The Small GTPaseRalA Controls Exocytosis of Large Dense Core Secretory Granules by Interacting with ARF6-dependent Phospholipase D1*

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RalA and RalB constitute a family of highly similar Ras-related GTPases widely distributed in different tissues. Recently, active forms of Ral proteins have been shown to bind to the exocyst complex, implicating them in the regulation of cellular secretion. Since RalA is present on the plasma membrane in neuroendocrine chromaffin and PC12 cells, we investigated the potential role of RalA in calcium-regulated exocytotic secretion. We show here that endogenous RalA is activated during exocytosis. Expression of the constitutively active RalA (G23V) mutant enhances secretagogue-evoked secretion from PC12 cells. Conversely, expression of the constitutively inactive GDP-bound RalA (G26A) or silencing of the RalA gene by RNA interference led to a strong impairment of the exocytotic response. RalA was found to co-localize with phospholipase D1 (PLD1) at the plasma membrane in PC12 cells. We demonstrate that cell stimulation triggers a direct interaction between RalA and ARF6-activated PLD1. Moreover, reduction of endogenous RalA expression level interfered with the activation of PLD1 observed in secretagogue-stimulated cells. Finally, using various RalA mutants selectively impaired in their ability to activate downstream effectors, we show that PLD1 activation is essential for the activation of secretion by GTP-loaded RalA. Together, these results provide evidence that RalA is a positive regulator of calcium-evoked exocytosis of large dense core secretory granules and suggest that stimulation of PLD1 and consequent changes in plasma membrane phospholipid composition is the major function RalA undertakes in calcium-regulated exocytosis.

Ral proteins have the potential to be activated by many different extracellular signals. One of the best known Ral-activating pathways is via Ral-specific guanine nucleotide exchange factors that become activated by binding GTP-bound Ras in response to many types of upstream signals, including almost all tyrosine kinase receptors and several G-protein-linked receptors (3–5). However, Ras-independent mechanisms of Ral activation occur as well. For instance, in platelets and in fibroblasts, elevation of intracellular calcium levels directly induces Ral activation without a contribution of Ras (6). The small GTPase Rap has been also identified as a Ral activator under some conditions (7).

Ral interacts with several protein effectors through two protein-protein interaction sites. The first binds phospholipase D1 (PLD1) via an N-terminal 11-amino acid sequence. Ral weakly stimulates PLD1 activity but operates synergistically with small GTPases of the ARF family (8, 9). The second is an effector-binding loop, which mediates interaction with Rap-binding protein 1 (RalBP1, also known as RLIP76) and filamin. RalBP1 was the first Ral effector to be identified and was originally distinguished by its GTPase-activating protein domain, which has the potential to regulate Rac and Cdc42 GTPases negatively (10–12). Filamin is an actin cross-linking protein that mediates filopodia formation (13). More recently, Ral was found associated in a GTP-dependent manner with the mammalian exocyst (14–16), a multiprotein complex that functions in polarized cells in membrane delivery to specific domains of the plasma membrane (17–19).

Several evidence support the idea that Ral proteins are intimately linked to vesicular trafficking events at the plasma membrane. First, RalBP1 regulates recycling of epidermal growth factor and insulin receptors by interacting with epsin homology domain proteins involved in endocytosis (20–22). RalBP1 also binds to the AP2 adaptor complex (23), which plays a key role in clathrin-mediated endocytosis. Second, Ral has been shown to participate in receptor-mediated endocytosis through a process that involves PLD (24). Third, activated Ral proteins have been implicated in the targeting of Golgi-derived vesicles to the basolateral membrane in epithelial cells (15, 25). This possibility arose from the discovery that two subunits of the exocyst complex are downstream binding partners of active RalA and RalB (14–16, 26). In budding yeast, the exocyst directs targeting of secretory vesicles to sites of rapid membrane growth (17). Consistent with a function in membrane

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§ The abbreviations used are: PLD, phospholipase D; siRNA, small interference RNA; shRNA, small hairpin RNA; GH, growth hormone.
addition, the analogous mammalian complex has been found at the tight junctions of epithelial cells, where it has been implicated in basolateral secretion (15, 27) and in axonal growth cones of developing neurons (28). Thus, by promoting assembly of the exocyst complex (26), Ral proteins have been proposed to regulate exocyst-mediated vesicle delivery to appropriate fusion sites at the plasma membrane.

