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Role of Phosphoinositide Signaling in the Control of Insulin Exocytosis

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Phosphoinositides (PI) are important signaling molecules involved in the regulation of vesicular trafficking. We found that phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-biphosphate [PI(4,5)P_2] increase the secretory response triggered by 10 μM Ca^{2+} in streptolysin-O-permeabilized insulin-secreting INS-1E cells. In addition, nutrient-induced exocytosis was diminished in intact cells expressing constructs that sequester PI(4,5)P_2 and in cells transfected with constructs that reduce by RNA interference the level of two enzymes involved in PI(4,5)P_2 production, type III PI4-kinase β and type I phosphatidylinositol 4-bisphosphate 5-kinase-γ. To clarify the mechanism of action of PI, we investigated the involvement in the regulation of insulin exocytosis of three potential PI targets, phospholipase D1, the Ca^{2+}-dependent activator protein for secretion 1, and Munc18-interacting protein 1. Transfection of insulin-secreting cells with plasmids that direct the synthesis of small interfering RNAs capable of reducing the endogenous levels of these proteins inhibited hormone release elicited by glucose- and cAMP-elevating agents without affecting basal release. Our data indicate that the production of PI(4,5)P_2 is necessary for proper control of β-cell secretion and suggest that at least part of the effect of PI on insulin exocytosis could be exerted through the activation of phospholipase D1, Ca^{2+}-dependent activator protein for secretion 1, and Munc18-interacting protein 1. (*Molecular Endocrinology* 19: 3097–3106, 2005)

THE RELEASE OF insulin by pancreatic β-cells plays a key role in the control of blood glucose levels. Indeed, insufficient supply of this hormone leads to hyperglycemia, glucose intolerance, and, eventually, to diabetes mellitus. Insulin exocytosis is mainly determined by plasma glucose levels and the concentrations of amino acids and fatty acids. However, a variety of neural and hormonal factors modulate the amplitude of the secretory response to nutrients (1–3). Early manifestations of β-cell dysfunction include delayed and blunted responses to glucose and loss of tight stimulus-secretion coupling (4). To define the causes of β-cell failure, a basic understanding of the physiology of normal insulin secretion is essential. Despite considerable progress in identification of the protein machinery required for insulin secretion (3, 5), the contribution of lipids in the regulation of β-cell exocytosis is still largely unknown. The fusion of secretory granules with the plasma membrane necessitates the rearrangement of lipid bilayers. This rearrangement is facilitated by the presence at the site of fusion of lipids with noncylindrical shape, such as fatty acids and phosphatidic acid (6). The lipid compositions of the secretory granule membrane and the plasma membrane are also crucial for the binding of proteins involved in the control of exocytosis. In fact, accumulation of negatively charged phospholipids at the cytoplasmic leaflet of the membranes allows interaction with positively charged protein surfaces and selective recruitment of regulatory components of the exocytotic machinery. Phosphoinositides (PI) constitute an important class of negatively charged phospholipids that serve as signaling molecules in several intracellular vesicular trafficking steps, including exocytosis and endocytosis (7–10). Generation of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] at defined locations in the cellular membranes permits selective recruitment of proteins endowed with specific PI(4,5)P_2 binding modules, such as the pleckstrin homology (PH) domain (7–10).

The major pathway for the generation of PI(4,5)P_2 involves two sequential phosphorylations, at positions 4 and 5 of the inositol ring of phosphatidylinositol, by PI4- and phosphatidylinositol 4-bisphosphate (PIP) (PIP4) 5-kinases, respectively (7–10). In pancreatic

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Abbreviations: CAPS1, Ca^{2+}-dependent activator protein for secretion 1; GFP, green fluorescence protein; h, human; HA, hemagglutinin; Mint1, Munc18-interacting protein 1; PH, pleckstrin homology; PI, phosphoinositide; PI4K, PI4-kinase; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P_2, phosphatidylinositol 4,5-biphosphate; PIP4, phosphatidylinositol 4-bisphosphate; PLCδ, phospholipase Cδ; PLD1, phospholipase D1; siRNA, small interfering RNA.

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β-cells, appropriate levels of $PI(4,5)P_2$ at the plasma membrane are required to sustain insulin secretion, and a type III PI4-kinase (PI4K) has been proposed to serve as a metabolic sensor, coupling glucose metabolism to hormone release (11).

In this study we attempted to identify the PI4K and PI5K involved in the control of insulin exocytosis and to determine the roles played in this process by proteins interacting with $PI(4,5)P_2$: phospholipase D-1 (PLD1), the Ca^{2+}-dependent activator protein for secretion 1 (CAPS1), and Mint-1 (Munc18-interacting protein).

RESULTS

To evaluate the role of PIs in the regulation of β-cell exocytosis, INS-1E cells, permeabilized with the pore-forming toxin streptolysin-O, were incubated with 10 μM PI(4)P, $PI(4,5)P_2$, or $PI(3,4,5)P_3$. None of the inositol-containing phospholipids affected hormone secretion occurring under resting conditions (0.1 μM free Ca^{2+}; not shown), but the secretory response elicited by 10 μM free Ca^{2+} was enhanced in cells incubated with PI(4)P or $PI(4,5)P_2$ (Fig. 1). $PI(3,4,5)P_3$ had no significant effect on Ca^{2+}-induced exocytosis. To assess the involvement of PIs in nutrient-induced secretion, INS-1E cells were transiently transfected with green fluorescence protein (GFP)-tagged constructs, leading to expression of the PH domain of phospholipase Cδ (PLCδ). The PH domain of PLCδ specifically binds and sequesters $PI(4,5)P_2$ (12). To monitor the effects of the PH domains on exocytosis, the cells were cotransfected with a second plasmid encoding human GH (hGH). Because hGH is targeted to β-cell secretory granules and is coreleased with insulin, the secretion of this hormone serves as a reporter for exocytosis in transiently transfected cells. As shown in Fig. 2, overexpression of the $PI(4,5)P_2$ binding domain led to a strong reduction in stimulus-induced secretion. Basal secretion was not affected (not shown). Overexpression of a mutant of PH-PLCδ that is unable to bind $PI(4,5)P_2$ (12) displayed no effect on exocytosis (Fig. 2). These data indicate that the presence of appropriate levels of $PI(4,5)P_2$ is needed to sustain insulin exocytosis in INS-1E cells.

