The Coffin–Lowry syndrome-associated protein RSK2 is implicated in calcium-regulated exocytosis through the regulation of PLD1

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Exocytosis of neurotransmitters and hormones occurs through the fusion of secretory vesicles with the plasma membrane. This highly regulated process involves key proteins, such as SNAREs, and specific lipids at the site of membrane fusion. Phospholipase D (PLD) has recently emerged as a promoter of membrane fusion in various exocytotic events potentially by providing fusogenic cone-shaped phosphatidic acid. We show here that PLD1 is regulated by ribosomal S6 kinase 2 (RSK2)-dependent phosphorylation. RSK2 is activated by a high K^+-induced rise in cytosolic calcium. Expression of inactive RSK2 mutants or selective knockdown of endogenous RSK2 dramatically affects the different kinetic components of the exocytotic response in chromaffin cells. RSK2 physically interacts with and stimulates PLD activity through the phosphorylation of Thr-147 in the PLD1 amino-terminal phox homology domain. Expression of PLD1 phosphomimetic mutants fully restores secretion in cells depleted of RSK2, suggesting that RSK2 is a critical upstream signaling element in the activation of PLD1 to produce the lipids required for exocytosis. We propose that PLD-related defects in neuronal and endocrine activities could contribute to the effect observed after the loss-of-function mutations in *Rsk2* that lead to Coffin–Lowry syndrome, an X-linked form of growth and mental retardation.

Exocytosis or fusion of membrane-bound vesicles with the plasma membrane is a fundamental cellular process involved in many physiological functions, including cell migration, cell repair, secretion, and neurotransmission. SNARE proteins have been identified as the minimal protein machinery required for membrane fusion (1). The role of lipids is less well understood, but recent findings indicate that the shape of the lipids might be important for the fusion process. *In vitro*, cone-shaped lipids, which spontaneously form negative membrane curvatures, favor the formation of the hemifusion intermediates when present in the contacting leaflets of apposed membranes (2–5). Moreover, the lipid-modifying enzyme phosphoplipase D (PLD), which produces the cone-shaped phosphatidic acid (PA), emerges as a major actor in a varied set of cellular processes that have in common membrane fusion (6–12).

In neuroendocrine cells, the PLD1 isoform is activated in response to secretagogues to produce PA at the granule docking sites on the plasma membrane as step essential for the late stages of exocytosis (13). Interestingly, PLD1 is a multimodule protein that displays very low basal activity and requires interactions with small GTPases of the ARF, Ral, and Rho families and PKC to be fully activated (14, 15). This feature is appealing because it means that the formation of PA at the sites of exocytosis might be to some extent controlled by extracellular signals. Hence, we previously established that ARF6 and RalA are essential activators for PLD1 in the exocytotic machinery, linking PLD1 activation to extracellular agonists (16, 17). PLD1 is also regulated by phosphorylation events in many cellular processes (14, 15). Our previous experiments indicated that phorbol esters were unable to modify PLD activity in resting or stimulated chromaffin cells, ruling out the idea that PLD might be regulated by PKC during exocytosis (18). However, the PLD1 Thr-147 residue known to be phosphorylated by PKC also lies within a consensus phosphorylation site for another kinase family, namely the ribosomal S6 serine/threonine kinases (RSKs). The present study was undertaken to investigate a possible functional relationship between PLD1 and a member of the RSK family. Using chromaffin and PC12 cells, we demonstrate that RSK2 is an essential modulator of PLD1 activity in calcium-regulated exocytosis.

Results

RSK2 Is Involved in Calcium-Regulated Exocytosis. Activation of RSKs occurs by phosphorylation. Using antibodies specifically recognizing the phosphorylated forms of RSK, we found that RSK was phosphorylated in cells stimulated with a depolarizing concentration (59 mM) of K^+ (Fig. 1). Densitometry scanning showed that the amount of phosphorylated RSK increased by 131 ± 12% and 117 ± 8% in stimulated PC12 and chromaffin cells, respectively ($P < 0.001$, $n = 5$). In both cell types, removal of extracellular calcium completely inhibited K^+-induced RSK phosphorylation (Fig. 1). Thus, RSK is phosphorylated and activated by a stimulus known to trigger exocytosis.