Release of hormones and neurotransmitters occurs through a specialized form of exocytosis that is tightly regulated in time and space by extracellular signals and cytosolic calcium levels. The presence of Ral proteins at high levels on synaptic vesicles (29, 30) and secretory granules in platelets (31) has led to the speculation that Ral may play a role in calcium-regulated exocytosis. Hence, a role for Ral in a regulatory aspect of neurotransmitter release has been identified through the analysis of neuronal tissue from transgenic mice expressing a dominant inhibitory form of RalA (32). Release of glutamate from isolated synaptic endings after depolarization was found normal in the transgenic mice, but protein kinase C-mediated enhancement of glutamate secretion was suppressed. This suggested the participation of Ral in some forms of synaptic plasticity linked to the recruitment of synaptic vesicles but not in neuronal exocytosis per se. In neuroendocrine PC12 cells, Ral proteins have been implicated in exocytosis by regulating the assembly of exocyst complexes (15). However, a recent report by Wang et al. (33) indicates that RalA plays its exocyst-mediated function in GTP-dependent but not in calcium-dependent exocytosis. Thus, the participation and precise function of Ral proteins in calcium-regulated secretion remains unclear.

In chromaffin cells, we previously reported the presence of RalA on the plasma membrane, whereas RalB resided on some intracellular vesicles that were distinct from catecholamine-containing secretory granules (34). This observation led us to further explore the role of RalA in the calcium-regulated exocytotic process. In the present paper, we show that RalA is a positive regulator of dense core granule exocytosis. Moreover, we obtained a series of results indicating that ARF6-regulated PLD1, present on the plasma membrane and implicated in the late stages of exocytosis (35, 36), participates in the downstream pathway by which RalA promotes calcium-regulated exocytosis.

MATERIALS AND METHODS

Plasmids and Small Interference RNA (siRNA)—RalA wild type and variants were as previously described (15) and were expressed in PC12 cells using pRK5. The sequence encoding the Ral-binding domain of RalBP1/RLIP76 (amino acids 403–499) was inserted as a BamHI-EcoRI cassette containing the H1-RNA promoter and the silencing sequence encoding both small hairpin RNAs (shRNAs) and growth hormone (GH), separated from its reverse 19-nucleotide complement by a short spacer (AAGGCAGGTTTCTGTAGAA) derived from the target transcript and searched against the gene data bank. For mammalian expression vectors preparations.

Antibodies and Immunofluorescence—The following antibodies were used: monoclonal anti-RalA and rabbit polyclonal anti-RalB (Transduction Laboratories, Lexington, KY); polyclonal anti-PLD1 raised in rabbits against the N-terminal domain of PLD1 (QCBI, BIOSOURCE International, France); monoclonal anti-SNAP-25 antibodies (Stemberger Monoclonals Inc., Lutherville, MD); monoclonal anti-HA antibodies (Babo, Richmond, CA); monoclonal anti-Myc antibodies (Novocrosta Laboratories); polyclonal anti-GH (Dr. A. F. Parlow, NIDDK, National Institutes of Health, Bethesda, MD); Alexa-488-anti-mouse and Alexa-555-anti-rabbit (Molecular Probes). Rabbit polyclonal anti-chromogranin A antibodies were prepared in our laboratory (41). For immunocytochemistry, chromaffin or PC12 cells on coated glass coverslips were fixed and immunostained as described previously (36). Stained cells were visualized with a Zeiss confocal microscope LSM 510. Using the Zeiss CLSM instrument software 3.2, the amount of RalA labeling was measured and expressed as the average fluorescence intensity multiplied by the corresponding surface area and divided by the total surface of each cell. This allows a quantitative cell-to-cell comparison of the RalA immunoactivity detected in cells.