Next, we searched for the enzymes involved in the synthesis of the pool of $PI(4,5)P_2$ required for β-cell exocytosis. $PI(4,5)P_2$ is produced by the concerted action of two kinases, PI4K and PIP5K (7, 8). Several PI4Ks with different properties and functions have been described (13). Incubation of mouse islets with

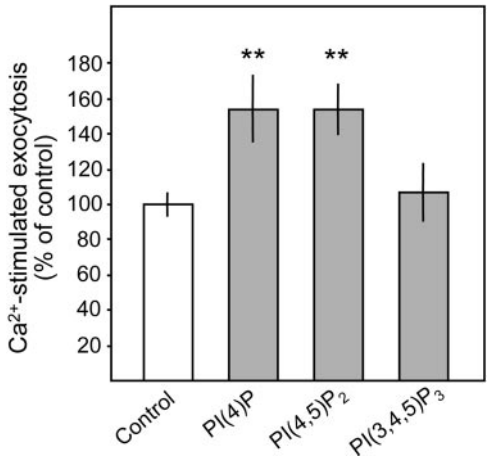

Fig. 1. PI(4)P and $PI(4,5)P_2$ Potentiate Ca^{2+}-Induced Exocytosis in Permeabilized INS-1E Cells

Cells of the insulin-secreting β-cell line INS-1E were permeabilized with streptolysin-O and preincubated for 7 min. The cells were then incubated for another 7-min period in either a buffer containing 0.1 μM free Ca^{2+} (basal condition) or a buffer containing 10 μM free Ca^{2+} (stimulatory condition). The indicated PIs were added at a concentration of 10 μM during the preincubation and incubation periods. At the end of the incubation, the supernatants were collected and analyzed by ELISA to assess exocytosis. Ca^{2+}-stimulated exocytosis was defined by the ratio between secretion at 10 and 0.1 μM Ca^{2+}. The results are the mean ± SEM of five to seven independent experiments performed in triplicate. In control cells, incubation at 10 μM free Ca^{2+} increased hormone release by 2.7 ± 0.3-fold. **, Significantly different from control ($P < 0.01$).

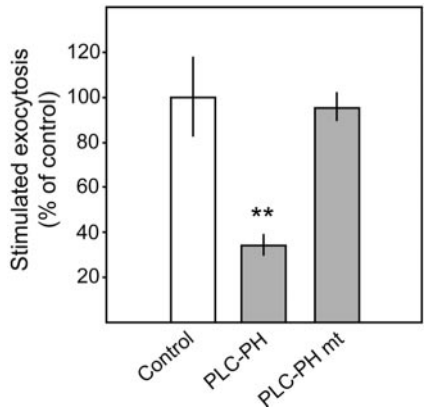

Fig. 2. Exocytosis of INS-1E Cells Is Inhibited by the Introduction of Protein Domains that Sequester $PI(4,5)P_2$

INS-1E cells were transiently transfected with an empty vector (control), a construct expressing the PH domain of PLCδ (PLC-PH), or a construct expressing a mutant of the PH domain of PLCδ unable to bind $PI(4,5)P_2$ (PLC-PH mut). Equal expression of the two GFP-tagged PH constructs was verified by analyzing the fluorescence of transfected cells. To monitor the effect on exocytosis, the cells were cotransfected with a plasmid encoding hGH. Three days later, the cells were incubated for 45 min in the presence or absence of 20 mM glucose, forskolin (1 μM), 3-isobutyl-1-methylxanthine (1 mM), and depolarizing concentrations of K^+. hGH release was measured by ELISA. Stimulated exocytosis was defined by the ratio of hGH release measured in the presence and absence of secretagogues. The results are the mean ± SEM of four independent experiments performed in triplicate. In control cells, the secretagogues increased hGH release 11.9 ± 2.5-fold. **, Significantly different from control ($P < 0.01$).

phenylarsine oxide, a pharmacological inhibitor of type III PI4Ks, has been reported to inhibit insulin secretion (11). In agreement with these findings, preincubation of INS-1E cells for 30 min in the presence of phenylarsine oxide (30 μM) severely hampered the secretory response triggered by glucose- and cAMP-elevating agents (18 $\pm$ 6%; n =3) compared with control cells. PI4Kβ is a type III kinase that is involved in different vesicular trafficking steps (14–17). To investigate the potential role of this protein in the regulation of insulin secretion, we attempted to diminish its expression level using the RNA interference process (18). To this end we generated plasmids that allow the synthesis of short, double-stranded RNA molecules, small interfering RNA (siRNAs), directed against sequences of PI4Kβ. The sequences chosen are conserved among humans, rats, and mice. To verify the efficacy of the constructs, each plasmid was transiently cotransfected in INS-1E cells with hemagglutinin (HA)-tagged PI4Kβ. As shown in Fig. 3A (*top panel*), coexpression of siPIK4β-a resulted in a very strong inhibition of HA-tagged PI4Kβ expression. In contrast, a second plasmid leading to the synthesis of a different siRNA (siPIK4β-i) was inactive. The expression of other unrelated proteins, such as GFP, was not affected by the silencers (not shown). We then verified the effects of the silencers on the expression of endogenous PI4Kβ. Under our experimental conditions, the transfection efficiency varied between 30–50%. Despite this relatively low transfection efficacy, we were able to detect a decrease in the endogenous level of PI4Kβ in homogenates of cells transfected with siPIK4β-a, but not with siPIK4β-i (Fig. 3A, *bottom panel*). The expression of other proteins participating in the control of insulin secretion, such as granuphilin and syntaxin-1, was not affected by the silencers (not shown). This observation demonstrates that our active construct can efficiently diminish the level of PI4Kβ in INS-1E cells. To determine whether silencing of PI4Kβ affects the synthesis of PI(4,5)P_2, INS-1E cells were transiently cotransfected with siPIK4β-a or siPIK4β-i and with GFP-tagged PH-PLCδ. After 3 d in culture, the cells were incubated for 45 min under basal conditions or in the presence of stimulatory concentrations of glucose- and cAMP-elevating agents. They were then fixed, and the distribution of GFP-tagged PH-PLCδ was determined by confocal microscopy. Under basal conditions, the ratio between the GFP fluorescence located at the plasma membrane and in the cytosol was about 3 (Fig. 3B). This ratio was not significantly different in cells transfected with siPIK4β-a and siPIK4β-i. Under stimulatory conditions, the plasma membrane to cytosol ratio in cells transfected with the inactive silencer was increased, whereas in cells in which PI4Kβ was silenced, the ratio tended to decrease (Fig. 3B). These data indicate that the activity of PI4Kβ is necessary to sustain PI(4,5)P_2 levels in the presence of insulin secretagogues. We then tested whether the impairment in PI(4,5)P_2 production is associated with a defect in secretion. Cotransfection of INS-1E cells with

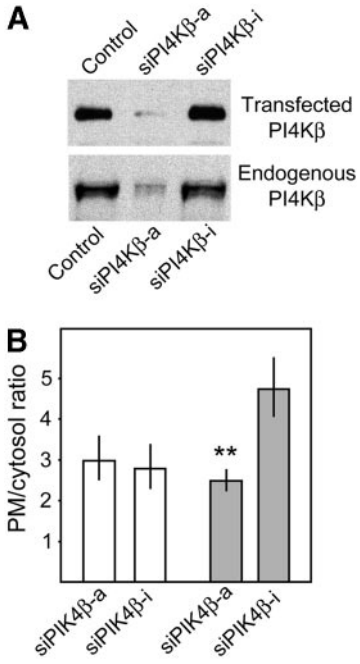

Fig. 3. Silencing of PI4Kβ by RNA Interference Leads to a Decrease in Plasma Membrane-Associated PI(4,5)P_2

