Regulated Exocytosis in Chromaffin Cells

TRANSLOCATION OF ARF6 STIMULATES A PLASMA MEMBRANE-ASSOCIATED PHOSPHOLIPASE D*

(Received for publication, September 24, 1997, and in revised form, October 30, 1997)

Anne-Sophie Caumont‡, Marie-Christine Galas‡, Nicolas Vitale§, Dominique Aunis‡, and Marie-France Bader‡¶

From the ‡INSERM, U-338 Biologie de la Communication Cellulaire, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France and §Pulmonary-Critical Care Medicine Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20814

The ADP-ribosylation factor (ARF) GTP-binding proteins have been implicated in a wide range of vesicle transport and fusion steps along the secretory pathway. In chromaffin cells, ARF6 is specifically associated with the membrane of secretory chromaffin granules. Since ARF6 is an established regulator of phospholipase D (PLD), we have examined the intracellular distribution of ARF6 and PLD activity in resting and stimulated chromaffin cells. We found that stimulation of intact chromaffin cells or direct elevation of cytosolic calcium in permeabilized cells triggered the rapid translocation of ARF6 from secretory granules to the plasma membrane and the concomitant activation of PLD in the plasma membrane. To probe the existence of an ARF6-dependent PLD in chromaffin cells, we measured the PLD activity in purified plasma membranes. PLD could be activated by a nonhydrolyzable analogue of GTP and by recombinant myristoylated ARF6 and inhibited by specific anti-ARF6 antibodies. Furthermore, a synthetic myristoylated peptide corresponding to the N-terminal domain of ARF6 inhibited both PLD activity and catecholamine secretion in calcium-stimulated chromaffin cells. The possibility that ARF6 participates in the exocytotic reaction by controlling a plasma membrane-bound PLD and thereby generating fusogenic lipids at the exocytotic sites is discussed.

ADP-ribosylation factors (ARFs)³ comprise a family of 20-kDa monomeric GTP-binding proteins that were discovered as one of several cofactors required in the cholera toxin-catalyzed ADP-ribosylation of the trimeric Gᵢ proteins (1). Six mammalian family members have been identified which have been classified into three groups according to their size and sequence homology. ARF1, ARF2, and ARF3 form class I, ARF4 and ARF5 form class II, and ARF6 forms class III (1). Members of the ARF family are subjected to myristoylation at the N-terminal glycine residue, a lipid co-translational modification that is essential for functional activity (2). ARFs are ubiquitously among eukaryotes with an amino acid sequence that is highly conserved across diverse species, suggesting a fundamental role in cellular physiology. Indeed, ARF proteins have been implicated in a wide range of vesicle transport and fusion steps along the secretory pathway (3–5). These include budding, transport, and fusion steps in the Golgi complex, in the endoplasmic reticulum and in the endocytic and exocytotic pathways.

The recent discovery that some members of the ARF family are effective activators of phospholipase D (PLD) has raised the possibility that a novel signal transduction pathway may regulate intracellular membrane traffic (6, 7). PLD is an enzyme that catalyzes the hydrolysis of phosphatidylcholine to produce membrane-localized phosphatic acid (PA) and soluble choline (8). In the presence of a primary alcohol, the enzyme can also catalyze a transphosphatidylation reaction that exchanges the polar headgroup of the phospholipid substrate with the given alcohol to form the corresponding phosphatidyl-alcohol (9). This unique and very useful property of PLD has been used to reveal PLD activation following agonist stimulation in many types of cells and tissues (10). Biochemical evidence suggests that multiple PLD isoenzymes with diverse mechanisms of activation occur in mammalian cells (11). An integral membrane-bound PLD that is highly specific for phosphatidylcholine as substrate and is activated by sodium oleate was recently purified (12). In addition, several forms of small G protein-dependent PLDs including ARF-sensitive (6, 7) and RhoA-sensitive (13) isoenzymes have been described. Phosphatidylinositol 4,5-bisphosphate (PIP₂), another important activator of PLD, seems to be generally required for the small G protein-dependent PLDs (6, 7) but not for the oleate-dependent PLD (12). Protein kinase C appears also as a major regulator of PLD since phorbol esters are among the most effective stimuli of PLD reported in many cell types (11). To date, several mammalian PLDs have been cloned and sequenced (11, 14).