To establish whether RSK2 isoform plays a role in exocytosis, we first examined in PC12 cells the effect of two dominant-negative mutants of RSK2 ($RSK2_{S227A}$ or $RSK2_{T577A}$) unable to be phosphorylated (19), by using growth hormone (GH) secretion as a reporter assay for exocytosis. Expression of both RSK mutants strongly inhibited K^+-evoked GH secretion (Fig. 2*A*). The functional importance of RSK2 in exocytosis was also addressed directly by RNA interference. Using a plasmid to express simultaneously GH and siRNAs targeted against the sequence of RSK2, we found that expression of the RSK2 silencer in PC12 cells significantly reduced the endogenous RSK2 level without affecting the expression of other RSK proteins or actin (Fig. 2*B*). The expression of RSK2 siRNAs did

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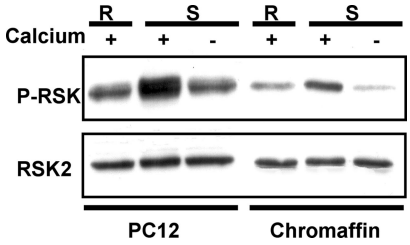

Fig. 1. Calcium-dependent phosphorylation of RSK in stimulated PC12 and chromaffin cells. Phospho-RSK antibodies (P-RSK) were used to detect the active forms of the kinase in resting (R) and 59 mM K^+-stimulated (S) PC12 and chromaffin cells. The absence (−) or presence (+) of 2.5 mM extracellular calcium is shown. Similar findings were obtained in three independent experiments.

also not reduce the expression level of GH (Fig. 2*B*) nor did it affect the distribution of GH-containing secretory vesicles (data not shown). Selective knockdown of RSK2 did not modify basal GH release, but it resulted in a significant decrease (≈75% inhibition) in the amount of GH secreted in response to 59 mM K^+ (Fig. 2*C*). A mutated siRNA sequence that did not affect endogenous RSK2 levels had no effect on secretion (Fig. 2*C*). Furthermore, expression of human RSK2 (insensitive to the siRNA) in RSK2-depleted PC12 cells completely rescued K^+-evoked GH release (Fig. 2*C*). Finally, we found that a 30-min preincubation with the RSK inhibitor BI-D1870 also inhibited in a dose-dependent manner GH secretion from PC12 cells stimulated with high K^+ [supporting information (SI) Fig. S1]. All together, these results suggest that RSK2 represents an important positive regulator of calcium-regulated exocytosis.

Depletion of RSK2 Affects the Different Kinetic Components of the Exocytotic Response in Chromaffin Cells. To further clarify the role of RSK2 in the successive steps underlying exocytosis, we examined the effects of RSK2 depletion on the kinetic components of secretion from chromaffin cells by using simultaneous membrane capacitance measurements and carbon fiber amperometry. Membrane capacitance measures membrane surface area of the cell, whereas amperometry detects catecholamine release from the fused secretory vesicles. GFP-positive chromaffin cells were recorded in whole-cell configuration for ≈2 min to allow dialysis of the photolabile Ca^{2+} chelator nitrophenyl (NP)-EGTA and the Ca^{2+} indicators in the cytoplasm (20). Secretion was triggered by a stepwise increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_I$) caused by flash photolysis of Ca^{2+} from NP-EGTA (Fig. 3*A*). Control chromaffin cells responded