A, *Upper panel*, INS-1E cells were transiently cotransfected with HA-tagged PI4Kβ and with an empty vector (control) or pSUPER constructs that led to the synthesis of siRNAs directed against PI4Kβ (siPIK4β-a and siPIK4β-i). Three days later, the cells were homogenized, and the expression of HA-tagged PI4Kβ was analyzed by Western blotting using an anti-HA antibody. *Lower panel*, INS-1E cells were transiently transfected with an empty vector (control), siPIK4β-a, or siPIK4β-i. Three days later, the cells were homogenized, and the expression of endogenous PIK4β was analyzed by Western blotting using an anti-PIK4β antibody. B, INS-1E cells were transiently transfected with a construct expressing the PH domain of PLCδ (PLC-PH) and with siPIK4β-a or siPIK4β-i. Three days later, the cells were analyzed by confocal microscopy, and the ratio between the fluorescence intensity at the plasma membrane and that in the cytosol was determined. Under stimulatory conditions, the plasma membrane/cytosol ratio of the cells transfected with siPIK4β-a was significantly different ($P < 0.01$) from that of the cells transfected with the inactive silencer (siPIK4β-i).

hGH and siPIK4β-a or siPIK4β-i revealed that silencing of PI4Kβ results in a defect in stimulus-induced secretion (Fig. 4A). As expected, transfection of siPIK4β-i had no significant effect on exocytosis. Very similar data were obtained when the same experiment was performed in the well-differentiated mouse insulin-secreting cell line MIN6 B1 (19) (Fig. 4B). These findings suggest that the activity of PI4Kβ is required for maintaining an appropriate pool of PI(4)P during insulin secretion.

Type I PIP5Kγ is enriched in brain and pancreatic β-cells (20, 21) and has very recently been involved in synaptic transmission and dense core vesicle fusion (22, 23). To evaluate the role of this enzyme in insulin exocytosis, we generated different plasmids driving

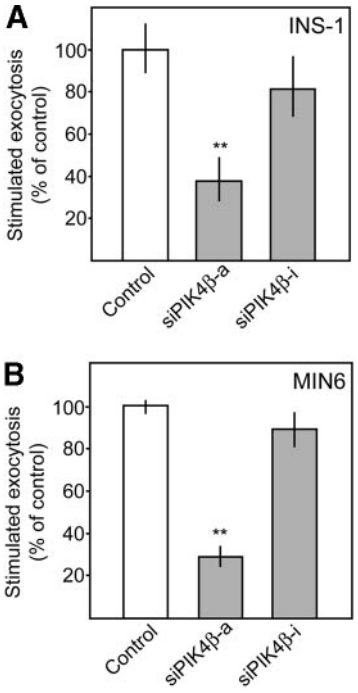

Fig. 4. Silencing of PI4Kβ by RNA Interference Leads to a Decrease in Stimulus-Induced Exocytosis in INS-1E and MIN6 Cells

INS-1E (*upper panel*) or MIN6 (*lower panel*) were transiently cotransfected with the plasmid encoding hGH and with an empty vector (control), siPIK4β-a or siPIK4β-i. Three days later, the cells were incubated in the presence or absence of 20 mM glucose, 1 μM forskolin, 1 mM 3-isobutyl-1-methylxanthine, and depolarizing concentrations of K^+. Exocytosis was monitored by ELISA. Stimulated exocytosis was defined as the ratio between the amount of hGH released in the presence and absence of secretagogues. The results are the mean $\pm$ SEM of three or four experiments. In control INS-1E cells, the secretagogues increased hGH release 8.2 $\pm$ 1.0-fold. In control MIN6 cells, the secretagogues increased hGH release 15.3 $\pm$ 0.6-fold. **, Significantly different from controls ($P < 0.01$).

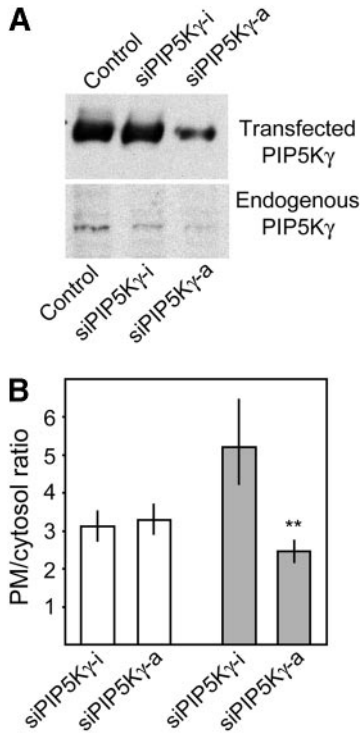

Fig. 5. Silencing of PIP5Kγ by RNA Interference Causes a Reduction in Plasma Membrane-Associated PI(4,5)P_2

A, *Upper panel*, INS-1E cells were transiently cotransfected with HA-tagged PIP5Kγ and with an empty vector (control) or siRNA constructs directed against PIP5Kγ (siPIP5Kγ-i and siPIP5Kγ-a). After 3 d, the expression of HA-tagged PIP5Kγ was determined by Western blotting using an anti-HA antibody. *Lower panel*, INS-1E cells transiently transfected with an empty vector (control), siPIP5Kγ-i, or siPIPK5γ-a were homogenized and processed by Western blotting to assess the expression of endogenous anti-PIP5Kγ using a specific antibody. B, INS-1E cells were transiently transfected with the PLC-PH construct and with siPIP5Kγ-i and siPIP5Kγ-a. Three days later, the cells were analyzed by confocal microscopy, and the ratio between the fluorescence intensity at the plasma membrane and that in the cytosol was determined. Under stimulatory conditions, the plasma membrane/cytosol ratio of the cells transfected with siPIP5Kγ-a was significantly lower ($P < 0.01$) than that of the cells transfected with siPIP5Kγ-i.

the synthesis of siRNAs specifically directed against PIP5Kγ. As shown in Fig. 5A, one of the plasmids (siPIP5Kγ-a) lowered the expression of epitope-tagged PIP5Kγ (*upper panel*) as well as that of the endogenous enzyme (*lower panel*). A second plasmid (siPIP5Kγ-i), in contrast, was without effect. PIP5Kγ activity was found to be required to sustain PI(4,5)P_2 levels under stimulatory conditions. In fact, in the presence of insulin secretagogues, the plasma membrane to cytosol ratio of GFP-tagged PH-PLC was significantly lower in cells transfected with siPIP5Kγ-a compared with cells transfected with the inactive PIP5Kγ silencer (Fig. 5B). Analysis of the secretory capacity of INS-1E and MIN6 cells transfected with these plasmids revealed that reduced levels of PIP5Kγ are associated with impairment in stimulus-induced secretion (Fig. 6). Basal secretion, in contrast, was not affected (not shown).