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RESULTS

Intracellular Distribution of RalA in Resting and Stimulated Chromaffin and PC12 Cells—We investigated the intracellular distribution of native RalA in cultured chromaffin cells by immunofluorescence and confocal microscopy. As illustrated in Fig. 1, RalA was restricted to the cell periphery. Double labeling with antibodies against the plasma membrane marker SNAP25 or against the chromaffin granule protein chromogranin A (CGA) indicated its association with the plasma membrane but not with the membrane of secretory granules (Fig. 1A). Endogenous RalA was similarly present at the plasma membrane in the chromaffin cell tumor derivatives, PC12 cells (see Fig. 6A). Next, we compared the distribution of RalA in resting and secretagogue-stimulated cells. Stimulation of chromaffin cells with nicotine (Fig. 1B) or stimulation of PC12 cells with a depolarizing concentration of potassium (59 mM K\(^+\)) (Fig. 6A) reduced the level of peripheral RalA immunoreactivity by ~85% without apparently increasing it in the cytosol or in other intracellular compartments. One possible explanation for this observation is that the monoclonal antibody recognized an epitope that was masked in stimulated cells due to the formation of a RalA-protein complex.

Subcellular fractionation experiments were performed on chromaffin cell homogenates to separate the cytosol, the Triton X-100-soluble fraction representing the membrane-bound compartment, and the Triton X-100-insoluble fraction representing the cytoskeleton and some detergent-resistant lipid microdomains. RalA was primarily detected in the Triton X-100-soluble fraction (Fig. 1C), suggesting that its presence in the cell periphery was most likely due to a binding to the plasma membrane rather than to an association with the actin filaments concentrated in the subplasmalemmal region. The distribution of RalA in the fractions prepared from nicotine-stimulated cells remained largely unchanged except for a slight increase detected in the Triton X-100-insoluble fraction (Fig. 1C). Taken together, these results suggest that RalA resides at the plasma membrane in resting cells. Since the protein was hardly detectable in stimulated cells, we concluded that RalA might be engaged in a putative complex with a regulator/effecter protein formed in response to secretagogue-evoked stimulation.

Secretagogue-evoked Stimulation Activates RalA in PC12 Cells—To probe the role of RalA in the control of dense core granule exocytosis, we first investigated whether RalA might be activated in response to a secretagogue that triggers exocytosis. Therefore, PC12 cells were maintained under resting conditions or stimulated for various periods of time with 59 mM K\(^+\), and RalA activation was assessed using the Ral-binding domain ofRalBP1 (Myc-RBD) as a bait to trap the endogenous GTPase in its GTP-bound form (Fig. 2). We found that the amount of GTP-bound RalA is relatively low in resting cells. However, 2–10 min of stimulation with 59 mM K\(^+\) increased the level of cellular RalA-GTP by 3–10-fold, respectively (Fig. 2, A and B). Importantly, RalA-GTP rapidly decreased to basal levels as cells returned to the resting condition, revealing a tight coupling between membrane depolarization and RalA activation. Since the immediate consequence of membrane depolarization or stimulated for various periods of time with 59 mM K\(^+\), cells were then lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM MgCl\(_2\), 5 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and mammalian protease inhibitors) (Sigma). Lysates were clarified by centrifugation and Myc-tagged proteins were immunoprecipitated from the supernatant of each sample using agarose-coupled mouse anti-Myc IgG. Precipitated proteins were resolved on 12% polyacrylamide-SDS gels and immunoblotted with anti-Myc (1:500) and anti-RalA (1:1000) antibodies. Blots were processed using the “Western-Light Plus” chemiluminescent detection system (Tropix, Bedford, MA).