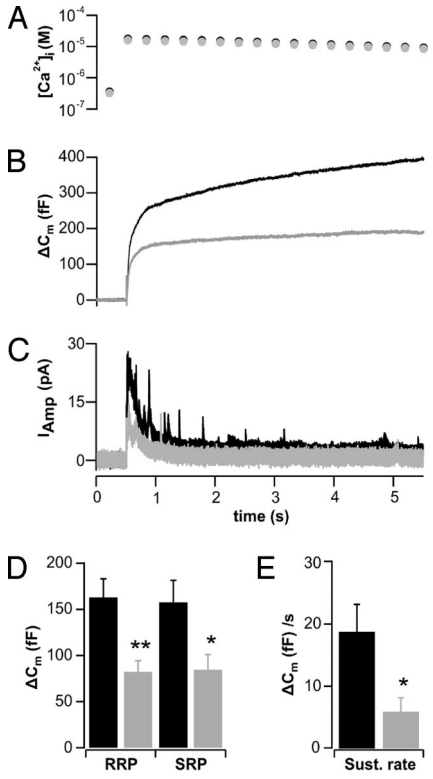

Fig. 3. Depletion of endogenous RSK2 levels by siRNA expression inhibits all kinetic components of the exocytotic response in chromaffin cells. (*A–C*) Average $[Ca^{2+}]_I$ (*A*), membrane capacitance change (*B*), and amperometric currents (*C*) of chromaffin cells stimulated by flash photolysis of Ca^{2+}-loaded NP-EGTA. Cells expressed GFP (black traces; $n = 18$) or GFP and the RSK2 siRNA (gray traces; $n = 20$). The burst and sustained components of the capacitance response were significantly reduced in RSK2-depleted cells. The amperometric response was also reduced in cells expressing RSK2 siRNA. (*D*) Effect of RSK2 depletion on the two kinetic components of the burst phase of the capacitance response. The fast component of the burst represents fusion of vesicles from the RRP, and the slow component represents fusion of vesicles from the SRP. Control cells, black bars; RSK2 siRNA-expressing cells, gray bars. *, $P < 0.02$; **, $P < 0.001$. (*E*) Rate of release during the sustained component of the capacitance response from control cells (black bars) and RSK2 siRNA-expressing cells (gray bars). *, $P < 0.017$.

to the flash stimulation with a typical biphasic increase in membrane capacitance, in which an exocytotic burst was followed by a sustained phase of secretion (Fig. 3*B*). The fast

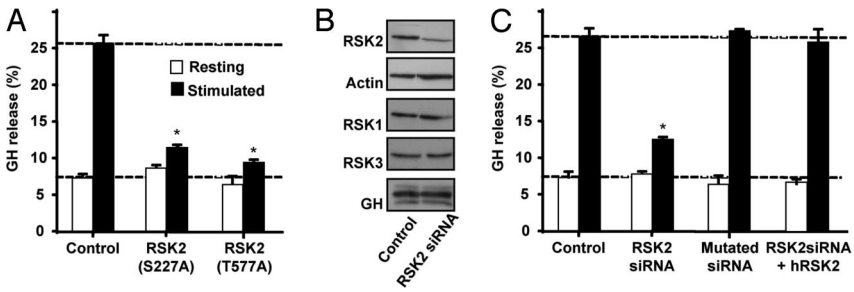

Fig. 2. RSK2 is involved in calcium-regulated exocytosis. (*A*) PC12 cells were cotransfected with the pGH and pTL1 vectors (control) or the pGH vector and pTL1 vectors expressing dominant negative mutants of RSK2 (S227A and T577A). Forty-eight hours after transfection, cells were incubated for 10 min in calcium-free Locke's solution (resting; open bars) or stimulated for 10 min with 59 mM K^+ (stimulated; closed bars) ($n = 3$; *, $P < 0.001$). (*B*) PC12 cells were transfected with either the pGHsuper vector (Control) or the pGHsuper-RSK2 siRNA vector (RSK2 siRNA). Seventy-two hours after transfection, proteins were extracted and analyzed by Western blot. (*C*) PC12 cells were transfected with pGHsuper (Control), pGHsuper-RSK2 siRNA or pGHsuper-mutated RSK2 siRNA, with or without pTL1 or pTL1-human RSK2 (hRSK2). Basal and 59 mM K^+-evoked GH release was measured 72 h after transfection as in *A*. In *A* and *C*, GH release is expressed as the percentage of total GH present in the cells before stimulation. Data are given as the mean values ± SEM obtained in three experiments from different cell cultures ($n = 4$; *, $P < 0.001$).