Having demonstrated the importance of PI(4,5)P_2 production for insulin secretion, we attempted to assess the contributions of known PI-binding proteins to the regulation of β-cell exocytosis. PLD1 is the main source of phosphatidic acid, a lipid that favors vesicle fusion (24). Several studies have demonstrated that the activity of PLD1 is enhanced by PI(4,5)P_2 (25, 26). We constructed two plasmids producing siRNAs specifically directed against PLD1. Transfection of siPLD1-a potently reduced the expression of exogenous GFP-tagged PLD1 (Fig. 7A, *upper panel*) and endogenous PLD1 (Fig. 7A, *lower panel*). In contrast, siPLD1-i was without effect. Taking into account that Western blots in Fig. 7A were performed with samples

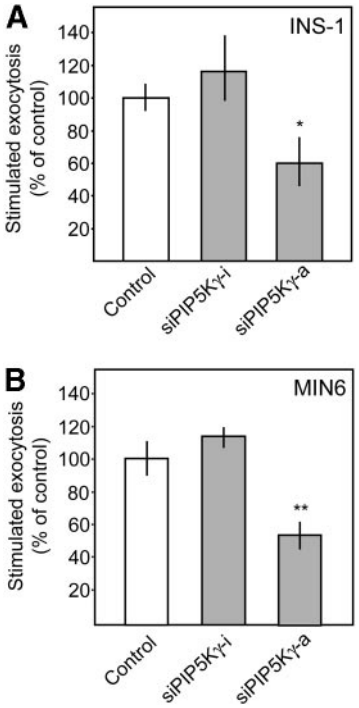

Fig. 6. Silencing of PIP5Kγ by RNA Interference Causes a Reduction of Stimulus-Induced Exocytosis

INS-1E (*upper panel*) or MIN6 (*lower panel*) was transiently transfected with the hGH plasmid together with either an empty vector (control) or the siRNA-encoding constructs. After 3 d in culture, the cells were incubated in the presence or absence of 20 mM glucose, 1 μM forskolin, 1 mM 3-isobutyl-1-methylxanthine, and depolarizing concentrations of K^+. Stimulated exocytosis was defined as the ratio between the amount of hGH released in the presence and absence of secretagogues. The results are the mean $\pm$ SEM of three independent experiments. In control INS-1E cells, the secretagogues increased hGH release 9.2 $\pm$ 0.7-fold. In control MIN6 cells, the secretagogues increased hGH release 16.1 $\pm$ 1.7-fold. *Asterisks* indicate a significant difference from controls (*, $P < 0.05$; **, $P < 0.01$).

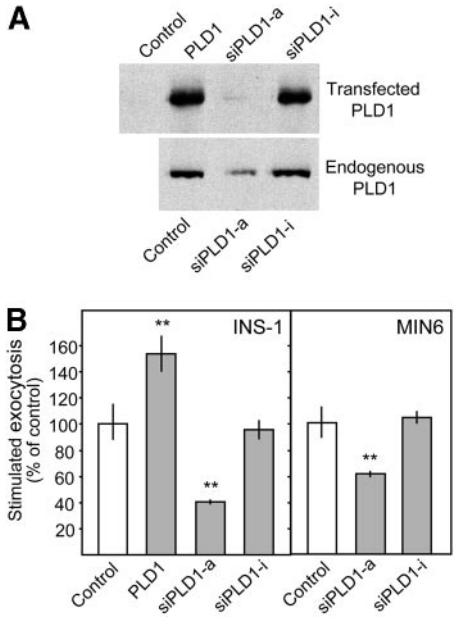

Fig. 7. Involvement of PLD1 in Insulin Exocytosis

A, *Upper panel*, INS-1E cells were transiently transfected with GFP-tagged PLD1 together with an empty pSUPER vector or with plasmids directing the synthesis of PLD1-specific siRNAs (siPLD1-a and siPLD1-i). Control cells were transfected with an empty pSUPER vector alone. The expression of GFP-tagged PLD1 was analyzed 3 d later by Western blotting using an anti-GFP antibody. *Lower panel*, Homogenates of INS-1E cells transiently transfected with an empty vector (control) with siPLD1-a or siPLD1-i were analyzed by Western blotting using a PLD1-specific antibody. B, INS-1E cells (*left panel*) and MIN6 (*right panel*) were cotransfected with the plasmid encoding hGH and with an empty vector (control) or vectors directing the synthesis of GFP-tagged PLD1, siPLD1-a, and siPLD1-i. After 3 d in culture, the cells were incubated in the presence or absence of secretagogues. Stimulated exocytosis was determined by calculating the ratio between the amount of hGH released in the presence and absence of stimuli. The results are the mean $\pm$ SEM of three independent experiments. In control INS-1E cells, the secretagogues increased hGH release 11.2 $\pm$ 0.2-fold. In control MIN6 cells, the secretagogues increased hGH release 15.6 $\pm$ 2.0-fold. **, Significantly different from controls ($P < 0.01$).

in which only part of the cells received siPLD1-a, silencing of endogenous PLD1 in INS-1E cells is probably nearly complete. To evaluate the role of PLD1 in insulin exocytosis, the PLD1 silencers were transiently cotransfected with the plasmid encoding hGH. As shown in Fig. 7B, stimulus-induced secretion was inhibited by siPLD1-a, but not by siPLD1-i. Overexpression of PLD1 enhanced hormone release, confirming the involvement of this enzyme in insulin exocytosis. Overexpression of PLD2, another PLD isoform with a different subcellular localization and function, was without effect (not shown).

CAPS1 is a PI-binding protein associated with insulin-containing granules that participates in the control of exocytosis in endocrine and neuroendocrine cells (27–32). The contribution of this protein to the regulation of the exocytotic process of β-cells was investigated using an approach similar to that used for PLD1. Overexpression of wild-type CAPS1 did not significantly modify hormone secretion of INS-1E cells (Fig. 8B). However, transfection of insulin-secreting cells with a plasmid (siCAPS-a) directing the synthesis of siRNAs capable of reducing the level of exogenous HA-tagged and of endogenous CAPS1 (Fig. 8A) led to a decrease in exocytosis elicited by glucose- and cAMP-elevating agents (Fig. 8B). This indicates that the presence of a critical level of CAPS1 is required to sustain insulin exocytosis.

Mint1 is a PI-binding protein involved in synaptic transmission (33, 34). This adaptor protein is thought to recruit, at specific sites at the plasma membrane, Munc-18, a key component required for vesicle fusion (35). Mint1 is mainly expressed in neurons, but has recently also been found in pancreatic β-cells (36). To