Studies in neutrophils (15), pancreatic B cells (16), and pheochromocytoma PC12 cells (17) have suggested a role for PLD in exocytosis. In chromaffin cells, however, the occurrence of an agonist-regulated PLD activity remains a controversial issue (18, 19). We recently described a secretory granule-associated ARF6 protein that may represent a key component of the exocytotic pathway in chromaffin cells (20). Since ARF6 is an established regulator of PLD (21), we examine here the PLD activity in resting and stimulated chromaffin cells. We found that stimulation of chromaffin cells triggered the rapid translocation of ARF6 from secretory granules to the plasma membrane and the concomitant activation of PLD in the plasma membrane. Both calcium-evoked PLD activation and calcium-induced catecholamine secretion could be inhibited by a synthetic peptide corresponding to the N-terminal domain of myristoylated ARF6. We propose that ARF6 may participate in the exocytotic reaction by controlling a plasma membrane-bound PLD and thereby contributing to the generation of fusogenic lipids at the exocytotic sites.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. Tel.: 33-3-88-45-67-13; Fax: 33-3-88-60-08-06; E-mail: bader@neurochem.u-strasbg.fr.
‡ The abbreviations used are: ARF, ADP-ribosylation factor; PLD, phospholipase D; PA, phosphatic acid; PIP₂, phosphatidylinositol 4,5-bisphosphate; GTP-γS, guanosine 5’-3-D-thiotriphosphate; SLO, streptolysin O; PIPES, 1,4-piperazinediethanesulfonic acid; DJbR, dopamine-β-hydroxylase; PET, 1-palmitoyl-2-oleoyl-sn-3-phosphethanolam; PC, phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate.
**Experimental Procedures**

**Culture of Chromaffin Cells—** Chromaffin cells were isolated from fresh bovine adrenal glands and maintained in primary culture, essentially as described previously (22). Cells were usually cultured as monolayers either on 24 multiple 16-mm Costar plates (Cambridge, MA) at a density of 2.5 × 10^5 cells/well or on 100-mm Costar plates at a density of 5 × 10^5 cells/plate. To trigger exocytosis, chromaffin cells were washed twice with Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2, 1.2 mM KH_2PO_4, 1.2 mM MgSO_4, 11 mM glucose, 0.56 mM ascorbic acid, and 15 mM Hepes, pH 7.2) and then stimulated 10 min with Locke's solution containing either 10 μM nicotine or 59 mM K+ (made by decreasing NaCl isosmotically). Experiments were carried out at 37 °C on 3–7-day-old cultures.

**Permeabilization with Streptolysin O (SLO) and Cell Stimulation—** Cultured chromaffin cells were washed four times with Locke's solution and twice with Ca^{2+}-free Locke's solution (containing 1 mM EGTA). Permeabilization was performed with 15 units/ml SLO (Institut Pasteur, Paris, France) for 2 min in 200 μl/16-mm well or in 5 ml/100-mm plate Ca^{2+}-free KG medium (150 mM K+—glutamate, 10 mM PIPES, pH 7.0, 5 mM nitritrolactiuric acid, 0.5 mM EGTA, 5 mM MgATP, 4.5 mM magnesium acetate, 0.2% bovine serum albumin). Cells were subsequently stimulated for 10 min in the presence of the compound to be tested in KG medium containing 20 μM free Ca^{2+} and 1 mM free Mg^{2+} (22).