RalA Regulates Exocytosis of Large Dense Core Secretory Granules in PC12 Cells—To establish that RalA plays a role in exocytosis, we first examined the effect of expressing the Ral-binding domain of RalBP1 (Myc-RBD) in PC12 cells using
growth hormone as a reporter for exocytosis (35). This protein fragment specifically binds to activated Ral-GTP and thereby interferes through competitive association with the ability of endogenous Ral proteins to interact with and stimulate effector molecules. As shown in Fig. 3, expression of Myc-RBD did not affect cell viability or GH expression level as judged by the GH immunodetected on nitrocellulose or by the total cellular GH content measured by radioimmunoassay. However, expression of Myc-RBD strongly reduced the K+-evoked GH secretion (~60% inhibition), whereas expression of RalBP1 deleted of the RBD and unable to bind to GTP-loaded Ral was ineffective. These results suggest that interactions of endogenous Ral-GTP proteins with downstream effectors are required for exocytosis.

To directly address the role of RalA in PC12 cell exocytosis, we selectively inhibited native levels of RalA by taking advantage of the possibilities offered by the RNA interference process. RNA interference is based on short double-stranded RNA molecules called siRNAs that trigger specific silencing of gene expression in a sequence-specific manner (42, 43). siRNAs can be generated in mammalian cells using plasmids that direct expression in a sequence-specific manner (42, 43). siRNAs can be generated in mammalian cells using plasmids that direct expression in a sequence-specific manner (42, 43). siRNAs can be generated in mammalian cells using plasmids that direct expression in a sequence-specific manner (42, 43).

Fig. 2. GTP-loaded RalA pull-down assay in resting and stimulated PC12 cells. A, PC12 cells transfected with pRK-Myc-RBD were stimulated for the indicated periods of time with 59 mM K+ and/or maintained in Locke’s solution for 10 min. Cells were then immediately lysed by the addition of ice-cold lysis buffer, and the lysate was used for immunoprecipitation of Myc-RBD using an anti-Myc antibody. Pulled-down Myc-RBD and GTP-loaded RalA were analyzed by gel electrophoresis and Western blots. Top panel, Western blot performed with anti-Myc antibody, revealing the amount of Myc-RBD present in each sample. Lower panel, RalA-GTP levels co-precipitated with Myc-RBD and revealed with an anti-RalA antibody. B, semi-quantitative analysis of RalA activation upon cell stimulation. Values obtained by scanning densitometry analysis are expressed as arbitrary units (A.U.) and given as the mean of values obtained from three independent experiments performed on different cell preparations ± S.E.

Fig. 3. Expression of the Ral-binding domain (RBD) of RalBP1 inhibits GH secretion from PC12 cells. PC12 cells were transfected (4 μg/well of each plasmid) with either pRK (empty vector), pRK-Myc-RBD, or pRK-MycRalBP1RBD plasmids along with the plasmid (4 μg/well) encoding GH. A, 48 h after transfection, the expressed proteins were detected by Western blots using anti-Myc and anti-GH antibodies. B, transfected cells were washed and subsequently stimulated for 10 min with 59 mM K+ (closed bars) or incubated in calcium-free Locke's solution (open bars). Extracellular fluids were then collected, and GH present in solutions and in cells was estimated by radioimmunoassay. The total GH content per well was 5.81 ± 0.18 ng in control experiments with the empty plasmid, 5.94 ± 0.25 ng in cells transfected with pRK-Myc-RBD, and 5.73 ± 0.19 ng in cells transfected with pRK-MycRalBP1RBD. GH release is expressed as the percentage of total GH present in the cells before the 10-min stimulation period. Data are given as the mean values ± S.E. (n = 3). Similar results were obtained in three independent experiments performed with different cell cultures.

did it affect the distribution of GH in secretory granules (Fig. 4B). Selective knock down of RalA by siRNA also did not modify basal secretion, but it resulted in a significant decrease (~50% inhibition) in the amount of GH released in response to stimulation by 59 mM K+ (Fig. 4C). Overall, these findings are consistent with the idea that RalA plays a positive role in the exocytotic pathway of large dense core granules.