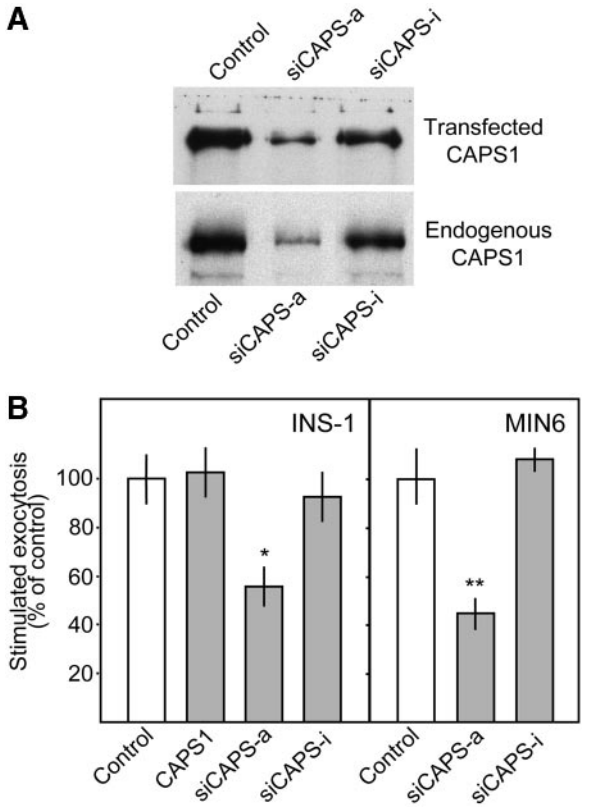

Fig. 8. CAPS1 Is Required for Insulin Exocytosis

A, *Upper panel*, INS-1E cells were transiently transfected with HA-tagged CAPS1 and an empty vector (control) or pSHAG1 vectors enabling the expression of CAPS1-specific siRNAs (siCAPS-a and siCAPS-i). The expression of HA-tagged CAPS1 was determined by Western blotting using an anti-HA antibody. *Lower panel*, Homogenates of INS-1E cells transiently transfected with an empty vector (control) with siCAPS-a or siCAPS-i were analyzed by Western blotting using a CAPS1-specific antibody. B, INS-1E cells (*left panel*) or MIN6 (*right panel*) were cotransfected with the plasmid encoding hGH and with an empty plasmid (control) or plasmids controlling the expression of CAPS1, siCAPS-a, or siCAPS-i. Three days later, the cells were exposed to basal or stimulatory conditions, and hGH release was measured by ELISA. Stimulated exocytosis was defined as the ratio between the amount of hGH secreted in the presence and absence of stimuli. The results are expressed as the mean ± SEM of three to five independent experiments performed in triplicate. In control INS-1E cells, the secretagogues elicited an increase of 11.6 ± 1.6-fold in hGH release. In control MIN6 cells, the secretagogues elicited an increase of 15.6 ± 1.9-fold in hGH release. *Asterisks* indicate a significant difference from controls (*, $P < 0.05$; **, $P < 0.01$).

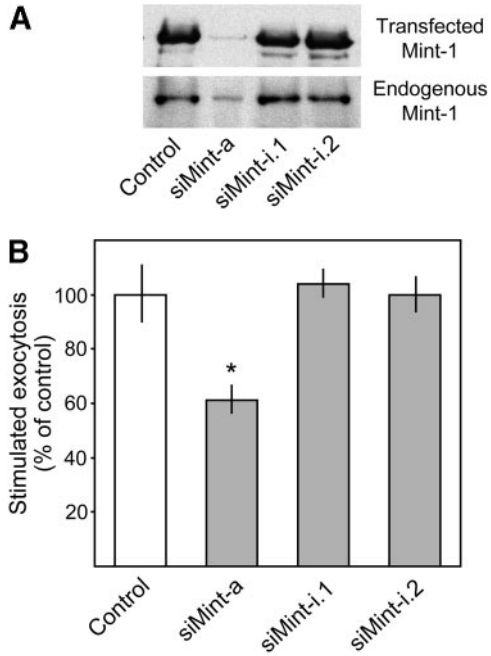

Fig. 9. Silencing of Mint1 Results in a Decrease in INS-1E Exocytosis

A, *Upper panel*, INS-1E cells were transiently transfected with Mint1 and an empty pSUPER (control) or with constructs leading to the production of Mint1-specific siRNAs (siMint-a, siMint-i.1, and siMint-i.2). The expression of Mint1 was determined by Western blotting. *Lower panel*, The expression of Mint1 in homogenates of INS-1E cells transiently transfected with pSUPER (control), siMint-a, siMint-i.1, or siMint-i.2 was examined by Western blotting using a Mint1 antibody. B, INS-1E cells were cotransfected with the plasmid expressing hGH and with pSUPER (control), siMint-a, siMint-i.1, or siMint-i.2. hGH release under basal or stimulatory conditions was assessed 3 d later by ELISA. The results are the mean ± SEM of three independent experiments performed in triplicate. In this series of experiments, control cells displayed an increase in hGH release of 7.1 ± 0.8-fold in the presence of glucose and cAMP-elevating agents. *, Significantly different from control ($P < 0.05$).

assess the role of this protein in insulin exocytosis, we generated three plasmids directing the synthesis of siRNAs against Mint1. Transfection of siMint-a damped the expression of both exogenous and endogenous Mint1 (Fig. 9A) and significantly reduced hormone release triggered by secretagogues (Fig. 9B). In contrast, the other two plasmids (siMint-i.1 and siMint-i.2), which are unable to affect Mint1 expression, did not alter the secretory response of the cells. The rat sequence targeted by siMint-a is not conserved in mice. For this reason, the effect of this construct was not tested in mouse MIN6 cells.

DISCUSSION

Insulin exocytosis is a central event in blood glucose homeostasis. The molecular mechanisms underlying this process are beginning to be elucidated, but the contribution of lipids to the regulation of secretory granule fusion is still poorly understood. $PI(4,5)P_2$ is a negatively charged phospholipid, nonuniformly distributed on cellular membranes, that controls spatially confined membrane events, including vesicle exo- and endocytosis (7–10). Exposure of pancreatic β-cells to

secretagogues is associated with increased PI turnover, and maintenance of an appropriate $PI(4,5)P_2$ pool is a prerequisite for regulated secretion (37). Our data are consistent with a positive role for $PI(4,5)P_2$ in the control of insulin release. In fact, in INS-1E cells exocytosis triggered by secretagogues is enhanced by introduction of $PI(4,5)P_2$ and is impaired if the pool of this lipid is reduced by either sequestering the molecule or preventing its synthesis. The enzymes responsible for the replenishment of the pool of $PI(4,5)P_2$ in secretagogue-stimulated β-cells have been unknown to date. In this study we identify two PI kinases, PI4Kβ and PIP5Kγ, that are necessary to sustain insulin exocytosis.

PI4Kβ is a type III kinase involved in different vesicular trafficking events (14–17). The activity of PI4Kβ is positively regulated by neuronal Ca^{2+} sensor 1 and is a rate-limiting factor in the synthesis of the $PI(4,5)P_2$ pool for stimulus-induced secretion in neuroendocrine and mast cells (15–17). Glucose, the main physiological stimulus for insulin release, promotes exocytosis by generating metabolic signals that lead to membrane depolarization and activation of the secretory machinery (1–3). In β-cells, a type III PI4K has been proposed to function as a metabolic sensor, coupling glucose metabolism to insulin release (11). Moreover, in a very recent study published during the revision of this manuscript, neuronal Ca^{2+} sensor 1 was reported to activate PI4Kβ in pancreatic β-cells and to increase insulin release (38). Our data are fully consistent with these observations and support the idea of an important role for PI4Kβ in the control of β-cell exocytosis.

In streptolysin-O-permeabilized INS-1E cells, addition of PI(4)P has effects on exocytosis similar to those of $PI(4,5)P_2$. The most likely explanation for this finding is that PI(4)P is rapidly converted to $PI(4,5)P_2$. PIP5Kγ, is a cytosolic enzyme that is activated by the small GTPase ADP-ribosylation factor 6 (39). Depletion of this enzyme from plasma membrane is associated with inhibition of dense core granule exocytosis in PC12 cells (39). In addition, PIP5Kγ knockout mice display multiple defects in the synaptic vesicle cycle, including exo- and endocytosis and alteration in vesicle fusion dynamics (22, 23). The role of PIP5Kγ in pancreatic β-cell secretion has been unknown to date. In this study we show that silencing of PIP5Kγ in INS-1E and MIN6 cells results in a decrease in hormone release, indicating a critical role for this enzyme in insulin secretion. ADP-ribosylation factor 6 activity is required to sustain $PI(4,5)P_2$ levels during β-cell stimulation and is necessary for proper response to insulin secretagogues (37). Our findings suggest that at least part of the effect of the guanosine phosphatase could be achieved by activation of PIP5Kγ.