{[^3H]Noradrenaline and Endogenous Catecholamine Release from Permeabilized Chromaffin Cells—} Catecholamine stores were labeled by incubating chromaffin cells with {[^3H]noradrenaline (14.68 Ci/mmol, NEN Life Science Products) for 60 min. Cells were then washed four times, permeabilized with SLO, and stimulated with 20 μM free Ca^{2+} as described above. The release of {[^3H]noradrenaline (made by Bligh and Dyer (25))} from the cell preparation was estimated as described previously (23).

**Subcellular Fractionation of Cultured Chromaffin Cells—** Cultured chromaffin cells were collected in 0.32 M sucrose, Tris 10 mM, pH 7.4, homogenized, and then centrifuged at 900 × g for 15 min. After centrifugation at 20,000 × g for 20 min, the pellet containing the crude membrane fraction was resuspended in 0.32 M sucrose (10 mM Tris, pH 7.4), layered on a continuous sucrose density gradient (1–2.2 M sucrose, 10 mM Tris, pH 7.4), and centrifuged for 90 min at 100,000 × g. Twelve 1-ml fractions were collected from the bottom to the middle and analyzed for PLD activity and protein content by the Bradford procedure. The gradient was estimated as described previously (24).

**Permeabilized Cells—** Plasma membranes were purified from fractions 2 and 3, which contained the highest Na^+/K^-ATPase activity. Fractions were diluted 10 times in 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol and membranes were collected by centrifugation for 30 min at 100,000 × g.

**Assay for Phospholipase D Activity in Cultured Chromaffin Cells—** Chromaffin cells were labeled with 1 μCi/ml [9,10^2H]-myristic acid for 24 h at 37 °C. Labeled cells were then washed, stimulated with nicotine or 59 mM K^+ in the presence of 1% ethanol or permeabilized with SLO, and then stimulated with 20 μM free Ca^{2+} in the presence of 1% ethanol. Cells were subsequently collected and lipids extracted and separated out in a final volume of 60 μl. All assays contained 0.4 μM MgCl_2 and 0.4 mM NaCl to favor the nucleotide exchange on ARF. The reaction was carried out in a final volume of 60 μl. All assays contained 6.25 μg of plasma membrane proteins in buffer A (50 mM Na-Hepes, pH 7.5, 5 mM EGTA, 80 mM KCl, 2 mM MgATP, 400 mM NaCl, 4.5 mM MgCl_2, 5 mM CaCl_2, and 1 mM dithiothreitol). When indicated, 30 μM GTP-S and 1 μM recombinant myristoylated ARF6 were included. The membranes and the above constituents in a volume of 34 μl were preincubated for 30 min at 37 °C. The reaction was subsequently started by the addition of 24 μl of lipid substrate and 1% ethanol. The final concentration of lipids in the assay were 135 μM PE, 12 μM PIP_2, 8 μM phosphatidylylycerol (PG), and 1 μM GTP-S. When indicated, 30 μM GTP-S and 1 μM recombinant myristoylated ARF6 were included. After incubation and centrifugation (2000 × g for 5 min), 400 μl of the aqueous phase was extracted and analyzed as described above.

**Antibodies, Peptides, and Proteins—** Polyclonal anti-ARF6 antibodies were raised in rabbits against bacterially overexpressed ARF6 protein. This antibody was a generous gift from Dr. J. B. Helms (Ruprecht-Karls-Universität, Heidelberg, Germany). The rabbit polyclonal anti-dopamine β-hydroxylase (EC 1.14.17.1) antiserum was prepared in our laboratory, and its specificity has been demonstrated (28).

**Myristoylation (myrARF6–2–13) and non-myristoylated (ARF6–2–13) N-terminal ARF6 peptides (GKVLSKFGKNE) and myristoylated (myrARF6–2–17) N-terminal ARF1 peptide (GNIFANLFGKFGKKE) were synthesized in our laboratory using the 432 peptide Synthesizer SYNERGY (Applied Biosystems, Warrington, UK). Purity was checked by high performance liquid chromatography. Sequence analysis was performed by Edman degradation on an automated Applied Biosystems 475A gas phase protein microsequencer (Applied Biosystems, Warrington, UK), and mass spectrometry was used to assess the structure of the final product.

