The C2 Domains of Rabphilin3A Specifically Bind Phosphatidylinositol 4,5-Bisphosphate Containing Vesicles in a Ca\(^{2+}\)-dependent Manner

IN VITRO CHARACTERISTICS AND POSSIBLE SIGNIFICANCE* 

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In the present study we investigated the lipid binding characteristics of the C2 domains of Rabphilin3A. We found that the tandem C2 domain of Rabphilin3A specifically bound lipid vesicles containing phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)\(_{2}\)) in a Ca\(^{2+}\)-dependent manner. There was little binding to vesicles containing PtdIns(3,4)\(_{2}\) in the presence or absence of Ca\(^{2+}\). Binding to phosphatidylinositol 3,4,5-triphosphate-containing vesicles was similar to binding to PtdIns(4,5)\(_{2}\)-containing vesicles. The presence of physiological amounts of phosphatidylserine (PS) greatly potentiated the ability of PtdIns(4,5)\(_{2}\) to cause vesicle binding. As with the C2 domains together, the binding of individual C2 domain of Rabphilin3A was much greater to PtdIns(4,5)\(_{2}\)-containing vesicles than PtdIns(3,4)\(_{2}\)-containing vesicles. Both C2 domains also bound 29 mol % PS-containing vesicles in a Ca\(^{2+}\)-dependent manner. Because of the importance of the C2B domain in the enhancement of secretion from chromaffin cells by Rabphilin3A, its biochemistry was further investigated. The mutation of aspartates 657 and 659 to asparagines in C2B decreased Ca\(^{2+}\)-dependent and increased Ca\(^{2+}\)-independent vesicle binding, indicating the Ca\(^{2+}\)-dependence of the domain is provided by aspartic acid residues in the putative Ca\(^{2+}\)-binding pocket. A peptide from the COOH-terminal region of the C2B domain specifically inhibited ATP-dependent secretion from permeabilized chromaffin cells and the binding of Rabphilin3A to phosphatidylcholine/PS/PtdIns(4,5)\(_{2}\)-containing lipid vesicles, suggesting a role of this sequence in secretion through its ability to interact with acidic lipid vesicles.

The Rab3 family is part of a large class of low molecular weight GTPases that are essential for vesicular trafficking in the protein secretory and endocytic pathways. Rab3a and Rab3c are expressed mainly in tissues with a highly differentiated Ca\(^{2+}\)-dependent secretory pathway, including neurons and chromaffin cells, suggesting a role for Rab3a in secretion. Indeed, several types of experiments indicate that Rab3a is a negative modulator of secretion (1–3) and may be involved in neuronal plasticity (4). Rabphilin3A was identified in brain as a possible effecter protein for Rab3a (5). It binds specifically to the GTP-bound form of Rab3a and Rab3c (5, 6). Cloning of Rabphilin3A revealed a 704-amino acid protein in bovine brain (5) and a 710-amino acid splice variant in bovine chromaffin cells (7). Rab3 and Rabphilin3A are found on synaptic vesicles and chromaffin granules where they can interact (6, 8–10).

Rabphilin3A is composed of at least two functionally distinct domains: the amino-terminal region (1–286 amino acids) which contains the Rab3a-binding domain (51–190 amino acids) (5, 11–13), and the carboxy-terminal region (287–710 amino acids) which contains two C2 domains (see Fig. 1). Rabphilin3A and the COOH-terminal fragment that contains two C2 domains bind phosphatidylserine (PS)\(^{1}\) in the presence of Ca\(^{2+}\) (14). The NH\(_{2}\)-terminal fragment without the C2 domains does not bind phospholipid (14). Overexpression of Rabphilin3A by transient transfection of CDNA enhanced secretion and transfection with antisense Rabphilin3A CDNA-inhibited secretion, suggesting that Rabphilin3A is likely to be a positive regulator of secretion (7). Deletion of C2B or both C2A and C2B transformed Rabphilin3A from a protein that enhanced secretion to one that profoundly inhibited secretion. The results indicated that the C2B domain is critical for the normal function of Rabphilin3A in secretion.

C2 domains are Ca\(^{2+}\) and acidic phospholipid-binding domains of approximately 130 amino acid residues (for review, see Ref. 15) which were first identified in the Ca\(^{2+}\)-dependent isoforms of protein kinase C (16). Subsequently, it was discovered that not all C2 domains support Ca\(^{2+}\)-dependent lipid binding. Tandem C2 domains (e.g. C2A and C2B) are found in Rabphilin3A, synaptotagmin, and Doc2. All three proteins have been implicated in neurotransmitter release (7, 17, 18). The characteristics of the C2 domains of synaptotagmin I has been especially well characterized. C2A binds phospholipid and syn-

1 The abbreviations used are: PS, phosphatidylserine; GST, glutathione S-transferase; [H]IPC, [\(^{3}H\)]phosphatidylcholine–[\(^{3}H\)]phosphatidylycholine; hGH, human growth hormone; PIPES, 1,4-piperazinediethanesulfonic acid; PC, phosphatidylcholine; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,4)P\(_{2}\), phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P\(_{2}\), phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P\(_{3}\), phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4)P 5-kinase, phosphatidylinositol 4-phosphate 5-kinase; Rp, Rabphilin3A; CAPS, Ca\(^{2+}\)-dependent activator protein for secretion.
taxin in a Ca2+-dependent manner (19, 20), whereas C2B binds the clathrin assembly protein AP2 (21), β-SNAP (22), and inositol polyphosphates (23, 24) irrespective of the presence of Ca2+. A 32-residue peptide of the base-rich binding motif of the synaptotagmin II C2B domain was established by photoaffinity labeling as necessary and sufficient for binding of inositol polyphosphates (25). The C2B domain is also responsible for Ca2+-dependent synaptotagmin I dimerization (26).