The signaling role of $PI(4,5)P_2$ is mediated through interactions with proteins involved in membrane trafficking that are endowed with $PI(4,5)P_2$-binding modules, such as the PH domain. In this study we investigated the roles of three $PI(4,5)P_2$-binding proteins: PLD1, CAPS1, and Mint1. PLD1 is a key factor in the regulation of neuroendocrine secretion (40). $PI(4,5)P_2$ binding is necessary for the activity of the enzyme, and a mutant of PLD1 that cannot interact with PIs is unable to control exocytosis (40). In β-cells, PLD activity is increased in response to a variety of secretagogues, and blockade of phosphatidic acid formation inhibits secretion (24). We found that in INS-1E cells, alteration of endogenous levels of PLD1, the predominant PLD isoform in β-cells (24), is paralleled by corresponding changes in hormone release elicited by secretagogues. PLD1 is associated with different cellular compartments (24–26). Our experiments do not determine which pool of PLD1 is involved in exocytosis. Indeed, insulin exocytosis could potentially be promoted by the generation of phosphatidic acid at the secretory granules membrane, the plasma membrane, or both.

CAPS1 was initially discovered as a cytosolic brain protein required for calcium-dependent dense core vesicle exocytosis in permeabilized PC12 cells (27). This protein is expressed in several neural and endocrine tissues, including pancreatic islets, indicating a more widespread role in regulated exocytosis (11, 27). Our data support an important role for CAPS1 in the control of insulin secretion, and complement results obtained in electrophysiology set ups in which CAPS1 antibodies were used to inactivate the protein (11). CAPS1 is required for a prefusion step preceding Ca^{2+}-induced exocytosis (32), but its precise mode of action is not yet fully elucidated. The protein has two distinct membrane association domains permitting concomitant binding to plasma membrane and secretory granules (41). This property could favor the approach of the two membranes during the final steps of exocytosis. CAPS1 possesses at least two distinct $PI(4,5)P_2$-binding domains capable of guiding the protein to specific membrane compartments in which complexes required for vesicle fusion assemble. In addition, $PI(4,5)P_2$ binding to CAPS1 induces conformational changes that may somehow modulate the interaction with other components of the exocytotic apparatus during the final steps of the secretory pathway.

Mint1 is a multidomain adaptor protein involved in synaptic vesicle exocytosis (33–35). Mint1 was initially identified in yeast two-hybrid screens for proteins interacting with Munc18-1, one of the main syntaxin-1 partners and a key component of the exocytotic machinery (33). Mint1 was later demonstrated to associate with a number of other neuronal proteins, including amyloid precursor protein, presenilins, and Ca^{2+} channels (35). In addition, the phosphotyrosine-binding domain of Mint1 specifically binds $PI(4,5)P_2$. The expression of Mint1 was thought to be restricted to neurons, but a recent study reported the presence of the protein in pancreatic islets (36). In this study, we not only confirm the presence of Mint1 in INS-1E cells, but, taking advantage of the RNA interference approach, we demonstrate that this adaptor protein is an important player in β-cell exocytosis. In neurons,

Mint1 was proposed to function by recruiting Munc18-1 in the vicinity of the site of exocytosis and to favor docking of synaptic vesicles at the plasma membrane. Because Munc18-1 is required for insulin secretion (42), it is tempting to speculate that a similar mechanism might operate in β-cells. Although the functional impact of PI binding is not yet established, the capacity to interact with $PI(4,5)P_2$ could help in restricting the distribution of Mint1 and, consequently, of Munc18-1 to specific sites at the plasma membrane.

In this study we investigated the role of the PI signaling pathway in the regulation of insulin secretion. Silencing or overexpression of the proteins involved in PI signaling affected stimulated exocytosis, but not basal secretion. This suggests that these components are dispensable to sustain a relatively low fusion rate, but become limiting when the cells are stimulated. Our data demonstrate that PI4Kβ and PIP5Kγ are necessary to provide appropriate levels of $PI(4,5)P_2$ to support insulin exocytosis in response to glucose- and cAMP-elevating agents. In addition, we have identified three proteins whose activity requires PI binding that are directly involved in the exocytotic process of pancreatic β-cells: PLD1, CAPS1, and Mint1. Each of these proteins appears to be needed for a full response to secretagogues. However, almost complete knockdown of these components results in partial inhibition of insulin secretion. It is probably not possible to directly correlate a decrease in the expression of a given protein with an impact on exocytosis. Indeed, if the amount of the protein is not rate limiting, an effect on hormone release would be apparent only below a given threshold. Moreover, other isoforms of CAPS and Mint present in β-cells may in part compensate for the loss of CAPS1 and Mint1.

In conclusion, we have demonstrated the importance of PI signaling in the control of insulin exocytosis. Our findings have certainly not exhausted the potential targets of $PI(4,5)P_2$ and do not rule out other possible actions mediated by these signaling molecules on cytoskeletal proteins, ion channels, or other components of the exocytotic machinery that could influence the secretory process of pancreatic β-cells.

MATERIALS AND METHODS

The antibody against type I PI5Kγ was provided by Dr. G. Di Paolo (Yale University, New Haven, CT). Antibodies against PI4Kβ, PLD1, and Mint1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), BioSource International (Camarillo, CA), and BD Transduction Laboratories (San Diego, CA), respectively. The antibody against CAPS1 has been described previously (27). Secondary antibodies coupled to horseradish peroxidase were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Phosphatidyl-D-myo-inositol-4-phosphate, phosphatidyl-D-myo-inositol-4,5-bisphosphate, and phosphatidylinositol-3,4,5-trisphosphate were obtained from Calbiochem (San Diego, CA). Phenylarsine oxide was purchased from Sigma-Aldrich Corp. The pSUPER plasmid (43) was provided by Dr. Agami (Netherlands Cancer Institute, Amsterdam, The Netherlands). The pSHAG1 vector was obtained from Dr. G. J. Hannon (Cold Spring Harbor Laboratory, Plainview, NY). The plasmids encoding GFP-tagged, wild-type PH-PLCδ and PH-PLCδ (S34T, R40L) were gifts from Dr. R. Holz (Michigan University, Ann Arbor, MI). HA-tagged PI4Kβ, GFP-tagged PLD1, and Mint1 were provided by Dr. M. A. De Matteis (Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy), Dr. M. A. Frohman (State University of New York, New York, NY), and Dr. T. Südhof (Texas University, Dallas, TX), respectively. Generation of HA-tagged PIP5Kγ and HA-tagged CAPS1 expression vectors has been described previously (39, 41).