Recombinant myristoylated ARF6 protein (rARF6) was produced in a bacterial expression system containing the N-myristoyltransferase in the plasmid pACYC177/ET3, and purified according to the procedure previously described by Hsu et al. (27).

**Protein Determination, Electrophoresis, and Immunoblotting—** Protein concentration was routinely determined using the Bradford procedure with Bio-Rad dye reagent and bovine serum albumin as standard. One dimensional SDS-polyacrylamide gel electrophoresis was performed on 12% acrylamide gel in Tris-glycine buffer (24). Proteins were transferred to nitrocellulose sheets at a constant current of 120 mA for 1 h. Blots were developed with secondary antibodies coupled to horse-radish peroxidase (Amersham, Les Ulis, France), and immunoreactive bands were detected with the ECL system (Amersham).

**Results**

**Activation of Phospholipase D in Response to Chromaffin Cell Stimulation—** To measure PLD activity, we made use of the specific transphosphatidylation reaction in which the phospholipid headgroup is exchanged for ethanol, producing PEt at the expense of PA. Therefore, cultured chromaffin cells were labeled with [3H]myristic acid. Preliminary time-course experiments indicated that maximal incorporation of the radioactivity in total lipids occurred after 20 h of incubation. [3H]Myristic acid was predominantly incorporated into phosphatidylycerolipids (data not shown), which represents the major PLD substrate (10). Stimulation of [3H]myristic acid labeled chromaffin cells with nicotine or with a depolarizing concentration of potassium triggered the formation of [3H]PA and [3H]PEt in the presence of 1% ethanol (Table I). Both secretagogues elicited a similar increase in PA and PEt levels, suggesting that an agonist-stimulated phospholipase D activity was present in chromaffin cells.

We investigate the mechanism of activation of PLD, chromaffin cells with permeabilized with SLO, chromaffin cell cytosolic Ca^{2+} at known values and to control the cytosolic levels of nucleotides. Permeabilized cells were maintained under resting conditions or stimulated with 20 μM Ca^{2+} in the presence of 5 mM MgATP and 1% ethanol. We found that calcium strongly stimulated the formation of both [3H]PEt (Fig 1) and [3H]PA (data not shown), suggesting that PLD activation might be a component of the calcium signaling cascade in stimulated chromaffin cells.
PLD can be activated by protein kinase C in many cell types (11). We looked, therefore, at the effect of phorbol 12-myristate 13-acetate (PMA) at 100 nM, a concentration known to give maximal stimulation of protein kinase C in chromaffin cells (28). However, as seen in Fig. 1, PMA was unable to significantly modify the basal or the calcium-evoked formation of \(^{3}\text{H}\)PEt in SLO-permeabilized chromaffin cells, indicating that PLD is not modulated by a protein kinase C-dependent pathway. In agreement, Purkiss et al. (18) previously reported that neither bradykinin nor 12-O-tetradecanoylphorbol-13-acetate stimulated PLD in intact chromaffin cells.

Current evidence supports the existence of two types of PLD activities: an oleate-dependent form and a form modulated by monomeric G protein (21). To probe the involvement of a GTP-binding protein in the calcium-evoked PLD activation, we introduced a nonhydrolyzable analogue of GTP into SLO-permeabilized chromaffin cells. Fig. 1 shows that GTPγS (20 μM) produced a large increase in the Ca\(^{2+}\)-dependent PLD activity, revealing that a G protein probably regulates PLD in chromaffin cells. In contrast, AlF\(_4^-\), which selectively activates trimeric G proteins (29), inhibited the Ca\(^{2+}\)-dependent PLD activity in SLO-permeabilized cells by more than 80% (Fig. 1). Taken together, these findings suggest that the Ca\(^{2+}\)-dependent PLD activity in chromaffin cells might be regulated by both monomeric and heterotrimeric G proteins.