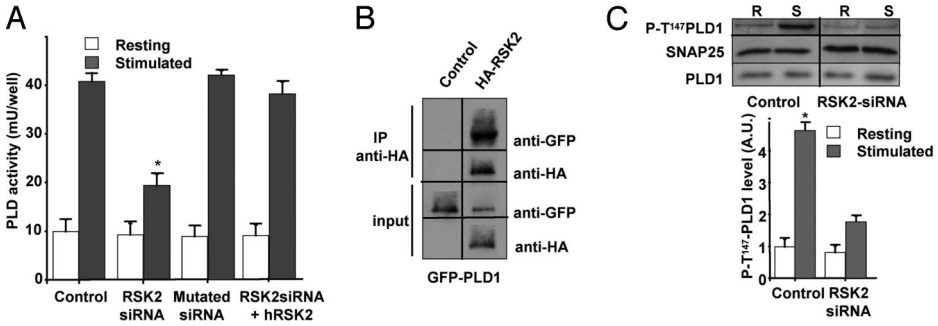

Fig. 4. RSK2 phosphorylates PLD1 and regulates its activity in secretagogue-stimulated PC12 cells. (*A*) PC12 cells expressing GFP (Control), GFP and RSK2 siRNA, or mutated RSK2 siRNA, with or without human RSK2 are shown. Resting or 59 mM K^+-stimulated cells were collected and assayed for PLD activity. Data are given as the mean values ± SEM obtained from different cell preparations ($n = 4$; *, $P < 0.001$). (*B*) PC12 cells were cotransfected with pGFP-PLD1 and pTL1 (Control) or pTL1-HA-RSK2. After 48 h, immunoprecipitation was performed with anti-HA antibodies. The input represents 10% of the total extract. Similar observations were obtained with five different cell cultures. (*C*) Resting or 59 mM K^+-stimulated PC12 cells expressing GFP (Control) or GFP and RSK2 siRNA were scrapped and analyzed by Western blotting using anti-SNAP25, anti-PLD1, and anti-phospho-Thr-147-PLD1 antibodies. Densitometry scanning analysis of three independent experiments is also shown. Stimulation triggered a 4.7- ± 0.5-fold increase in Thr-147-phosphorylated PLD1 in control cells ($n = 3$; *, $P < 0.001$) but only a 1-fold increase in cells transfected with RSK2 siRNA. Note that in these experimental conditions 65% of the total cell population expressed the RSK2 siRNA.

exocytotic burst represents the fusion of two populations of release-competent (primed) vesicles, the readily releasable pool (RRP) and slowly releasable pool (SRP) of vesicles. The sustained component is attributable to fusion of docked vesicles that undergo priming on the run (21–23). As illustrated in Fig. 3*A*, the $[Ca^{2+}]_I$ was similar in RSK2 siRNA transfected cells and control cells before flash (322 ± 27 and 360 ± 24 nM, respectively) and after flash photolysis of Ca^{2+} (15.9 ± 0.6 and 17.8 ± 0.4 μM, respectively). However, the observed membrane capacitance increase was reduced by a factor of two in RSK2-depleted cells in comparison with control (Fig. 3*B*). This effect was not caused by an increase in endocytosis but by a reduction in exocytosis as the amperometric current measured by carbon fiber was reduced to a similar degree (Fig. 3*C*). By analyzing the different kinetic components of the capacitance response, we found that the burst phase of release was reduced by a factor of two, from 320 ± 37 femtofarads (fF) in control cells to 167 ± 21 fF in RSK2 siRNA-transfected cells (Fig. 3*B*), and that both the RRP and SRP were equally decreased (Fig. 3*D*). However, the time constants of release of the SRP and RRP were not changed (data not shown). The rate of release during the sustained component was reduced by a factor of three from 18.7 ± 4.4 $fF \cdot s^{-1}$ in control cells to 5.9 ± 2.2 $fF \cdot s^{-1}$ in siRNA-transfected cells (Fig. 3E). Thus, depletion of endogenous RSK2 affects all kinetic components of exocytosis to a similar degree, suggesting that RSK2 is most likely implicated in a late step in the exocytotic pathway close to the ultimate fusion process.

RSK2 Interacts with PLD1 and Regulates PA Production in Cells Undergoing Exocytosis. PLD1 has been identified as an essential lipid-modifying enzyme for exocytotic fusion (6, 7, 13). It contains two potential membrane association domains, namely a pleckstrin homology (PH) domain and a phox homology (PX) domain; both of them are required for recruitment to the plasma membrane (24). Because Thr-147 in the PX domain lies within a consensus site of phosphorylation by RSKs, we thought that RSK2 could be involved in a late phase of exocytotis by regulating PLD1 activity. To probe this idea, we examined whether the reduction of endogenous RSK2 affects PLD1 activation in PC12 cells stimulated with a secretagogue. Fig. 4*A* shows PLD activity measured in homogenates prepared from resting and K^+-stimulated PC12 cells. Reduction of endogenous RSK2 level by siRNA resulted in a strong inhibition of K^+-induced PLD stimulation compared with control cells. This inhibition could be completely rescued by expression of the siRNA-insensitive human RSK2 (Fig. 4*A*). In contrast, expression of the mutated RSK2 siRNA did not modify the levels of PLD activation after stimulation (Fig. 4*A*).