Preparation of Silencing Vectors for PI4Kβ, PI5Kγ, PLD1, Mint1, and CAPS1

Mammalian expression vectors directing the synthesis of siRNAs targeted against PI4Kβ, PIP5Kγ, PLD1, or Mint1 were prepared according to the method of Brummelkamp *et al.* (43). Two cDNA fragments encoding a 19-nucleotide sequence derived from the target transcript and separated from its reverse 19-nucleotide complement by a short spacer were synthesized by MWG Biotech Co. (Ebersberg, Germany), annealed, and cloned in front of the H1-RNA promoter in the pSUPER vector (43). The silencers were generated using the following sequences: rat PI4Kβ [accession no. NM_031083; nucleotides 875–893 (active) and 1869–1887 (inactive)], rat PIP5Kγ [accession no. XM_234925; nucleotides 1908–1926 (active) and 1077–1095 (inactive)], rat PLD1 [accession no. NM_030992; nucleotides 2604–2622 (active) and 369–387 (inactive)], and Mint1 [accession no. AF029105; nucleotides 1366–1384, siMint-a nucleotides 1398–1416, iMint-i.1; nucleotides 1753–17771, iMint-i.2]1 silencers were constructed by inserting short hairpin oligonucleotides including sequences 3902–3930 (active) and 75–102 (inactive) of rat CAPS1 (accession no. NM_013219) in pSHAG1. This vector contains a U6 promoter and *Bse*RI and *Bam*HI cloning sites in a pENTR/D topo backbone (44). Each silencer was tested by Western blotting for its effect on the expression of its specific target and other unrelated proteins, such as GFP, granuphilin, and syntaxin-1.

Cell Culture and Transfection

The rat pancreatic β-cell line INS-1E was cultured in RPMI 1640 medium supplemented with 50 μM β-mercaptoethanol, 100 μM sodium pyruvate, and 5% fetal calf serum as previously described (45, 46). The mouse pancreatic β-cell line MIN6 B1 was cultured as previously described (19). Transient transfection was performed using the Effectene reagent (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. In all experiments the DNA/Effectene ratio was 1:25. Under our experimental conditions, transfection efficiency, estimated by counting the fraction of fluorescent cells after transfection with a plasmid encoding GFP, ranged from 30–50%.

Secretion Assay

INS-1E and MIN6 B1 cells were transiently cotransfected with a plasmid encoding hGH and with plasmids encoding either the RNAi silencers or, in some cases, constructs leading to overexpression of the proteins under study. None of the plasmids used in this study affected the total amount of hGH produced by INS-1E or MIN6 cells. Thus, in each experiment the total cellular content of hGH was equivalent for all transfectants. Three days later, the cells were preincubated for 30 min in 20 mM HEPES (pH 7.4), 128 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.7 mM $CaCl_2$. The cells were then incubated for 45 min at 37 C either in the same buffer (basal) or in a buffer containing 20 mM HEPES (pH 7.4), 53 mM NaCl, 80 mM KCl,

1 mM $MgCl_2$, 2.7 mM $CaCl_2$, 20 mM glucose, 1 μM forskolin, and 1 mM 3-isobutyl-1-methylxanthine (stimulated). Exocytosis from transfected cells was assessed by measuring by ELISA the amount of hGH released in the medium during the incubation period (Roche, Rotkreuz, Switzerland). For the experiments with streptolysin-O, INS-1E cells were rendered permeable in potassium glutamate buffer [20 mM HEPES (pH 7.0), 140 mM potassium glutamate, 5 mM NaCl, 7 mM $MgSO_4$, 5 mM Na_2ATP, and 10.2 mM EGTA] containing 1.5 IU/ml streptolysin-O for 7 min at 37 C. After permeabilization, the cells were preincubated for 7 min in potassium glutamate buffer containing 0.1 μM free Ca^{2+}. The medium was then replaced with a potassium glutamate buffer containing either 0.1 μM (basal) or 10 μM (stimulated) free Ca^{2+} for another 7-min period at 37 C. PI(4)P, PI(4,5)P_2, or PI(3,4,5)P_3 (final concentration, 10 μM) was added during the preincubation and incubation periods. PI(4)P and PI(4,5)P_2, which are water soluble, were dissolved directly in potassium glutamate buffer. PI(3,4,5)P_3 was first dissolved in dimethylsulfoxide at a concentration of 10 mM and then diluted to the final concentration in potassium glutamate buffer.

Immunoblotting

The cells were transfected and grown on 24-well plates under the same conditions used for the secretion assays. After 3 d, the cells were washed twice in ice-cold PBS and harvested. Cells from one well were solubilized in 200 μl sodium dodecyl sulfate sample buffer and boiled for 10 min. Equal amounts of protein were separated on polyacrylamide gels and transferred to nitrocellulose membranes. Immunostaining with specific antibodies and horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 was carried out using the enhanced chemiluminescence technique (Amersham Biosciences, Piscataway, NJ).

Assessment of PI(4,5)P_2 Levels at the Plasma Membrane

INS-1E cells were transiently cotransfected with GFP-tagged PH-PLC and the indicated silencers. After 3 d in culture, the cells were washed and incubated under the same conditions as those used for the secretion assays. After 45 min, they were fixed and analyzed by confocal microscopy (Leica model TCS NT, Lasertechnik, Heidelberg, Germany). Images acquired through the center of the transfected cells were used to determine the relative fluorescence intensity of GFP-tagged PH-PLC at the plasma membrane and in the cytosol using Scion Image software (Scion Corp., Frederick, MD).

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REFERENCES

1. Straub SG, Sharp GW 2002 Glucose-stimulated signaling pathways in biphasic insulin secretion. Diabetes Metab Res Rev 18:451–463
2. Wollheim CB, Maechler P 2002 β-Cell mitochondria and insulin secretion: messenger role of nucleotides and metabolites. Diabetes 51:S37–S42
3. Rorsman P, Renstrom E 2003 Insulin granule dynamics in pancreatic β cells. Diabetologia 46:1029–1045
4. Pratley RE, Weyer C 2001 The role of impaired early insulin secretion in the pathogenesis of type II diabetes mellitus. Diabetologia 44:929–945
5. Easom RA 2000 β-Granule transport and exocytosis. Semin Cell Dev Biol 11:253–266
6. Chernomordik LV, Kozlov MM 2003 Protein-lipid interplay in fusion and fission of biological membranes. Annu Rev Biochem 72:175–207
7. Martin TF 1997 Phosphoinositides as spatial regulators of membrane traffic. Curr Opin Neurobiol 7:331–338
8. Takenawa T, Itoh T 2001 Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. Biochim Biophys Acta 1533:190–206
9. De Matteis MA, Godi A 2004 PI-loting membrane traffic. Nat Cell Biol 6:487–492
10. Wenk MR, De Camilli P 2004 Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals. Proc Natl Acad Sci USA 101:8262–8269
11. Olsen HL, Hoy M, Zhang W, Bertorello AM, Bokvist K, Capito K, Efanov AM, Meister B, Thams P, Yang SN, Rorsman P, Berggren PO, Gromada J 2003 Phosphatidylinositol 4-kinase serves as a metabolic sensor and regulates priming of secretory granules in pancreatic β cells. Proc Natl Acad Sci USA 100:5187–5192
12. Varnai P, Balla T 1998 Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[^{3}H]inositol-labeled phosphoinositide pools. J Cell Biol 143:501–510
13. Balla T 1998 Phosphatidylinositol 4-kinases. Biochim Biophys Acta 1436:69–85
14. Godi A, Pertile P, Meyers R, Marra P, Di Tullio G, Iurisci C, Luini A, Corda D, De Matteis MA 1999 ARF mediates recruitment of PtdIns-4-OH kinase-β and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. Nat Cell Biol 1:280–287
15. Taverna E, Francolini M, Jeromin A, Hilfiker S, Roder J, Rosa P 2002 Neuronal calcium sensor 1 and phosphatidylinositol 4-OH kinase β interact in neuronal cells and are translocated to membranes during nucleotide-evoked exocytosis. J Cell Sci 115:3909–3922
16. Rajebhosale M, Greenwood S, Vidugiriene J, Jeromin A, Hilfiker S 2003 Phosphatidylinositol 4-OH kinase is a downstream target of neuronal calcium sensor-1 in enhancing exocytosis in neuroendocrine cells. J Biol Chem 278:6075–6084
17. Kapp-Barnea Y, Melnikov S, Shefler I, Jeromin A, Sagi-Eisenberg R 2003 Neuronal calcium sensor-1 and phosphatidylinositol 4-kinase β regulate IgE receptor-triggered exocytosis in cultured mast cells. J Immunol 171: 5320–5327
18. Hannon GJ 2002 RNA interference. Nature 418:244–251
19. Lilla V, Webb G, Rickenbach K, Maturana A, Steiner DF, Halban PA, Irminger JC 2003 Differential gene expression in well-regulated and dysregulated pancreatic β-cell (MIN6) sublines. Endocrinology 144:1368–1379
20. Ishihara H, Shibasaki Y, Kizuki N, Wada T, Yazaki Y, Asano T, Oka Y 1998 Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and