Subcellular Localization of the Phospholipase D Activity in

**FIG. 1. Activation of a G protein-dependent phospholipase D activity in stimulated chromaffin cells.** Chromaffin cells labeled with \(^{3}\text{H}\)myristic acid were permeabilized with SLO and then incubated for 10 min with 20 μM GTPγS (open columns) or stimulated for 10 min with KG medium containing 20 μM free Ca\(^{2+}\) (closed columns) in the presence of 1% ethanol. GTPγS (20 μM), AlF\(_4^-\) (20 mM NaF + 50 μM AlCl\(_3\)), and PMA (100 nM) were added during the stimulation period. Phospholipids were subsequently extracted and analyzed by thin layer chromatography. The formation of \(^{3}\text{H}\)PEt is expressed as a percentage of total counts recovered in the extracted lipids. Data are the mean values of triplicate determinations ± S.E. Similar results were obtained in three independent experiments.

PLD can be activated by protein kinase C in many cell types (11). We looked, therefore, at the effect of phorbol 12-myristate 13-acetate (PMA) at 100 nM, a concentration known to give maximal stimulation of protein kinase C in chromaffin cells (28). However, as seen in Fig. 1, PMA was unable to significantly modify the basal or the calcium-evoked formation of \(^{3}\text{H}\)PEt in SLO-permeabilized chromaffin cells, indicating that PLD is not modulated by a protein kinase C-dependent pathway. In agreement, Purkiss et al. (18) previously reported that neither bradykinin nor 12-O-tetradecanoylphorbol-13-acetate stimulated PLD in intact chromaffin cells.

Current evidence supports the existence of two types of PLD activities: an oleate-dependent form and a form modulated by monomeric G protein (21). To probe the involvement of a GTP-binding protein in the calcium-evoked PLD activation, we introduced a nonhydrolyzable analogue of GTP into SLO-permeabilized chromaffin cells. Fig. 1 shows that GTPγS (20 μM) produced a large increase in the Ca\(^{2+}\)-dependent PLD activity, revealing that a G protein probably regulates PLD in chromaffin cells. In contrast, AlF\(_4^-\), which selectively activates trimeric G proteins (29), inhibited the Ca\(^{2+}\)-dependent PLD activity in SLO-permeabilized cells by more than 80% (Fig. 1). Taken together, these findings suggest that the Ca\(^{2+}\)-dependent PLD activity in chromaffin cells might be regulated by both monomeric and heterotrimeric G proteins.

Subcellular Localization of the Phospholipase D Activity in

**FIG. 2. Distribution of ARF6 and phospholipase D activity in subcellular fractions from resting and stimulated chromaffin cells.** A, chromaffin cells labeled with \(^{3}\text{H}\)myristic acid were permeabilized with SLO and subsequently incubated in KG medium containing 1% ethanol and 20 μM GTPγS in the presence (stimulated cells) or absence (resting cells) of 20 μM free Ca\(^{2+}\). Cells were then collected and processed for subcellular fractionation. Fractions collected from the continuous sucrose density gradient layered with the crude plasma membrane pellet were assayed for Na\(^{+}\)/K\(^{-}\)-ATPase for plasma membranes (open circles) and DjBH for chromaffin granules (open triangles). Fraction 3 contained most of the plasma membranes, and fractions 10 and 11 were enriched in chromaffin granules. Phospholipids were then extracted from each fraction, and the amount of \(^{3}\text{H}\)PEt formed was determined by thin layer chromatography (closed triangles, PLD activity in resting cells; closed circles, PLD activity in stimulated cells). Most of the PLD activity is detected in plasma membrane-containing fractions prepared from calcium-stimulated chromaffin cells. B, chromaffin cells were maintained under resting conditions or stimulated for 5 min with 10 μM nicotine. Cells were subsequently collected and processed for subcellular fractionation. Fractions from sucrose density gradients (10 μg of protein) were subjected to gel electrophoresis and immunodetection on nitrocellulose sheets using anti-ARF6 antibodies. In resting cells, ARF6 protein is essentially present in fractions 10 and 11 containing chromaffin granules. Stimulation with nicotine triggers the translocation of ARF6 to fraction 3 enriched in plasma membranes.