Evidence has accumulated that the polyphosphoinositides are necessary for steps in vesicular trafficking (for a review paper, see Ref. 27). Indeed, several years ago we demonstrated that the requirement of ATP for secretion in bovine chromaffin cells could be partially explained by the need to maintain polyphosphoinositides (28). These findings have since been strongly corroborated by findings that phosphatidylinositol transfer protein and phosphatidylinositol 4-phosphate (PtdIns(4)P) 5 kinase are both required for ATP-dependent secretion from PC12 cells (29, 30).

Because of the ability of the carboxyl-terminal domain of Rabphilin3a to bind vesicles with acidic phospholipids and the requirement for the polyphosphoinositides in secretion, we have investigated the ability of Rabphilin3a to bind lipid vesicles containing various polyphosphoinositides. As this work was in progress, it was found that the C2B domain of synaptotagmin I interacts with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) (31). In the present study we found that two domains of Rabphilin3a specifically bind vesicles containing PtdIns(4,5)P2 in a Ca2+-dependent manner and that the presence of physiological amounts of PS greatly potentiates the ability of PtdIns(4,5)P2-containing vesicles to bind. Furthermore, a peptide Rp(676–695) specifically inhibits ATP-dependent secretion from permeabilized chromaffin cells and the binding of Rabphilin3a to PC/PS/PtdIns(4,5)P2-containing lipid vesicles, consistent with a role of the sequence in Rabphilin3a in secretion through its ability to interact with acidic lipid vesicles.

**Experimental Procedures**

**Materials—** α-L-Phosphatidylcholine (brain PC) and α-L-phosphatidylserine (brain PS) were obtained from Avanti Polar Lipids (Alabaster, AL). α-L-phosphatidylinositol 4-monophosphate (PtdIns(4)P), 1,2-diacyl-sn-glycero-3-phosphate-1-D-myoinositol 4-phosphate) and α-L-phosphatidylinositol 4,5-diphosphate (PtdIns(4,5)P2, 1,2-diacyl-sn-glycero-3-phospho-1-D-myoinositol 4,5-bisphosphate) were purchased from Sigma. 1-(Cys) Dipalmitoyl-[methylycholine]-H-phosphatidylinositol (HPC, specific activity, 60 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Dipalmitoyl derivatives of PtdIns(4)P, PtdIns(4,5)P2, PtdIns(3,4,5)P3, and PtdIns(3,4)P2 were synthesized as described (32–35). 1-[7,8-3H]-Norephinephrine (specific activity, 12 Ci/mmol) was purchased from Amersham (United Kingdom). Peptides that are homologous to various regions of Rabphilin3a and synaptotagmin were a gift from Dr. Teresa L. Burgess (Amgen, Thousand Oaks, CA). They were synthesized with biotin on the NH2 terminus and purified determined by high pressure liquid chromatography and mass spectral analysis. The peptide corresponding to RP676–695 was also synthesized at the University of Michigan Protein and Carbohydrate Structure Facility and had the same effects on secretion as the peptide synthesized by Amgen.

**Construction of Expression Vectors and Purification of Recombinant Proteins—** The deletion mutants of Rabphilin3a were made by polymerase chain reaction from plasmid containing full-length bovine chromaffin cell Rabphilin3a (7). Primers were designed to generate 5′-BamHI and 3′-EcoRI restriction sites. Polymerase chain reaction products were digested with BamHI and EcoRI and subcloned into pGEX-2T (Pharmacia) and pCMV-hemagglutinin (pCMV-MA), a mammalian expression vector that is under the control of human cytomegalovirus immediate-early promoter and contains the hemagglutinin epitope (YPDPDYFV-DYA). The construction of mutant protein GST-RpC2B,DE57N,DE59N was made using the Altered Sites in vitro mutagenesis system (Promega) and the sequence was confirmed by a Thermo Sequenase radio-labeled terminator cycle sequencing kit (Amersham). The pGEX-2T vectors containing the cDNA sequences encoding C2 domains of Rabphilin3a were expressed in *Escherichia coli* HB 101 cells as GST fusion proteins by isopropyl-β-D-thiogalactoside induction. Bacterial lysates were incubated with glutathione-Sepharose 4B beads (Pharmacia) for 2 h at 4 °C and washed several times with 1 × phosphate-buffered saline and then with buffer A (100 mM KCl, 20 mM PIPES, pH 7.0). Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). They were synthesized with biotin on the NH2 terminus and purity determined by high pressure liquid chromatography.