deletion/substitution analysis of members of this novel lipid kinase family. J Biol Chem 273:8741–8748
21. Wenk MR, Pellegrini L, Klenchin VA, Di Paolo G, Chang S, Daniell L, Arioka M, Martin TF, De Camilli P 2001 PIP kinase Iγ is the major PI(4,5)P(2) synthesizing enzyme at the synapse. Neuron 32:79–88
22. Di Paolo G, Moskowitz HS, Gipson K, Wenk MR, Voronov S, Obayashi M, Flavell R, Fitzsimonds RM, Ryan TA, De Camilli P 2004 Impaired PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking. Nature 431:415–422
23. Gong LW, Di Paolo G, Diaz E, Cestra G, Diaz ME, Lindau M, De Camilli P, Toomre D 2005 Phosphatidylinositol phosphate kinase type Iγ regulates dynamics of large dense-core vesicle fusion. Proc Natl Acad Sci USA 102: 5204–5209
24. Hughes WE, Elgundi Z, Huang P, Frohman MA, Biden TJ 2004 Phospholipase D1 regulates secretagogue-stimulated insulin release in pancreatic β-cells. J Biol Chem 279:27534–27541
25. Brown FD, Thompson N, Saqib KM, Clark JM, Powner D, Thompson NT, Solari R, Wakelam MJ 1998 Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. Curr Biol 8:835–838
26. Du G, Altshuller YM, Vitale N, Huang P, Chasserot-Golaz S, Morris AJ, Bader MF, Frohman MA 2003 Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. J Cell Biol 162:305–315
27. Walent JH, Porter BW, Martin TFJ 1992 A novel 145 kd brain cytosolic protein reconstitutes Ca^{2+}-regulated secretion in permeable neuroendocrine cells. Cell 70: 765–775
28. Loyet KM, Kowalchyk JA, Chaudhary A, Chen J, Prestwich GD, Martin TF 1998 Specific binding of phosphatidylinositol 4,5-bisphosphate to calcium-dependent activator protein for secretion (CAPS), a potential phosphoinositide effector protein for regulated exocytosis. J Biol Chem 273:8337–8343
29. Tandon A, Bannykh S, Kowalchyk JA, Banerjee A, Martin TFJ, Balch WE 1998 Differential regulation of exocytosis by calcium and CAPS in semi-intact synaptosomes. Neuron 21:147–154
30. Elhamdani A, Martin TFJ, Kowalchyk JA, Artalejo CR 1999 Ca^{2+}-dependent activator protein for secretion is critical for the fusion of dense-core vesicles with the membrane in calf adrenal chromaffin cells. J Neurosci 19:7375–7383
31. Rupnik M, Kreft M, Sikdar SK, Grilc S, Romih R, Zupancic G, Martin TF, Zorec R 2000 Rapid regulated dense-core vesicle exocytosis requires the CAPS protein. Proc Natl Acad Sci USA 97:5627–5632
32. Grishanin RN, Kowalchyk JA, Klenchin VA, Ann K, Earles CA, Chapman ER, Gerona RR, Martin TF 2004 CAPS acts at a prefusion step in dense-core vesicle exocytosis as a PIP2 binding protein. Neuron 43:551–562
33. Okamoto M, Sudhof TC 1997 Mints, Munc18-interacting proteins in synaptic vesicle exocytosis. J Biol Chem 272: 31459–31464
34. Ho A, Morishita W, Hammer RE, Malenka RC, Sudhof TC 2003 A role for Mints in transmitter release: Mint 1 knockout mice exhibit impaired GABAergic synaptic transmission. Proc Natl Acad Sci USA 100:1409–1414
35. Biederer T, Sudhof TC 2000 Mints as adaptors. Direct binding to neurexins and recruitment of munc18. J Biol Chem 275:39803–39806
36. Zhang W, Lilja L, Bark C, Berggren PO, Meister B 2004 Mint1, a Munc-18-interacting protein, is expressed in insulin-secreting β-cells. Biochem Biophys Res Commun 320:717–721
37. Lawrence JT, Birnbaum MJ 2003 ADP-ribosylation factor 6 regulates insulin secretion through plasma membrane phosphatidylinositol 4,5-bisphosphate. Proc Natl Acad Sci USA 100:13320–13325
38. Gromada J, Bark C, Smidt K, Efanov AM, Janson J, Mandic SA, Webb DL, Zhang W, Meister B, Jeromin A, Berggren PO 2005 Neuronal calcium sensor-1 potentiates glucose-dependent exocytosis in pancreatic β cells through activation of phosphatidylinositol 4-kinase β. Proc Natl Acad Sci USA 102:10303–10308
39. Aikawa Y, Martin TF 2003 ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis. J Cell Biol 162:647–659
40. Vitale N, Caumont AS, Chasserot-Golaz S, Du G, Wu S, Sciorra VA, Morris AJ, Frohman MA, Bader MF 2001 Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. EMBO J 20:2424–2434
41. Grishanin RN, Klenchin VA, Loyet KM, Kowalchyk JA, Ann K, Martin TF 2002 Membrane association domains in Ca^{2+}-dependent activator protein for secretion mediate plasma membrane and dense-core vesicle binding required for Ca^{2+}-dependent exocytosis. J Biol Chem 277: 22025–22034
42. Zhang W, Efanov A, Yang SN, Fried G, Kolare S, Brown H, Zaitsev S, Berggren PO, Meister B 2000 Munc-18 associates with syntaxin and serves as a negative regulator of exocytosis in the pancreatic β-cell. J Biol Chem 275:41521–41527
43. Brummelkamp TR, Bernards R, Agami R 2002 A system for stable expression of short interfering RNAs in mammalian cells. Science 296:550–553
44. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS 2002 Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 16:948–958
45. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB 1992 Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology 130:167–178
46. Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P 2004 Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. Endocrinology 145:667–678

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