Interaction between RSK2 and PLD1 was assessed by immunoprecipitation using PC12 cells expressing GFP-PLD1 and HA-RSK2. Fig. 4*B* shows that RSK2 coprecipitates with PLD1 in PC12 cell protein extracts. Conversely, PLD1 coprecipitates with both HA-RSK2 and endogenous RSK2 (Fig. S2). These results support a physical interaction between RSK2 and PLD1 in neuroendocrine cells. This interaction was not significantly modified in protein extracts prepared from K^+-stimulated cells (data not shown). Using an antibody that specifically recognizes the Thr-147-phosphorylated form of PLD1, we were able to detect a significant increase in Thr-147 phosphorylation of PLD1 in cells stimulated with 59 mM K^+ (Fig. 4*C*). Depletion of endogenous RSK2 by siRNA dramatically reduced the amount of the Thr-147-phosphorylated form of PLD1, indicating that RSK2 is the upstream kinase that phosphorylated PLD1 in stimulated cells (Fig. 4*C*). Accordingly, 30 min of preincubation with the RSK inhibitor BI-D1870, but not with the PKC inhibitor staurosporine, inhibited Thr-147 phosphorylation of PLD1 (Fig. S3). Taken together, these results reveal that PLD1 is phosphorylated and activated by RSK2 in cells undergoing exocytosis.

Thr-147 Phosphorylation of PLD1 Is Required for PA Production and Proper Exocytotic Function. To determine the significance of the Thr-147 phosphorylation event for the function of PLD1 in exocytosis, we examined the effect on GH secretion of various PLD1 proteins carrying phosphorylation-deficient (T147A) or phospho-mimetic mutations (T147D and T147E) on Thr-147. Therefore, PLD activity and GH release were measured in parallel in resting and secretagogue-stimulated PC12 cells expressing PLD1 siRNAs to reduce endogenous PLD1 and the various PLD1 mutants additionally mutated at wobble codons within the siRNA target sequence for siRNA-resistant expression. As expected, inhibition of endogenous PLD1 expression resulted in a strong decrease in K^+-stimulated PLD activity and GH release from PC12 cells. This inhibition could be completely rescued by expression of a WT PLD1 protein insensitive to siRNA-mediated degradation (Fig. 5 *A* and *B*). Expression of the Thr-147 phosphorylation-deficient mutant (T147A) was not able to restore normal levels of PLD activity and secretion in K^+-stimulated cells expressing PLD1 siRNAs, whereas expression of the Thr-147 phospho-mimetic mutants T147D and T147E resulted in slightly higher than normal levels of PLD1 activation and GH secretion (Fig. 5 *A* and *B*). Note that both WT and

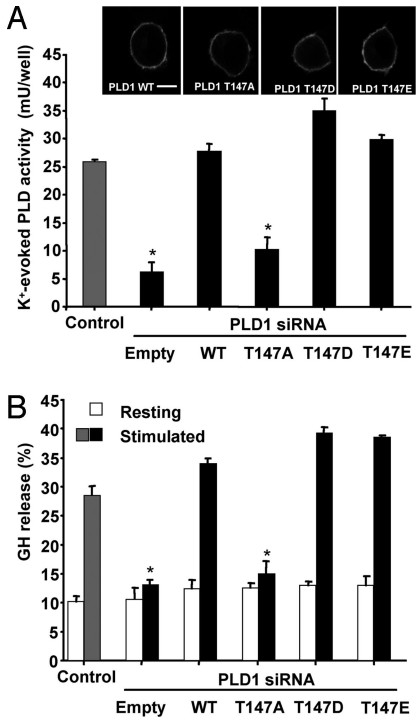

Fig. 5. Phosphorylation on Thr-147 is required for PLD1-mediated PA production and exocytosis. PC12 cells were cotransfected with pEGFP-N2 (Control) and pCGN, or pEGFP-N2-PLD1 siRNA and either pCGN, pCGN-PLD1 rescue WT, pCGN-PLD1 rescue T147A, pCGN-PLD1 rescue T147D or pCGN-PLD1 rescue T147E. (*A*) Cells were stimulated for 10 min with 59 mM K^+, and PLD activity was assayed. K^+-evoked PLD activity was obtained by subtracting the PLD activity detected in resting cells from the PLD activity measured in the K^+-stimulated cells. Data are normalized to the transfection efficiency (55–65%). $n = 4$; *, $P < 0.001$. (*Inset*) Distribution of PLD1 rescue WT, PLD1 rescue T147A, PLD1 rescue T147D, and PLD1 rescue T147E. (Bar: 5 μm.) (*B*) GH release measured in parallel experiments from resting (open bars) and 59 mM K^+-stimulated (closed bars) cells. $n = 4$; *, $P < 0.001$.