**Assay for the Binding of Lipid Vesicles to Recombinant Proteins—** In all experiments measuring phospholipid binding, phospholipid vesicles contained [3H]PC. Phospholipids were mixed (total 180 μg/ml) and dried under a stream of nitrogen. Dried phospholipids were resuspended in 1–5 ml of buffer A (100 mM KCl, 20 mM PIPES, pH 7.0) by vortexing and sonicated for 30 s using a probe sonicator. The lipid vesicles were briefly centrifuged before use to remove aggregates, stored at 4 °C, and used within 1 week. In most of the figures and the text, the relative amounts of lipids in the vesicles is expressed as mole %.

Lipid vesicles of 0.1 μm diameter prepared by extrusion through Nucleopore filter gave similar results to those prepared by sonication. GST or GST-Rp mutants containing the C2 domain of Rabphilin3a attached to glutathione-Sepharose 4B beads (1–4 μg protein) were incubated at room temperature with [3H]PC-labeled phospholipid vesicles (total 18 μg/assay, approximately 200,000 cpm except for peptide competition experiments which used 3 μg/assay) in 200 μl of buffer A containing 5 mM EGTA and 0.5 mM Ca2+. After 20 min shaking, the beads were washed three times with 1 ml of the buffer A in the continuing presence or absence of Ca2+. Lipid binding associated with the beads was quantified by liquid scintillation counting. Because there was quantitative variation between batches of protein, results were confirmed with several independent protein preparations.

**Chromaffin Cell Preparation, Transfection, Secretion, and Immunocytochemistry—** Chromaffin cell preparation, transfection, secretion, and immunocytochemistry were performed as described previously (1, 36). Digitonin-permeabilized secretion experiments were performed 4–7 days after the plating of cells in 96-well plates. Immediately before secretion experiments cells were incubated for 3–5 h in culture medium containing [3H]norephinephrine and 0.5 mM ascorbate. Cells were washed for at least 30 min with a phosphate-buffered saline containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, 15 mM HEPES (pH 7.4), 0.5 mM ascorbate, and 0.5% bovine serum albumin. To permeabilize cells, chromaffin cells were treated for 4–6 min with 20 μM digitonin in potassium glutamate solution (KGEP, 139 mM potassium glutamate, 5 mM EGTA, 20 mM PIPES, pH 6.8) containing 2 mM MgATP (unless otherwise specified) and 0.5% bovine serum albumin. Secretion was subsequently determined during 12–18 min incubation in KGEP without digitonin, with 2 mM MgATP (unless otherwise specified), and with or without buffered Ca2+ concentrations of 30 μM (for Ca2+ dose-response curve 1, 30, and 300 μM). To determine the peptide effect on secretion, varying concentrations of peptides were present during digitonin-permeabilization and following incubation. At the end of experiments, the incubation solution was removed, and the cells were lysed with 1% Triton X-100. The radioactivity released into the incubation solution and the radioactivity remaining in the cell were determined by liquid scintillation counting. Experiments were performed at 27 °C. Data are usually presented as mean ± S.E. of the mean with 3–4 samples per group.

**Results**

**The Carboxyl-terminal Region of Rabphilin3a Binds PtdIns(4,5)P2 in a Ca2+-dependent Manner That Is Potentiated by PS—** GST or GST-Rp(287–710) (with both C2 domains, Fig. 1) were attached to beads and incubated with PC vesicles containing various concentrations of PtdIns(4,5)P2 in the absence or presence of Ca2+. Rp(287–710) bound in a Ca2+-dependent manner to PC vesicles containing low amounts of PtdIns(4,5)P2 (Fig. 2A). Significant binding occurred at 1.4 mol % PtdIns(4,5)P2. This concentration of PtdIns(4,5)P2 is considerably less than the 29 mol % of PS necessary to support Ca2+-dependent binding of Rabphilin3a (Fig. 2B).

Addition of PtdIns(4,5)P2 to PC vesicles introduces negative surface charge. In order to reduce the relative contribution of PtdIns(4,5)P2 to the negative surface charge, the effect of PS on the binding of Rabphilin3a to PtdIns(4,5)P2-containing vesicles was investigated. The presence of 19 mol % PS, which alone does not support binding to Rabphilin3a (Fig. 2B), greatly
enhanced the ability of PtdIns(4,5)P₂-containing vesicles to bind Rp(287–710) (Fig. 2C). Substantial increases in binding occurred at 0.36 mol % or 0.72 mol % PtdIns(4,5)P₂, which without PS does not support binding to Rabphilin3a (Fig. 2A). Similar results were obtained with vesicles containing 9.5 mol % PS (data not shown). The experiments demonstrate that low concentrations of PtdIns(4,5)P₂ can regulate the ability of the carboxyl-terminal region of Rabphilin3a to bind to membranes.

The PtdIns(4,5)P₂ Binding of Rabphilin3a Is Specific—To determine whether Rabphilin3a binding to PtdIns(4,5)P₂-containing lipid vesicles is specific, we investigated the ability of Rabphilin3a to bind vesicles containing various polyphosphoinositides (3–5 mol %). Incorporation of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ into PC vesicles caused significant increases in both Ca²⁺-dependent and -independent binding of lipid vesicles to Rp(287–710) (Fig. 3). Vesicles containing PS, PtdIns(4)P, or PtdIns(3,4)P₂ did not bind significantly to Rp(287–710). The specificity of binding for PtdIns(4,5)P₂ compared with PtdIns(3,4)P₂-containing vesicles was also evident with vesicles containing 9.5 mol % PS and 0.71 mol % polyphosphoinositides (Fig. 3, inset). The much greater binding of Rabphilin3a to PtdIns(4,5)P₂-containing vesicles compared with PtdIns(3,4)P₂-containing vesicles indicates that the configuration of the phosphate groups on the inositol ring is important for interaction with Rabphilin3a.