**TABLE I**

<table>
<thead>
<tr>
<th></th>
<th>cpm</th>
<th>Ratio stimulated/ basal</th>
<th>cpm</th>
<th>Ratio stimulated/ basal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>[^3]H</strong>PA**[^3]H**PEt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locke</td>
<td>718 ± 85</td>
<td>100 ± 11</td>
<td>279 ± 71</td>
<td>100 ± 26</td>
</tr>
<tr>
<td>Nicotine (10 μM)</td>
<td>1430 ± 106</td>
<td>185 ± 6</td>
<td>497 ± 38</td>
<td>166 ± 6</td>
</tr>
<tr>
<td>K(^{+}) (59 mM)</td>
<td>1301 ± 23</td>
<td>171 ± 7</td>
<td>556 ± 20</td>
<td>189 ± 18</td>
</tr>
</tbody>
</table>

Phospholipase D and Exocytosis

Stimulated Chromaffin Cells—To determine the intracellular localization of the PLD activity in stimulated chromaffin cells, we analyzed the phospholipid content of subcellular fractions collected from a sucrose density gradient layered with a crude membrane preparation. Chromaffin cells labeled with \(^{3}\text{H}\)myristic acid were permeabilized with SLO and subsequently incubated in KG medium containing 1% ethanol and 20 μM GTPγS in the presence or absence of 20 μM free Ca\(^{2+}\). Cells were then collected and processed for subcellular fractionation. As expected, the \(^{3}\text{H}\)PEt levels detected in the fractions obtained from resting cells remained negligible (Fig. 2A). Interestingly, radioactive \(^{3}\text{H}\)PEt was essentially collected in fractions 2 and 3 in gradients prepared from stimulated cells (Fig. 2A). These fractions contained plasma membranes as estimated by the Na\(^{+}\)/K\(^{-}\)-ATPase activity. It is noteworthy that fractions 11 and 12, enriched in chromaffin granules revealed by the peak of DjBH, contain very little radioactive \(^{3}\text{H}\)PEt in both resting and stimulated cells. In other words, PLD activity in chromaffin cells is not associated with secretory granules. However, a rise in cytosolic calcium triggers the activation of a G protein-regulated PLD activity, essentially in the plasma membrane of chromaffin cells.
Translocation of ARF6 from Secretory Chromaffin Granules to the Plasma Membrane in Stimulated Chromaffin Cells—We previously reported that ARF6 is specifically associated with secretory granule membranes in chromaffin cells, most likely through an interaction with the β subunit of a granule-bound trimeric G protein (20). Stimulation of chromaffin cells with nicotine or direct elevation of cytosolic Ca²⁺ in permeabilized cells triggered the rapid dissociation of ARF6 from secretory granules (20). To identify the target membrane to which ARF6 translocates in stimulated cells, we compared here the distribution of ARF6 in membrane fractions collected from resting or nicotine-stimulated chromaffin cells. Fig. 2B illustrates an immunodetection analysis, using an anti-ARF6 antibody, of fractions collected from a sucrose density gradient layered with chromaffin cells, we first examined the effect of recombinant ARF6 on PLD activity associated with purified plasma membranes. Plasma membranes recovered from a sucrose density gradient were assayed for PLD activity using lipid vesicles labeled with [³H]dipalmitoyl phosphatidylcholine. As illustrated in Fig. 3, the presence of 30 μM GTPyS did not significantly increase the plasma membrane-associated PLD activity estimated by the formation of [³H]PA and [³H]PEt. However, addition of 1 μM myristoylated recombinant ARF6 together with 30 μM GTPyS stimulated the formation of [³H]PA and [³H]PEt by approximately 135%. These results indicate that the plasma membrane-associated PLD is essentially ARF6-sensitive in chromaffin cells. Data are given as mean values ± S.E. (n = 3). *, p < 0.01, and **, p < 0.02 when tested by Student’s t test.
Phospholipase D Activity in Chromaffin Cells—
Phospholipase D and Exocytosis