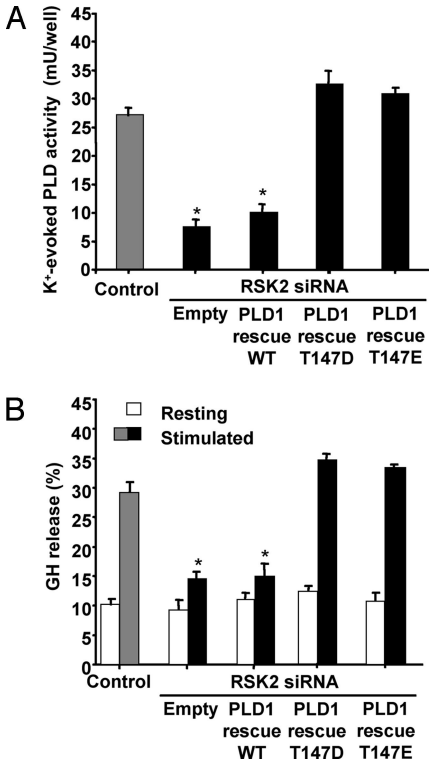

Fig. 6. Thr-147-phosphorylated PLD1 rescues secretagogue-evoked PLD activation and exocytosis in RSK2-depleted PC12 cells. PC12 cells were transfected with pEGFP-N2 (Control) and pCGN or with pEGFP-N2-RSK2 siRNA and either pCGN, pCGN PLD1 rescue WT, pCGN PLD1 rescue T147D, or T147E. (*A*) K^+-evoked PLD activity was obtained by subtracting the PLD activity detected in resting cells from the PLD activity measured in the 59 mM K^+-stimulated cells. Data are normalized to the transfection efficiency (55–65%). $n = 3$; *, $P < 0.001$. (*B*) GH release from resting or 59 mM K^+-stimulated PC12 cells. $n = 3$; *, $P < 0.001$).

mutated PLD1 proteins were found at the plasma membrane when expressed in PC12 cells, indicating that the inefficiency of the PLD1 T147A mutant was not caused by a mislocalization of the protein (Fig. 5*A Inset*). We verified by Western blotting that PLD1 T147A was not phosphorylated in stimulated cells (Fig. S4). In other words, phosphorylation of the Thr-147 residue is required for both secretagogue-evoked PLD1 activation at the plasma membrane and for the function that PLD1 fulfils in the exocytotic machinery.

Thr-147 Phosphorylation by RSK2 Mediates Secretagogue-Evoked PLD1 Activation and Exocytosis. To further probe the hypothesis that RSK2 is responsible for the Thr-147 phosphorylation and activation of PLD1 and is thereby required for exocytosis, we measured GH release from cells expressing RSK2 siRNAs and attempted to rescue secretion with the WT PLD1 or the T147D- and T147E-mutated PLD1 proteins. As previously mentioned and illustrated in Figs. 2*C* and 4*A*, reduction of endogenous RSK2 levels significantly inhibited secretagogue-mediated PLD1 activation and GH secretion (Fig. 6 *A* and *B*, respectively). In cells with reduced RSK2 levels, expression of WT PLD1 was unable to restore normal PLD activity and had no effect on secretion (Fig. 6). In contrast, both PLD1 phosphomimetic mutants were activated in stimulated cells and fully restored secretion despite the low level of endogenous RSK2. These results are in line with a sequence of events in which elevated K^+ and rise in cytosolic calcium activates RSK2, which in turn phosphorylates and stimulates PLD1 to produce the PA required for the exocytotic reaction. In other words, RSK2 appears to be a critical upstream signaling element for PLD1 activation and the production of fusogenic lipids required for exocytosis.

Discussion

Using chromaffin and PC12 cells, two well established cell models for the study of exocytosis, we show that RSK2 plays a role in the calcium-regulated exocytosis of large dense-core secretory granules. We found that RSK2 coprecipitates with PLD1 and through the phosphorylation of PLD1 Thr-147 stimulates its enzymatic activity. Moreover, we demonstrate that PLD1 phosphorylation by RSK2 is essential for the function of PLD1 in exocytosis.