Both C2 Domains of Rabphilin3a Separately Bind Acidic Phospholipid in a Ca²⁺-dependent Manner—Rabphilin3a contains tandem C2 domains (C2A and C2B). To determine the lipid specificity of binding of each of the C2 domains separately, GST, GST-RpC2A (Rp(402–536)), or GST-RpC2B (Rp(537–710)) was attached to beads and incubated with lipid vesicles of various compositions (Fig. 4). Each of the C2 domains alone showed the same specificity for the polyphosphoinositides as the carboxyl-terminal segment (Rp(287–710)) containing both C2 domains (Fig. 3). Both C2A and C2B bound most strongly in a Ca²⁺-dependent manner to lipid vesicles containing 3–4 mol % of either PtdIns(4,5)P₂ or PtdIns(3,4,5)P₃. There was little binding to PtdIns(3,4)P₂ and no Ca²⁺-dependent binding to PtdIns(4)P.

The characteristics of the lipid binding of the C2A and C2B were not identical. As shown in Fig. 4C, the Ca²⁺-dependent binding of C2A to lipid vesicles was stimulated by extremely low concentrations of PtdIns(4,5)P₂ (0.36 mol % in the absence of PS). The C2B domain requires approximately 10 times more PtdIns(4,5)P₂ for comparable Ca²⁺-dependent binding (data not shown). The lipid binding characteristics of C2A and C2B were further compared in Fig. 5. Both C2A and C2B bound lipid vesicles containing 29 mol % PS, although neither bound the vesicles as well as an equimolar amount of Rp(287–710) which contains both C2 domains (Fig. 5A). A consistent finding was that C2B bound more strongly than C2A to vesicles containing 29 mol % PS both in the absence and presence of Ca²⁺. C2A and C2B bound similarly vesicles containing 3.6 mol % PtdIns(4,5)P₂ (Fig. 5B) or containing 19 mol % PS and 0.72 mol % PtdIns(4,5)P₂ (Fig. 5C).

The Ability of the C2B to Bind to Acidic Phospholipids in a Ca²⁺-dependent Manner Requires Aspartic Acid Residues in a Putative Ca²⁺-binding Pocket—Because of the importance of the C2B domain in the enhancement of secretion from chromaffin cells by Rabphilin3a (7), the biochemistry of the C2B domain was further investigated. Analysis of calcium binding to C2B domain of synaptotagmin revealed a bipartate calcium-binding motif that involves the coordination of two calcium ions by five conserved aspartate residues located on separate loops (37, 38). To examine the importance of aspartic acid residues for Ca²⁺-dependent lipid binding of C2B domain of Rabphilin3a, Asp-657 and Asp-659 (see Fig. 1) were replaced with asparagine (Asn) by site-directed mutagenesis. These residues correspond to Asp-230 and -232, respectively, in the C2A domain of synaptotagmin, each of which coordinate two Ca²⁺ ions in the binding pocket. The GST-RpC2B,D657N,D659N mutant was well expressed in bacteria (Fig. 5D). The mutant protein bound acidic vesicles in the absence of Ca²⁺ but lipid binding was only minimally enhanced by Ca²⁺ (Table I). The results, therefore, suggest that the mutated aspartates are critical for providing Ca²⁺ dependence of lipid binding to the C2B domain.

To investigate the role of Asn-657 and Asn-659 in the ability of overexpressed Rabphilin3a to enhance secretion, a plasmid encoding HA1-Rp(1–710,D657N,D659N) was transiently transfected together with a plasmid encoding hGH in bovine chromaffin cells. No effect on hGH secretion was detected (data...
not shown). However, the mutated protein was not stable in the cell. Whereas at least 85% of the hGH-positive cells coexpressed HA1-tagged, wild type Rabphilin3a in similar experiments, only 30% of the hGH-positive cells transfected with the plasmid encoding Rp(1–710,D657N,D659N) coexpressed the mutant protein. Because of the poor expression in chromaffin cells, it is uncertain whether the mutant is without effect on secretion.

The Effects of Peptides Derived from Rabphilin3a Suggest a Specific Role for a Basic Peptide in C2B in Secretion and Binding to PS/PtdIns(4,5)P2-containing Lipid Vesicles—A large number of peptides of approximately 20 amino acids that are homologous to various regions in Rabphilin3a were generated. In addition, several peptides homologous to regions in synaptotagmin were also synthesized. These peptides were tested for their ability to inhibit Ca\(^{2+}\)-dependent secretion from digitonin-permeabilized cells. Of the 18 peptides tested, the only peptide that significantly inhibited secretion was Rp(676–695) (Table II). The peptide Rp(676–695) (abbreviated P676, AKGERLKHWECLKNKDKKI) significantly inhibited secretion from digitonin-permeabilized chromaffin cells with detectable inhibition by 100 \(\mu\)M peptide and substantial inhibition at 300 \(\mu\)M peptide (Fig. 6A). P676 is from the \(\beta\)-strand 8 region of C2B (15, 37) and has 8 positive and 3 negatively charged amino acids. The inhibition was not solely due to the basic nature of the peptide since a peptide with the identical amino acids but in a scrambled sequence was without effect on secretion (Fig. 6A).

