Annexin 2 Promotes the Formation of Lipid Microdomains Required for Calcium-regulated Exocytosis of Dense-Core Vesicles

Sylvette Chasserot-Golaz,* Nicolas Vitale,* Emeline Umbrecht-Jenck,* Derek Knight,† Volker Gerke,‡ and Marie-France Bader*

*Centre National de la Recherche Scientifique, Unité Propre de Recherche 2356, IFR 37 des Neurosciences, 67084 Strasbourg Cedex, France; †Biomedical Sciences, Kings College London, London SE1 1U, United Kingdom; and ‡Institute for Medical Biochemistry, Center for Molecular Biology of Inflammation, University of Münster, D-48149 Münster, Germany

Submitted July 23, 2004; Revised November 24, 2004; Accepted December 16, 2004
Monitoring Editor: Anthony Bretscher

Annexin 2 is a calcium-dependent phospholipid-binding protein that has been implicated in a number of membrane-related events, including regulated exocytosis. In chromaffin cells, we previously reported that catecholamine secretion requires the translocation and formation of the annexin 2 tetramer near the exocytotic sites. Here, to obtain direct evidence for a role of annexin 2 in exocytosis, we modified its expression level in chromaffin cells by using the Semliki Forest virus expression system. Using a real-time assay for individual cells, we found that the reduction of cytosolic annexin 2, and the consequent decrease of annexin 2 tetramer at the cell periphery, strongly inhibited exocytosis, most likely at an early stage before membrane fusion. Secretion also was severely impaired in cells expressing a chimera that sequestered annexin 2 into cytosolic aggregates. Moreover, we demonstrate that secretagogue-evoked stimulation triggers the formation of lipid rafts in the plasma membrane, essential for exocytosis, and which can be attributed to the annexin 2 tetramer. We propose that annexin 2 acts as a calcium-dependent promoter of lipid microdomains required for structural and spatial organization of the exocytic machinery.

INTRODUCTION

Annexins form an evolutionary conserved multigene family of proteins with members being expressed throughout the plant and animal kingdoms. The common characteristic of annexins is that they bind to negatively charged phospholipids in biological membranes in a Ca\textsuperscript{2+}-dependent manner (for review, see Creutz, 1992; Gerke and Moss, 2002). As such, annexins have been implicated in various membrane trafficking events, including exocytosis, endocytosis, and cell-to-cell adhesion (Lecat and Lafont, 1999). The most compelling evidence for involvement in calcium-regulated exocytosis has been reported for annexin 2. Endogenous annexin 2 exists in part as a soluble monomer, p36, and in part as a heterotetrameric complex, p90, with its specific ligand the S100A10 protein also called p11 (Schafer and Heizmann, 1996). When complexed, the central S100A10 dimer links two annexin 2 chains in a highly symmetrical manner, creating a scaffold that can bridge opposing membrane surfaces (Lambert et al., 1997; Rety et al., 1999; Lewit-Bentley et al., 2000). Quick-freeze, deep-etch electron microscopic analysis has documented that annexin 2 forms cross-links between secretory granules and the plasma membrane in stimulated neuroendocrine cells (Nakata et al., 1990; Senda et al., 1994).

In chromaffin cells, we (Sarafian et al., 1991) and others (Ali et al., 1989) have identified annexin 2 as one of the cytosolic proteins that can retard the rundown of secretory responsiveness to Ca\textsuperscript{2+} stimulation of permeabilized cells when added exogenously as a purified protein. In our assay, the tetrameric complex was more efficient than the monomeric annexin 2 protein, and phosphorylation by protein kinase C (PKC) was required (Sarafian et al., 1991). More recently, we demonstrated that a synthetic peptide corresponding to an NH\textsubscript{2}-terminal annexin 2 sequence containing the PKC phosphorylation site inhibits catecholamine secretion in response to nicotine when microinjected into chromaffin cells (Chasserot-Golaz et al., 1996). Together, these results strongly suggested, but did not prove, that annexin 2 plays an important role in calcium-regulated exocytosis. Hence, the functional implication of annexin 2 in exocytosis remains a controversial issue, because a peptide competing for the interaction of annexin 2 with p11 has no effect on secretion in permeabilized chromaffin cells (Ali and Burgoyne, 1990), although it significantly reduced Ca\textsuperscript{2+}-triggered exocytotic membrane incorporation in endothelial cells (Konig et al., 1998). Moreover, expression of a chimeric protein that leads to the formation of cytosolic annexin 2 aggregates does not affect secretion in PC12 cells, another argument against the participation of annexin 2 in exocytosis (Graham et al., 1997).