The implication of RalA in calcium-evoked exocytosis has been recently questioned based on the observation that wild-type RalA has no effect on calcium-dependent noradrenaline release when introduced into the cytosol of permeabilized PC12 cells (33). We therefore examined the effect of expressing wild-type RalA, the RalAG23V mutant defective in GTP hydrolysis and its corresponding dominant inactive mutant, RalA(G26A), preferentially binding GDP on GH secretion from PC12 cells. Consistent with the results of Wang et al. (33), overexpression of wild-type RalA did not modify GH secretion in response to high potassium (Fig. 5). However, expression of the constitutively active RalA(G23V) mutant increased GH release, whereas the dominant negative RalA(G26A) significantly decreased it (Fig. 5). These results emphasize the positive control that RalA is able to exert in calcium-regulated exocytosis.
RalA Binds to ARF6-activated PLD1 in Stimulated PC12 Cells—Because Ral proteins are known regulators of ARF-dependent PLD1 (8, 9), a protein that plays a key role in exocytosis in chromaffin and PC12 cells (35, 44), we investigated whether PLD1 might be an effector for RalA in the exocytotic pathway. Therefore, we first compared the distribution of RalA and PLD1 in resting and stimulated PC12 cells. In agreement with the localization of endogenous PLD1 (35, 44), GFP-PLD1 was found at the plasma membrane in both resting and stimulated cells (Fig. 6A). Immunostaining with anti-RalA antibodies revealed the close co-localization of RalA and GFP-PLD1 at the plasma membrane in resting cells (Fig. 6A). However, since endogenous RalA was barely detected in stimulated cells, it was not possible to compare its distribution with PLD1 under stimulatory conditions. When overexpressed, wild type RalA was also present at the plasma membrane in both resting (Fig. 7A) and K+-stimulated cells (Fig. 6A), where it was found to co-localize with GFP-PLD1 (Fig. 6A), in line with the idea that the two proteins might be able to interact in cells undergoing exocytosis.

Direct interaction of RalA with PLD1 was assessed by immunoprecipitation. We previously described that ARF6 is the upstream activator of the plasma membrane-bound PLD1 in the exocytotic pathway (36). ARF6 forms a complex with PLD1 in secretagogue-activated cells that could be co-precipitated from cells expressing HA-ARF6 using anti-HA antibodies (36). Using a similar approach, we examined whether RalA might be part of this complex. Therefore, PC12 cells expressing HA-ARF6 or the PLD1-nonresponsive HA-ARF6(N48I) mutant were stimulated with 59 mM K+ or maintained in resting conditions, collected, and processed for immunoprecipitation. As illustrated in Fig. 6B, precipitation of wild type HA-ARF6 co-precipitated endogenous PLD1 and RalA from lysates obtained from K+-stimulated cells but not from lysates from resting cells. Moreover, immunoprecipitation of the ARF6(N48I) mutant, which can bind to PLD1 but is unable to activate it (36), similarly co-precipitated PLD1 from lysates of K+-stimulated cells but was unable to pull down RalA (Fig. 6B). Taken together, these results indicate that RalA forms a specific complex with the ARF6-activated PLD1 at the plasma membrane in secretagogue-stimulated cells.

We previously described that in chromaffin cells, secreta-
gogues stimulate an increase of PLD activity that was found to correlate in timing and calcium dependence with the exocytotic response (44). To unequivocally link RalA with this PLD activation, we measured PLD activity in control cells and in cells expressing RalA siRNA under resting and stimulated conditions. In agreement with our previous observations, we found that K/H11001-evoked stimulation enhanced PLD activity in PC12 cells (Fig. 4D). However, this PLD activation was significantly reduced in cells expressing the RalA siRNA (Fig. 4D). From the data calculated from four independent experiments (six determinations/experiment) and taking into account that ~50% of the cells were transfected, we estimated that PLD activation was inhibited by 72% in cells with reduced endogenous RalA level. Thus, RalA belongs to the upstream activators that mediate PLD activation in secretagogue-stimulated cells.