Furthermore, another highly basic peptide derived from the middle region of C2B, Rp(446–465), another strongly basic peptide, and Rp(611–630) did not alter secretion.

The experiment in Fig. 7A was performed in the presence of MgATP. We had previously found that secretion from chromaffin cells has two distinct components: a rapid, late, MgATP-independent step in which exocytosis is triggered by Ca\(^{2+}\) and an earlier, slower step in which secretion is primed by MgATP (39, 40). Fig. 7B demonstrates that P676 completely inhibited the ATP-dependent component of secretion with only a small inhibition of the ATP-independent component. In two experiments, the average inhibition of ATP-dependent secretion was 96% and of ATP-independent secretion was 34%. The specificity for inhibition of ATP-dependent secretion was also reflected in the Ca\(^{2+}\) dose-response curve in the presence of MgATP (Fig. 6A). Furthermore, another highly basic peptide derived from the middle region of C2B, Rp(611–630) (abbreviated P611, KPDMGKKAKHKTQIKKKTLN) with 9 positive and 1 negatively charged amino acids, also had no effect on secretion (Fig. 6B). A peptide composed of its scrambled amino acids was also without effect on secretion.

The effect of P676 was further investigated on secretion from permeabilized cells. The inhibition of secretion caused by P676 was not overcome by increasing the Ca\(^{2+}\) concentration (Fig. 7A). Rp(446–465), another strongly basic peptide, and Rp(611–630) did not alter secretion.
The lesser secretion stimulated by 300 μM compared to 30 μM Ca\(^{2+}\) in the absence of P676 is characteristic of the ATP-dependent component of secretion (39). (The mechanism for the inhibition is unknown.) The inability of 300 μM Ca\(^{2+}\) to reduce secretion in the presence of P676 is predicted if the peptide selectively inhibits ATP-dependent secretion.

We determined whether the specific inhibitory effect of P676 on secretion is related to the ability of the peptide to bind to C2A and C2B domains of Rabphilin3a individually bind PS- or PtdIns(4,5)P\(_2\)-containing vesicles in a Ca\(^{2+}\)-dependent manner. GST or GST fusion proteins of RpC2B (537–710), C2A (402–536), or C2B (537–710) was incubated with PC vesicles containing 29 mol % PS (A), 3.6 mol % PtdIns(4,5)P\(_2\) (B), or 19 mol % PS and 0.72 mol % PtdIns(4, 5)P\(_2\) (C) in the absence (open bars, 1 mM EGTA) or presence of Ca\(^{2+}\) (filled bars, 100 μM Ca\(^{2+}\)). D, expression of GST fusion constructs of Rabphilin3a. The indicated GST fusion proteins (1.5 μg/lane) were expressed in bacteria, purified, and subjected to 12% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue.

| Experiment | RpC2B [H]PC bound (cpm) | RpC2B[D657N,D659N] [H]PC bound (cpm) | ΔCa (cpm) | RpC2B[D657N,D659N] [H]PC bound (cpm) | ΔCa (cpm) |
|------------|--------------------------|--------------------------|------------|--------------------------|------------|
| 1          | 2,027                    | 14,305                   | 12,278     | 6,327                    | 9,575      |
| 2          | 5,521                    | 11,611                   | 6,090      | 9,473                    | 8,303      |
| 3          | 2,554                    | 14,726                   | 12,172     | 5,071                    | 12,136     |
| 4          | 6,376                    | 17,281                   | 10,905     | 12,183                   | 13,437     |
| Average ± S.E. | 4,120 ± 1,076 | 14,481 ± 1,161 | 10,361 ± 1,458 | 8,257 ± 1,601 | 10,653 ± 1,171 | 2,599 ± 1,741 |

The Table 1 shows the comparison of binding of RpC2B and RpC2B,D657N,D659N to PC/PS/PtdIns(4,5)P\(_2\) (80.2:19.1:0.7) lipid vesicles.

GST-RpC2B (537–710) or GST-RpC2B,D657N,D659N (Asp\(^{657}\) and Asp\(^{659}\) were replaced with asparagine by site-directed mutagenesis) attached to beads was incubated with the lipid vesicles consisting of PC/PS/PtdIns(4,5)P\(_2\) (80.2:19.1:0.7) in the absence (–Ca) or presence of 100 μM Ca\(^{2+}\)+(+Ca). Ca\(^{2+}\)-dependent binding (ΔCa) indicates the difference between binding in the presence of Ca\(^{2+}\) and binding in the absence of Ca\(^{2+}\). Values represent the mean of duplicate or triplicate determinations. Four experiments were shown, each using an independent preparation of proteins.