In the present study, using a variety of direct means, we have revisited the role of annexin 2 in calcium-regulated exocytosis. Using chromaffin cells deficient in endogenous annexin 2, we demonstrate that the presence of annexin 2 at the cell periphery is a prerequisite for the docking and subsequent fusion of secretory granules with the plasma
membrane. Our results suggest that the translocation of annexin 2 to the plasma membrane favors the formation of lipid microdomains that are required for granule exocytosis.

MATERIALS AND METHODS

Chromaffin Cells and [H]Noradrenaline Release

Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase, purified on self-generating Percoll gradients, and maintained in culture as described previously (Kader et al., 1986). Catecholamine stores were labeled by incubation of cultured chromaffin cells with [H]noradrenaline (13.3 Ci/mmol; Amersham Biosciences, LaÈrøe, Denmark) for 45 min at 37°C. Following exocytosis, chromaffin cells were washed twice with Locke’s solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 11 mM glucose, 0.56 mM ascorbic acid, and 15 mM HEPES, pH 7.2) and then stimulated 5 min with Locke’s solution containing either 10 μM nicotine or 59 mM K+ (made by decreasing NaCl isotonically). Permeabilization of chromaffin cells with streptolysin-O (SLO; Institut Pasteur, Paris, France) was performed as described previously (Sarafian et al., 1991). Briefly, cells were washed with calcium-free Locke’s solution (containing 1 mM EGTA) and then permeabilized for 2 min at 37°C with streptolysin-O (18 U/ml) in 200 μl of calcium-free permeabilizing medium (150 mM glutamate, potassium salt, 10 mM PIPES, 5 mM nitrilotriacetic acid [NTA], 0.5 mM EGTA, 0.2% bovine serum albumin [BSA], 5 mM Mg-ATP, and 4.5 mM magnesium). Secretion was induced for 5 min with permeabilizing media containing 50 μM free calcium concentration. [H]Noradrenaline release after stimulation was determined by measuring the radioactivity present in the incubation medium and in cells after precipitation with 10% (wt/vol) trichloroacetic acid (TCA). The amount of released [H]noradrenaline is expressed as a percentage of total radioactivity present in the cells before stimulation.

Construction and Expression of Viral Vectors

Construction of pSVE1EGFPubX was described previously (Knight, 1999). The first (natural) promoter is followed by enhanced green fluorescent protein (EGFP) and the second pSVE1 internal promoter by BanHI and Smal cloning sites. For sense and antisense constructs, the fragment 9–970 of annexin 2 was ligated in-frame in sense and antisense orientation into BanHI and Smal-cut pSVE1EGFPubX. The chimeric X construct corresponding to the 54 first base of annexin 2 fused to the entire p11 sequence was generated as described previously (Harder and Gerke, 1993; Harder et al., 1993). The recombinant viral vectors and the SFV Helper 2 vector were linearized with SphI, transfected, and transduced into 106 baby hamster kidney (BHK) cells essentially as described previously (Liljestrom and Garoff, 1991). After 24 h, the virus was harvested, concentrated by centrifugation on sucrose gradient, and suspended in OptiMEM containing 10% fetal calf serum (FCS). The viral stocks were aliquoted and stored at −80°C. Helper 2-packaged recombinant viruses were activated by α-chymotrypsin digestion (1/20) for 30 min at room temperature, followed by proinactivation of α-chymotrypsin. The titer of viral stocks was determined by infecting BHK cells in 3-cm plates with 0.2% FCS and cells expressing GFP, under conditions of single virus infection (i.e., <10% of BHK cells infected). The titer of the viral stocks was typically in the order of 109 infectious units. Chromaffin cells on coated glass coverslips in 24-well plates were routinely infected in 0.4 ml of OptiMEM containing 0.2% FCS and cells on 3-cm plates with 1 ml of solution containing 10 infectious units per cell. Cells were used for functional studies between 24 and 48 h after infection to detect EGFP-expressing cells.