**PLD1 Is a Molecular Partner for RalA in the Exocytotic Machinery**—To further probe the idea that PLD1 is an effector by which RalA regulates the exocytotic process, we expressed in PC12 cells various GTP-bound RalA(G23V) mutants previously shown to be selectively uncoupled from known RalA effectors, namely RalA(G23V,T46A) (unable to associate with RalBP1) (12), RalA(G23V,D49E) (unable to interact with the exocyst subunit Sec5) (15), and ΔNt-RalA(G23V) (deleted of the amino-terminal 11 amino acids, which are required for association of Ral with PLD) (8). Fig. 7A illustrates the intracellular distribution of these various RalA proteins. Double staining with anti-SNAP-25 antibodies confirmed that the mutants retained their ability to associate with the plasma membrane in resting (Fig. 7A) and stimulated PC12 cells (data not shown).

The effect of the RalA variants on K/H11001-evoked GH secretion is shown in Fig. 7B. RalA(G23V,T46A) uncoupled from RalBP1 retained a stimulatory activity comparable with the active RalA(G23V), excluding RalBP1 as an effector of RalA in the exocytotic process. Accordingly, RalBP1 has been described as a direct effector of Ral-mediated regulation of receptor endocytosis (23, 45). RalA(G23V,D49E) was also able to stimulate the
exocytic response, albeit slightly less efficiently than RalA(G23V). In contrast, ANT-RalA(G23V) uncoupled from PLD1 was unable to retain a significant stimulatory activity (Fig. 7B). These data are consistent with the proposal that PLD1 is a major partner for RalA in the exocytotic process of large dense core secretory granules.

**DISCUSSION**

Ral GTPases display two properties that could potentially serve to regulate exocytosis in neurosecretory cells; they interact with the exocyst (Sec6/8 complex), and they stimulate PLD activity. The exocyst complex comprises eight proteins originally identified in the budding yeast, where they have been shown to be essential for exocytosis (17, 46). Mammalian counterparts for each of these proteins have been identified (47), and it has been suggested that the function of the exocyst complex is to mark the plasma membrane as a delivery site for exocytotic vesicles (27, 48). Thus, Ral may participate in regulated exocytosis by controlling the exocyst assembly (26) and tether vesicles in proximity to the plasma membrane-bound SNAREs involved in docking and fusion. RalA has also been shown to interact directly with PLD1 and to enhance ARF-stimulated PLD1 activity (8, 9). PLD1 generates phosphatidic acid, a multifunctional lipid that has been involved in vesicular trafficking by altering membrane curvature and favoring fusion, serving as a protein attachment site, activating selected enzymes, or representing the starting material for the production of additional signaling lipids. We previously demonstrated that PLD1 constitutes a critical factor for regulated exocytosis in neuroendocrine cells (35) and neurons (49), electing PLD1 as another potential target by which Ral proteins may control the exocytotic machinery.