7A). The lesser secretion stimulated by 300 μM compared to 30 μM Ca\(^{2+}\) in the absence of P676 is characteristic of the ATP-dependent component of secretion (39). (The mechanism for the inhibition is unknown.) The inability of 300 μM Ca\(^{2+}\) to reduce secretion in the presence of P676 is predicted if the peptide selectively inhibits ATP-dependent secretion.

We determined whether the specific inhibitory effect of P676 on secretion is related to the ability of the peptide to bind to C2A and C2B domains of Rabphilin3a individually bind PS- or PtdIns(4,5)P\(_2\)-containing vesicles in a Ca\(^{2+}\)-dependent manner. GST or GST fusion proteins of RpC2B (537–710), C2A (402–536), or C2B (537–710) was incubated with PC vesicles containing 29 mol % PS (A), 3.6 mol % PtdIns(4,5)P\(_2\) (B), or 19 mol % PS and 0.72 mol % PtdIns(4, 5)P\(_2\) (C) in the absence (open bars, 1 mM EGTA) or presence of Ca\(^{2+}\) (filled bars, 100 μM Ca\(^{2+}\)). D, expression of GST fusion constructs of Rabphilin3a. The indicated GST fusion proteins (1.5 μg/lane) were expressed in bacteria, purified, and subjected to 12% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue.
**TABLE II**

Effects of peptides on Ca\(^{2+}\)-dependent secretion from digitonin-permeabilized chromaffin cells

| Peptide       | Sequence                                      | Relative secretion | Notes                     |
|---------------|-----------------------------------------------|--------------------|---------------------------|
| Rabphilin3a   |                                              |                    |                           |
| 54–73         | EKEIQVRIAAREKMEEMEQ                           | 1.03 (1)           | Rab3a-binding domain      |
| 61–80         | VIARAEKMEEMEQERIGRLV                         | 1.05 (1)           | Rab3a-binding domain      |
| 81–97         | DRLENMKNVAGDGVNR                             | 0.97 (1)           | Rab3a-binding domain      |
| 171–190       | MIPKKCLKQPPQVSEPVPAA                          | 0.98 (1)           |                           |
| 195–215       | EPKHPAPABTRVFSLACGDT                         | 1.10 (1)           |                           |
| 288–309       | RPAPASMQSAPPQPGQPGPG                          | 1.00 (1)           | Proline-rich domain       |
| 356–375       | AGTREDRAGHPPGYSYTQASA                        | 1.07 (2)           |                           |
| 366–405       | RQPFPPEEDEEEAANYSDE                          | 1.20 (2)           | Highly negatively charged  |
| 445–465       | PYVKLHLLPGASKSNKLRTK                         | 0.90 (2)           | Basic peptide in C2A (+6) |
| 611–630       | KPDFKKAKHTQIKKTLNPFENE                       | 0.91 ± 0.05 (4)    | Highly basic peptide in C2B (+8) |
| 617–636       | KAKHTQIKKTLNPFENE                            | 1.04 (1)           | PEP20 homologue in C2B (+4) |
| 676–695       | AKGERLKHWYELCNDKKIK                          | 0.40 ± 0.05 (6)    | Basic COOH-terminal peptide in C2B (+5) |
| 696–710       | IERWHQLQNENHVSSD                             | 0.93 (1)           | COOH-terminal peptide     |
| S611–630      | IKKTHKTPKLGKDNKMQA                            | 0.94 (1)           | Scrambled peptide for 611–630 (+8) |
| S676–695      | KERDRKWECKHLKNYAGK                           | 1.07 (2)           | Scrambled peptide for 676–695 (+5) |
| Synaptotagmin |                                              |                    |                           |
| hStgI(383–402)| STGAELRHWSDMLANPRPFI                        | 0.81 (2)           | Homologue for Rp(676–695) |
| StgIII(480–499)| LKKRTS1KKNTLNPYNEA                         | 1.05 ± 0.02 (3)    | PEP20 homologue in C2B (+5) |
| StgIII(539–558)| AADPHGREHWAELNPRK                          | 0.97 ± 0.08 (3)    | Homologue for Rp(676–695) |

**FIG. 6.** Effects of peptides on secretion from digitonin-permeabilized chromaffin cells. Bovine adrenal chromaffin cells, prelabeled with \([^{3}H]\)norepinephrine (NE), were permeabilized with 20 \(\mu\)M digitonin, 2 mM MgATP, and varying concentrations of peptide in KGEP for 6 min. Secretion was subsequently stimulated with various Ca\(^{2+}\) concentrations in the presence of 2 mM MgATP ± 300 \(\mu\)M peptide. ATP-dependent secretion (indicated by ATP-dep) is defined as the difference between secretion in the presence of MgATP (filled bars) and secretion in the absence of MgATP (open bars).

**FIG. 7.** Specificity and the Ca\(^{2+}\) and ATP dependence of the inhibition of secretion by Rp(676–695). A, bovine adrenal chromaffin cells, prelabeled with \([^{3}H]\)norepinephrine, were permeabilized with 20 \(\mu\)M digitonin, 2 mM MgATP, and varying concentrations of peptide. There were 3–4 wells/group. ATP-dependent secretion (indicated by ATP-dep) is defined as the difference between secretion in the presence of MgATP (filled bars) and secretion in the absence of MgATP (open bars).