RSK2 belongs to a kinase family (RSK1, RSK2, RSK3, and RSK4) that has been implicated in a number of cellular processes, including regulation of transcription, cell survival, and cell cycle (25). RSKs do so through phosphorylation of multiple substrates on Arg/Lys-X-Arg-X-X-Ser/Thr or Arg-Arg-X-Ser/Thr consensus sites (26). In humans, loss-of-function mutations in the gene encoding RSK2 lead to Coffin–Lowry syndrome (CLS) (27). CLS is characterized by severe mental retardation, growth retardation, and progressive skeletal deformations (28). CLS animal models obtained by inactivation of the *Rsk2* gene display poor coordination and reduced learning ability (29). These findings suggest that RSK2 is required for correct neuronal development and/or function and other RSKs cannot compensate for the loss of RSK2. At the molecular level, *RSK2*

knockout mice revealed that RSK2 is required for osteoblast differentiation and function through its ability to phosphorylate specific transcription factors and that this mechanism could underlie the skeletal abnormalities observed in CLS patients. The molecular bases of the other major CLS features such as growth and mental retardation remain elusive. The present report describes RSK2 as a critical regulator of PLD1 in the course of exocytosis. PLD1 has been implicated in the release process of a variety of hormones (6, 8–11), most likely at a late stage of exocytosis through the production of lipids that are essential for the fusion process. PLD1 seems to be also essential for fast neurotransmitter release (7). Thus, the functional link between RSK2 and PLD1 brings some clues for understanding the molecular mechanisms underlying the neuronal clinical features of CLS, by supporting the idea that part of the growth and mental retardation phenotypes observed in CLS patients could be associated to defects in hormone or neurotransmitter release.

The role of RSK2 as a modulator of PLD1 in the exocytotic machinery raises a question about the upstream signaling pathway that activates RSK2 in cells stimulated for exocytosis. Our results indicate that membrane depolarization that directly triggers a rise in cytosolic calcium through the activation of voltage-gated calcium channels is sufficient to stimulate phosphorylation and activation of RSK2. RSKs are classically activated by ERK-mediated phosphorylation events (30, 31). Using antibodies specifically recognizing the phosphorylated form of ERK1 and ERK2, we found that these kinases became phosphorylated and activated in cells stimulated with high potassium (Fig. S5). This finding is not really a surprise as it has been reported that ERK activation occurs after Ca^{2+} influx in many cell types, including neuroendocrine cells and neurons. To which extent ERKs are the upstream signal for RSK2-mediated PLD1 activation in secretory cells remains to be determined. Nevertheless, it is interesting to recall that in neurons signaling through ERKs has been implicated in several forms of synaptic plasticity, although little information is available on the pathways that are activated downstream of ERK. Thus, it is tempting to speculate that at least some of the ERK-mediated effects on synaptic activity may be mediated by RSK2/PLD1 and the possible modulation or activation of presynaptic exocytotic sites through the production of fusogenic lipids.

Finally, the mechanism targeting RSK2 to the hot spots of exocytosis presents another open question. It has been recently reported that RSKs contain C-terminal sequences that bind PDZ-containing proteins (32), which are in neurons often enriched in synaptic active zones (33). In PC12 and chromaffin cells, we recently described that the PDZ-containing protein Scribble present at the plasma membrane is critical for exocytosis (34). Using PC12 cell extracts, we found that Scribble coprecipitated with RSK2 (unpublished data), suggesting that Scribble might be implicated in the recruitment of RSK2 to the plasma membrane. Interestingly, Scribble is a scaffold that recruits also βPIX, a nucleotide guanine exchange factor for Rac1 and Cdc42, and GIT1 a GTPase-activating protein for ARF6 (35). Both Rac1 and ARF6 GTPases are upstream regulators of PLD1 in the exocytotic process (unpublished data and ref. 16). Thus, an interesting possibility is that protein scaffolds like Scribble may spatially integrate several signaling pathways to PLD1 to finely tune the production of fusogenic lipids at the active site of exocytosis.

Materials and Methods

Materials. Antibodies anti-RSK2 E1 and anti-RSK1 or RSK3 (Santa Cruz Biotechnology), anti-p44/42 MAPK (New England BioLabs), anti-phospho-p44/42 MAPK, anti-phospho-PLD1 (Thr-147) (Cell Signaling), anti-HA (Covance Research Products), anti-actin (Sigma-Aldrich), and mouse anti-GFP (Roche Applied Science) were used. Anti-phospho-RSK, anti-human GH, and secondary antibodies coupled to Alexa conjugates have been described (13, 36). The RSK inhibitor BI-D1870 was obtained from the Medical Research Council Protein Phosphorylation Unit, University of Dundee, Dundee, Scotland.