In this report, we present data indicating that RalA is found on the plasma membrane in chromaffin and PC12 cells, where it co-localizes with PLD1 upon cell stimulation. Co-precipitation experiments revealed that RalA interacts directly with ARF6-activated PLD1 in stimulated but not in resting cells, suggesting that RalA may be functionally linked to the plasma membrane-bound PLD1 in the course of exocytosis. Transfection experiments with RalA mutants or silencing of the RalA gene by an RNAi strategy confirm the involvement of RalA in the exocytotic process. Expression of the constitutively active RalA(G23V) strongly enhanced GH secretion from PC12 cells. Conversely, reduction of endogenous RalA inhibited secretion, favoring a positive role for RalA in large dense core granule exocytosis. Consistent with these results, active RalA has been recently reported to enhance basolateral membrane delivery and secretion in epithelial cells (25). How does GTP-loaded RalA facilitate exocytosis in PC12 cells? We found that a RalA(G23V) mutant truncated in the amino-terminal region and unable to interact with PLD1 completely lost its ability to enhance secretagogue-evoked GH release. Together with the observations that RalA interacts with ARF6 and PLD1 at the plasma membrane in stimulated cells and mediates PLD activation upon secretagogue-evoked stimulation, these results suggest that stimulation of the ARF6-dependent PLD1 is the major function RalA undertakes in regulated exocytosis in neuroendocrine cells. Hence, despite the presence of Sec6 on large dense core secretory granules (50), there is still no direct evidence for a function of the exocyst in regulated exocytosis in neuroendocrine cells. Moreover, exocyst subunits are down-regulated in mature synapses (28), an observation that has led to the idea that the complex participates in formation of synapses rather than in synaptic vesicle release in established synapses. Accordingly, in *Drosophila* with mutations in a central structural component of the complex, it was found recently that defects in the exocyst inhibited neurite outgrowth, but release of neurotransmitter at the synapse persisted (51). The present results indicating that PLD1 is a major molecular partner for RalA in the exocytotic pathway in neuroendocrine cells raise also the question of the actual implication of the exocyst in dense core granule exocytosis.

In chromaffin and PC12 cells, PLD1 is found at the plasma membrane and is activated by granule-associated ARF6 following secretagogue-induced targeting and docking of granules onto the plasma membrane (35, 36). Functional studies based on the expression or microinjection of various PLD1 mutants confirmed that ARF6-regulated PLD1 operates at a step that lies subsequent to the cytoskeleton-mediated recruitment of secretory granules to the exocytotic sites at the plasma membrane (35). Thus, through the stimulation of PLD1, RalA might enhance neuroendocrine secretion by influencing some late postdocking events in the exocytotic pathway. In neurons, however, RalA has been reported to control the refilling of the readily releasable pool of synaptic vesicles docked at the plasma membrane, most likely by modulating some synapsin/cytoskeleton-mediated events (32). Thus, despite the similarities between the exocytotic machinery underlying hormone and neurotransmitter release, RalA seems to play a distinct function, perhaps related to the observation that it is associated with synaptic vesicles in neurons (26, 29, 30, 52) but found on the plasma membrane in neuroendocrine cells. On the other hand, PLD1 plays also a role in late stages of neurotransmitter release, most likely by controlling the fusogenic status of presynaptic release sites (49). Whether RalA might be able to modulate this PLD1 activity in neurons has not yet been investigated.

The involvement of PLD1 in a late postdocking step of exocytosis implies a precise regulation of its enzymatic activity. This may be achieved through a tight spatial and temporal regulation of the granule-bound ARF6 activation/inactivation cycle. Hence, ARNO, the guanine nucleotide exchange factor controlling the activity of ARF6 (36, 53), activates ARF6 only after recruitment and docking of granules to the plasma membrane (36). However, exocytosis in neuroendocrine cells is above all a calcium-regulated event, and additional mechanisms are likely to occur at the plasma membrane to link ARF6-dependent PLD1 to variations in cytosolic calcium. With this in mind, it is interesting to recall that RalA is activated by cytosolic calcium (7). Accordingly, we show here that RalA activation occurs in response to membrane depolarization, which directly triggers calcium influx through voltage-gated calcium channels. Moreover, RalA contains a calmodulin-binding site at its C terminus, and Ca2+-calmodulin binding stimulates GTP binding to RalA, suggesting that calmodulin is an RalA activator (54, 55). Calmodulin is also an established regulator of exocytosis (56) that has been implicated in a late role in secretory granule release by signaling through membrane-attached molecules to increase exocytosis (57, 58). The possibility that RalA represents an additional checkpoint that integrates calcium/calmodulin signals to PLD1 and its lipid-modifying activity at the sites of exocytosis is an attractive line of future investigation.

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