Acidic vesicles containing PtdIns(4,5)P\(_2\), GST-Rp(287–710) attached to beads was incubated with PC vesicles containing 19 mol \% PS and 0.72 mol \% PtdIns(4,5)P\(_2\) in solutions containing varying concentrations of peptide. Fig. 8 shows that P676 specifically inhibited both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent binding of Rabphilin3a to PC/PS/PtdIns(4,5)P\(_2\) (80:2:19:1:0:7)-containing lipid vesicles. In contrast, the scrambled peptide (S676) caused little inhibition. P676 also specifically inhibited binding of Rabphilin3a to 29 mol \% PS-containing vesicles (data not shown), which indicates that the effect of
peptide was not specific for PtdIns(4,5)P$_2$. The above experiments are consistent with Rp(676–695) being able to bind to acidic phospholipids. The ability of the peptide to bind vesicles with acidic phospholipids was investigated. A short COOH-terminal peptide, GST-Rp(676–695), was expressed that included the basic domain Rp(676–695) but not the putative Ca$^{2+}$-binding domain of C2B. GST-Rp(676–710) bound PC/PS/PtdIns(4,5)P$_2$ (80.2:19.1:0.7)-containing lipid vesicles in a Ca$^{2+}$-independent manner (Fig. 8C).

To examine further the function of residues 676–695 in C2B, we attempted to express GST-Rp(537–675) without the basic moiety to examine its ability to bind acidic lipid vesicles. Because the peptide was proteolytically cleaved in the bacteria (Fig. 5D), the experiment could not be performed. We also attempted to express HA1-Rp(1–675) in chromaffin cells to test the function of the protein without the carboxyl-terminal basic sequence on secretion. The protein was unstable in the cells. Less than 5% of the hGH-positive cells coexpressed the transfected protein.

**DISCUSSION**

In a previous study we demonstrated that overexpression of full-length Rabphilin3a enhanced secretion in chromaffin cells and that removal of one or both of the C2 domains converted the protein from one that enhanced secretion to one that strongly inhibited secretion (7). The experiments highlighted the importance of the carboxyl C2 domains, especially C2B, for the function of Rabphilin3a in secretion. In the present study we investigated the lipid binding characteristics of the C2 domains together and individually. We found that both C2A and C2B domains of Rabphilin3a specifically bind PtdIns(4,5)P$_2$-containing vesicles in a Ca$^{2+}$-dependent manner, and the presence of PS greatly potentiated the ability of PtdIns(4,5)P$_2$ to cause binding. Furthermore, a peptide from the COOH-terminal region of the C2B domain specifically inhibited ATP-dependent secretion from permeabilized chromaffin cells and the binding of Rabphilin3a to PC/PS/PtdIns(4,5)P$_2$-containing lipid vesicles, suggesting a role of this sequence in secretion through its ability to interact with acidic phospholipids.

**The Carboxyl-terminal Region of Rabphilin3a with Tandem C2 Domains Binds PtdIns(4,5)P$_2$ Containing Vesicles in a Stereospecific Manner**—The tandem C2 domains (C2AB) of Rabphilin3a bound in a Ca$^{2+}$-dependent manner PC vesicles containing 1.4–3.6 mol % PtdIns(4,5)P$_2$ (Fig. 2A). Because there was little binding to vesicles containing 3.6 mol % PtdIns(3,4,5)P$_3$ (Fig. 3), the interaction of C2AB with polyphosphoinositides is likely to be caused by a stereospecific interaction. The same specificity for PtdIns(4,5)P$_2$ over PtdIns(3,4,5)P$_3$ was observed when the polyphosphoinositides were incorporated into vesicles containing 9.5–19 mol % PS (with negative charge) (Fig. 3, inset), further suggesting the importance of the 5-phosphate. Vesicles containing the more negatively charged lipid PtdIns(3,4,5)P$_3$ bound the C2 domains of Rabphilin3a no more strongly than vesicles containing PtdIns(4,5)P$_2$ (Fig. 3), thus indicating that the interaction does not simply increase with negative charge of the lipid.

The presence of the negatively charged PS in vesicles increased the ability of low concentrations of PtdIns(4,5)P$_2$ to enhance vesicle binding to the C2 domains. The addition of 0.36 mol % PtdIns(4,5)P$_2$ to 19 mol % PS-containing vesicles increased the average negative surface charge density of the vesicles by only ~5%, but greatly enhanced the ability of the vesicles to bind in a Ca$^{2+}$-dependent manner to the C2 domains of Rabphilin3a (Fig. 3C). The results highlight the importance of a specific interaction of PtdIns(4,5)P$_2$ with the C2 domains rather than a nonspecific electrostatic interaction of the vesicle surface with the protein.

The concentrations of PS and PtdIns(4,5)P$_2$ in the cytoplasmic leaflets of membranes are approximately 15–20 mol % and 0.05–0.25 mol %, respectively (41). Therefore, the above experiments suggest that the PS would render physiological membranes especially sensitive to PtdIns(4,5)P$_2$-dependent interactions with the C2 domains of Rabphilin3a.