Chromaffin cells labeled with [3H]noradrenaline were permeabilized with SLO and subsequently stimulated for 10 min with KG medium containing 20 μM free calcium in the presence of 1.5% ethanol, 1-butanol, or 2-butanol. Results are expressed as the net [3H]noradrenaline release obtained by subtracting the basal calcium-independent release. Basal release was not modified by alcohol concentration tested. Data are given as the mean values ± S.E. (n = 3).

<table>
<thead>
<tr>
<th>Control</th>
<th>7.30 ± 0.55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>4.16 ± 0.55</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>3.67 ± 0.36</td>
</tr>
<tr>
<td>2-Butanol</td>
<td>6.05 ± 0.52</td>
</tr>
</tbody>
</table>

Table II
Effect of ethanol, 1-butanol, and 2-butanol on Ca2+-evoked secretion in SLO-permeabilized chromaffin cells

The net [3H]noradrenaline release is given as the percentage of total catecholamines in the absence of calcium. Exocytotic events in chromaffin cells.

Correlation between Catecholamine Secretion and ARF6-Phospholipase D Activation in Chromaffin Cells—Fig. 5 illustrates the time course and calcium dose-response curve for secretion and PLD activation in SLO-permeabilized chromaffin cells. Secretion was estimated by measuring the release of endogenous catecholamines, and PLD activity was detected by measuring the formation of labeled PEt and PA. We found a close relationship between the effects of the various N-alkylamines on Ca2+-evoked catecholamine secretion and PLD activation in SLO-permeabilized chromaffin cells. Table II shows the effects of ethanol, 1-butanol, and 2-butanol on Ca2+-evoked catecholamine secretion in permeabilized chromaffin cells.
DISCUSSION

Dramatic advances have recently been made in our understanding of the protein machinery responsible for the formation, targeting, and fusion of vesicles along the secretory pathway. Most of the emerging models emphasize the convergence in protein molecules and mechanisms underlying the multiple steps of intracellular vesicular transport (30, 31). Since ARF proteins belong to the molecules that have been implicated as ubiquitous regulators in membrane traffic (3–5), we recently investigated the possible function of ARF in calcium-regulated exocytosis in chromaffin cells (20). We found that ARF6 is specifically associated with the membrane of secretory chromaffin granules, most likely through an interaction with βγ subunits of a trimeric G protein. Interestingly, nicotine-induced stimulation of intact cells or direct elevation of cytosolic calcium in permeabilized cells triggered the rapid dissociation of ARF6 from secretory granules (20). Although ARF proteins are generally believed to cycle on and off the plasma membrane in a manner that is tightly coupled to the binding and hydrolysis of GTP, we could not detect ARF6 in the cytosol in subcellular fractionation experiments (20) or among the cytosolic proteins released through the pores created in the plasma membrane of SLO-permeabilized cells. Thus, we postulated that ARF6 translocated from chromaffin granules to an unknown membrane-bound compartment upon cell stimulation. Interestingly, the subcellular distribution of human ARF proteins has recently been examined in detail in Chinese hamster ovary cells, and ARF6 was the only isoform that could not be detected in the cytosol (32). It is also noteworthy that both wild-type and mutant forms of ARF6 were exclusively localized in membrane compartments when overexpressed in fibroblasts (33). Thus, ARF6 seems to behave quite distinctly from other ARFs, at least regarding its membrane binding activity and intracellular localization. Based on subcellular fractionation techniques and immunological detection, we report here that agonist stimulation triggers the translocation of ARF6 from secretory granules to the plasma membrane in chromaffin cells. Moreover, we found a close correlation between the presence of ARF6 in the plasma membrane and the activation of a GTP-dependent PLD activity in the plasma membrane, suggesting that PLD may be a possible effector for ARF6 in the exocytotic pathway.

