicles persisted in this mutant (31). RNAi-mediated knock-down of Sec10 expression, another component of the exocyst, failed to show defects in neurotransmission, but knock-down of Sec10 expression did cause defects in hormonal secretion from endocrine cells (30). Our results suggest that the function of the exocyst is critical for GTP-dependent but not for Ca\(^{2+}\)-dependent exocytosis in neuroendocrine PC12 cells. In transgenic mice expressing a dominant inhibitory form of RaIA, Ca\(^{2+}\)-dependent glutamate release was normal, but refilling of the readily releasable pool was suppressed (29). Thus, the GTP-dependent interaction of RaIA and the exocyst may also contribute to modulation of the readily releasable pool of synaptic vesicles in neurons.

We found that both GTP-dependent exocytosis and Ca\(^{2+}\)-dependent exocytosis are SNARE-dependent. Even in the well studied Ca\(^{2+}\)-dependent exocytosis, how Ca\(^{2+}\)-sensors, such as synaptotagmins (38, 39, 41), transmit the signal to the SNAREs remains controversial. At least two scenarios may explain the communication between signaling sensors and the SNAREs. In the first scenario, either the Ral-exocyst or synaptotagmin-phospholipid/PIP\(_2\) physically interacts with the SNAREs to trigger the fusion (41). The identification of the mechanisms by which GTP-dependent Ral-exocyst interaction communicates with the SNAREs to trigger exocytosis/membrane fusion is an area for future study.

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