Plasmids and siRNA. Rat RSK2 cDNA fragments encoding the 21-nt siRNA sequence CTGAAGAAGGCAGTATCAAAG (identical in rat and bovine) derived from the target transcript or the mutated cDNA fragment CTCTAGAAGGCAGTATCAAAG, separated from their reverse 19-nt complement by a short spacer, were annealed and cloned in the BglII and HindIII sites in front of the H1 promoter of either the pEGFP-N2-RNAi plasmid or a modified pXGH5 plasmid encoding GH (pGHsuper) as described (13). PLD1 siRNA and PLD1 rescue plamids have been described (12). T147 mutants were obtained by site-directed mutagenesis as described (37). GFP-PLD1, RSK2, RSK2 S227A, and T577A tagged with HA in pTL1 vectors have been described (13, 19).

Cell Culture and GH Secretion. PC12 cells were cultured in DMEM (Invitrogen) supplemented with 4.5 μg/ml glucose and containing 30 mM NaHCO3, 5% FBS, 10% horse serum, and 100 units/ml of penicillin/streptomycin. Chromaffin cells were isolated from fresh bovine adrenal glands and maintained in primary culture as described (38). Cultured 80% confluent PC12 cells were transfected by using GeneReporter (Gene Therapy Systems), and 59 mM K^+-mediated GH release was monitored 48 or 72 h after transfection (13).

Determination of PLD Activity. Seventy-two hours after electroporation (290 V, 1,200 mF), PC12 cells were washed four times with Locke's solution and then incubated for 10 min in calcium-free Locke's solution or stimulated in Locke's solution with a depolarizing concentration of K^+. PLD activity was measured as described (13) and normalized to transfection efficiency. The PLD activity of the nontransfected cell population was subtracted, and the PLD activity value from the transfected cells was multiplied by 1/transfection efficiency to estimate the PLD activity in equal number of cells. In Figs 1–6, data are given as the mean of six determinations performed on at least three different cell preparations ± SEM.

Western Blot and Immunoprecipitation. Seventy-two hours after electroporation, cells were lyzed and proteins were resolved by SDS/10% PAGEs. Proteins were transferred to nitrocellulose membranes as described (39). Detection was performed by chemiluminescence by using the Super Signal West Dura Extended Duration Substrate (Pierce). Transfection efficiency was assessed in parallel by counting GH- or GFP-expressing cells. Protein extracts were prepared 48 h after transfection in lysis buffer (50 mM Hepes, 3 mM EGTA, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 80 mM KCl, 0.1% Triton X-100, 0.1% sodium deoxycholate, 1 mM sodium orthovanadate, 40 mM NaF, and protease inhibitor mixture) (Sigma-Aldrich). Five hundred micrograms of total protein was used for immunoprecipitation.

Immunocytochemistry. Resting and stimulated PC12 cells were fixed and further processed for immunofluorescence as described (40, 41). Stained cells were visualized with a Zeiss confocal microscope LSM 510.

Amperometry and Capacitance Measurements. Conventional whole-cell recordings were performed with 3- to 4-MΩ pipettes and an EPC-9 patch-clamp amplifier together with Pulse software (HEKA). Capacitance measurements were performed by using the Lindau-Neher technique implemented as the sine + dc mode of the software lock-in extension of Pulse software. Chromaffin cells were voltage-clamped at a holding potential of −70 mV, and a sine wave voltage command with an amplitude of 35mV was applied. The extracellular solution contained 145 mM NaCl, 2.4 mM KCl, 10 mM Hepes, 4 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose. The intracellular solution contained 100 mM Cs-glutamate, 2 mM Mg-ATP, 0.3 mM GTP, 40 mM Cs-Hepes, 5 mM NP-EGTA, 4 mM $CaCl_2$, 0.4 mM Furaptra, and 0.4 mM Fura-4F, adjusted to pH 7.2 with CsOH. For ratiometric determination of intracellular calcium, a calibration was performed in chromaffin cells by using patch pipettes filled with solutions of known calcium concentrations. Secretion was elicited by an increase in intracellular calcium that resulted from flash photolysis of calcium bound to NP-EGTA. Amperometric measurements were carried out simultaneously by using 5-μm carbon fiber electrodes. The sensitivity of carbon fibers was checked on stimulated chromaffin cells before each experiment.

Statistics. Statistical analysis was performed with Student's *t* test.

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