The participation of ARF in exocytosis in endocrine and neuroendocrine cells has been previously postulated (34–36). To probe the importance of ARF6 in agonist-stimulated PLD activity and exocytotic response in chromaffin cells, we used here synthetic N-terminal ARF peptides described to block ARF activities in various cellular processes (37, 38). We found that myrARF6-(2–13), a peptide corresponding in sequence to the myristoylated N-terminal domain of ARF6, specifically inhibited calcium-evoked catecholamine release and PLD activation in stimulated chromaffin cells. By comparison, the non-myristoylated ARF6-(2–13) peptide had little effect, an observation that may be related to the presumed importance of the myristoyl group in the binding of ARF6 to membranes (2, 33). Dose-response experiments indicated that myrARF6-(2–13) was able to block almost completely the calcium-evoked secretory response in permeabilized chromaffin cells (>90% inhibition). Thus, ARF6 activation of PLD may represent a key event in the exocytotic pathway in neuroendocrine cells.

Interestingly, we found that AlF₄⁻, which activates specifically heterotrimeric G proteins (29), inhibited the calcium-induced PLD activity in permeabilized chromaffin cells. This observation correlates well with our previous findings that AlF₄⁻ can prevent the calcium-induced uncoupling of ARF6-Gβγ on the secretory granule membrane (20) and strongly reduce the calcium-evoked exocytotic activity in stimulated cells (22). The regulation of ARF activities in the Golgi complex (39) and in the endocytotic pathway (40) by trimeric G proteins has already been reported. In chromaffin cells, activation of the secretory granule-associated Gα subunit inhibits the ATP-dependent priming step of exocytosis (22, 23). This suggests that activated Gα₉ blocks the exocytotic machinery when the α₉ subunit is dissociated from βγ. Our data support a model in which exocytosis requires the inactivation of the granule-bound Gα₉, leading to the reassociation of α₉ with βγ. Gβγ interacting with Gα₉ is then unable to retain ARF6, which translocates to the plasma membrane and activates PLD. We recently identified another putative effector of Gα₉ in the exocytotic pathway, namely the

2 M.-C. Galas and M.-F. Bader, unpublished data.
monomeric GTP-binding protein Rho, which seems to regulate the peripheral actin network (41). Interestingly, several reports describe a reciprocal regulatory relationship between actin reorganization and PLD activity (42–44). Thus, an attractive speculation is that the granule-bound Gs plays a dual control of the plasma membrane-associated PLD in the exocytotic pathway: through $\beta y$ and ARF6, which directly activate the enzyme and through $\alpha_p$ and Rho, which may modulate PLD by a specific cytoskeleton reorganization.

PLD hydrolyzes PC to generate PA and choline. PA seems to play an important role for the synthesis of peptides, and Dr. Nancy Grant for revising the manuscript.

Acknowledgments—We gratefully acknowledge Dr. Joel Moss for the generous gift of the bacterial expression system for myrARF6 and Dr. J. Bernd Helms for kindly providing us with anti-ARF6 antibodies. We thank Danièle Thiéssé for culturing chromaffin cells, Gérard Nullans for the synthesis of peptides, and Dr. Nancy Grant for revising the manuscript.

REFERENCES

Marie-France Bader
Anne-Sophie Caumont, Marie-Christine Galas, Nicolas Vitale, Dominique Aunis and Marie-France Bader

Regulated Exocytosis in Chromaffin Cells: TRANSLOCATION OF ARF6 STIMULATES A PLASMA MEMBRANE-ASSOCIATED PHOSPHOLIPASE D

doi: 10.1074/jbc.273.3.1373

Access the most updated version of this article at http://www.jbc.org/content/273/3/1373

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 27 of which can be accessed free at http://www.jbc.org/content/273/3/1373.full.html#ref-list-1