Each C2 Domain of Rabphilin3a Can Bind Acidic Phospholipid Vesicles—It had previously been demonstrated that constructs containing both C2 domains of Rabphilin3a or C2A alone bind phosphatidyserine-containing lipid vesicles in a Ca$^{2+}$-dependent manner (14, 23). We have confirmed these findings and also found that the second C2 domain binds phosphatidyserine-containing vesicles (Fig. 5A). This finding is in contrast to an earlier study which suggested that C2B of Rabphilin3a is incapable of binding acidic phospholipid vesicles (23). We believe that the discrepancy is explained by the truncated C2B domain used in the previous study that did not contain a basic amino acid sequence critical for Ca$^{2+}$-dependent phospholipid binding (see below).

As with the combined C2AB domain, each C2 domain independently bound PtdIns(4,5)P$_2$-containing lipid vesicles in a Ca$^{2+}$-dependent manner (Fig. 5B) with specificity for PtdIns(4,5)P$_2$ over PtdIns(3,4,5)P$_3$. However, the lipid binding characteristics of C2A and C2B domains of Rabphilin3a differ in some respects. For example, Ca$^{2+}$-dependent binding of vesicles to Rabphilin3a C2A was stimulated by as little as 0.36 mol % PtdIns(4,5)P$_2$ (in the absence of PS), which was 10-fold less than for comparable Ca$^{2+}$-dependent binding of vesicles to Rabphilin3a C2B. In addition, Rabphilin3a C2B bound 29 mol

![Fig. 8. COOH-terminal peptide effects on lipid binding. A and B, Rp(676–695) specifically inhibits binding of C2AB to PS/PtdIns(4,5)P$_2$-containing vesicles, C, a related peptide directly binds lipid vesicles. GST-Rp(287–710) was incubated with the lipid vesicles composed of PC/PS/PtdIns(4,5)P$_2$ (80.2:19.1:0.7), with or without Ca$^{2+}$ (+1 mM EGTA) in the presence of various concentrations of Rp(676–695) (A) or its scrambled sequence, S(676–695) (B and C). GST-Rp(2CB/537–710), or GST-Rp(676–710) attached to beads was incubated with the lipid vesicles composed of PC/PS/PtdIns(4,5)P$_2$ (80.2:19.1:0.7) in the absence (+1 mM EGTA, open bars) or presence of Ca$^{2+}$ (100 μM Ca$^{2+}$, filled bars).](image-url)
% PS-containing vesicles more strongly than Rabphilin3a C2A.

Because of the importance of the C2B domain of Rabphilin3a in the enhancement of secretion, we explored the basis for the Ca\(^{2+}\) dependence of the domain. Based upon the analogy with the C2A domain of synaptotagmin I for which the structural basis of Ca\(^{2+}\) binding is partially understood, we mutated two aspartates, which would be predicted to coordinate Ca\(^{2+}\). Indeed, we found that mutation of aspartates 657 and 659 to asparagines in C2B greatly decreased its Ca\(^{2+}\)-dependent lipid binding (Table I). There was a concomitant increase in Ca\(^{2+}\)-independent binding of vesicles. Rp(676–710), which includes the basic amino acid motif of P676 (see below) but without the putative Ca\(^{2+}\)-binding domain, bound PC/PS/PtdIns(4,5)P\(_2\)-containing vesicles in a Ca\(^{2+}\)-independent manner (Fig. 8C). It is therefore, possible that this basic motif is necessary for lipid binding and is exposed in the mutant C2B (D657N and D659N) in the absence of Ca\(^{2+}\) and becomes exposed in the wild type C2B only in the presence of Ca\(^{2+}\).

Comparison with the Lipid Binding Characteristics of Synaptotagmin—The tandem C2 domains of synaptotagmin and Rabphilin3a differ in their lipid binding characteristics. C2A enhances the binding of synaptotagmin C2AB to PtdIns(4,5)P\(_2\)-containing vesicles but inhibits the binding to PtdIns(3,4,5)P\(_3\)-containing vesicles (31). We have confirmed these results (data not shown). In contrast, C2AB of Rabphilin3a bound both PtdIns(4,5)P\(_2\) and PtdIns(3,4,5)P\(_3\)-containing vesicles in a Ca\(^{2+}\)-dependent manner (Fig. 3). In synaptotagmin I, the C2A domain binds PS or PtdIns(4,5)P\(_2\)-containing vesicles in a Ca\(^{2+}\)-dependent manner, whereas the C2B domain binds either type of vesicle in a Ca\(^{2+}\)-independent manner (31). In Rabphilin3a, both C2A and C2B bind PS or PtdIns(4,5)P\(_2\)-containing vesicles in a Ca\(^{2+}\)-dependent manner (Fig. 5, A and B).

A Specific Role of Rp(676–695) in C2B Domain in Secretion and Binding to PC/PS/PtdIns(4,5)P\(_2\)-Containing Lipid Vesicles—Of numerous peptides tested, Rp(676–695) was the only one that significantly inhibited Ca\(^{2+}\)-dependent secretion from digitonin-permeabilized cells (Table II). Its sequence, KHWYE-the C2A domain of synaptotagmin I for which the structural putative Ca\(^{2+}\) in the basic amino acid motif of P676 (see below) but without the independent binding of vesicles. Rp(676–710), which includes in the mutant C2B (D657N and D659N) in the absence of Ca\(^{2+}\) and becomes exposed in the wild type C2B only in the presence of Ca\(^{2+}\).

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