Electrochemical Measurement of Catecholamine Secretion from Single Chromaffin Cells

Cells cultured on 35-mm plates at a density of 7.5 × 105 cells/plate were washed with ascorbate-free Locke’s solution and placed on the stage of an inverted microscope. A carbon fiber electrode was positioned in tangent contact with a single chromaffin cell by using a three-dimensional microinjection setup (Steinberg et al., 1986). Chromaffin cell secretion was evoked by applying nicotine (100 μM) in ascorbate-free Locke’s solution for 5 s to single cells by means of a glass micropipette (Femtotips; Eppendorf, Hamburg, Germany), and the amperometric response was measured as described previously (Viale et al., 2003). The latency of the response was quantified by measuring the area below the current curve by using the MacLab system.

Immunoblotting, Immunofluorescence, and Confocal Microscopy

One-dimensional SDS-gel electrophoresis was performed on 10% acrylamide gels in Tris-glycine buffer. The proteins were transferred to nitrocellulose sheets at a constant current of 120 mA for 1 h. Blots were developed using secondary antibodies coupled to horseradish peroxidase (HRP) (Amersham Biosciences) and the immunocomplex detected using the enhanced chemiluminescence (ECL) system (Amersham Biosciences). For immunocytochemistry, chromaffin cells on coated glass coverslips were fixed as described previously (Chasserot-Golaz et al., 1996). The transcytoplasmic activity of dopamine β-hydroxylase (DBH) on the plasma membrane of stimulated chromaffin cells was tested by incubating cells for 5 min in Locke’s solution containing 10 μM nicotine in the presence of anti-DBH antibodies diluted to 1:500. Staining for F-actin was performed with tetramethylrhodamine B isothiocyanate-conjugated phalloidin (0.5 μg/ml; Sigma-Aldrich, St. Louis, MO) for 15 min in the dark at room temperature.

Stained cells were visualized using a Zeiss Axioskop microscope LSM 510. Using the Zeiss CLSM instrument software 2.8, the amount of cholera toxin associated with the plasma membrane or the amount of phalloidin detected in the cell was measured and expressed as the average fluorescence intensity normalized to the corresponding surface area and divided by the total surface of each cell. This allows a quantitative cell-to-cell comparison of the fluorescence detected in cells.

Antibodies

Rabbit polyclonal antibodies raised against annexin 2 (p36) purified from bovine aorta were used at 1:200 dilution (generous gift from J. C. Cava- dor, National de la Santé et de la Recherche Medicale U-249, Montpellier, France). Mouse monoclonal antibodies against XM (H21) were used at a 1:5 dilution (Osborn et al., 1988; Harder and Gerke, 1993). Mouse monoclonal antibodies against p11 were used at a 1:50 dilution (BD Transduction Laboratories, Lexington, KY). Rat polyclonal antibodies against DBH (EC.1.14.17.1) were used at a 1:50 dilution to specifically label secretory granules in chromaffin cells (Pollard et al., 1982; Perrin and Aunis, 1985). Rabbit polyclonal anti-chromogranin A antibodies were prepared in our laboratory (Ehrhart et al., 1986) and used at a 1:2000 dilution. Mouse monoclonal antibodies anti-synaptotagmin (mAb1D12) were used at a 1:2000 dilution (generous gift from Dr. M. Takahashi, Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo, Japan). Monoclonal anti-SNAP-25 antibodies were used at a 1:2000 dilution (Stettenberger Monoclonals, Lutherville, MD). Mouse monoclonal antibodies anti-flotillin were used at a 1:500 dilution (BD Transduction Laboratories, Lexington). Mouse monoclonal antibodies anti-transferrin receptor was used at a 1:1000 dilution (Zymed Laboratories, South San Francisco, CA). Cy3-anti-mouse, Cy3-anti-rabbit, and Cy3-anti-mouse were obtained from Amer- sham Biosciences.

Subcellular Fractionation

For subcellular fractionation, cultured chromaffin cells were collected and homogenized in 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, and then centrifuged at 800 × g for 15 min. The supernatant (cell lysate) was further centrifuged at 20 000 × g for 20 min. The 20 000-g pellet containing the crude membrane fraction was resuspended in sucrose 0.32 M (10 mM Tris/HCl, pH 7.4), layered onto a sucrose 1.2–2 M gradient, and centrifuged for 90 min at 100 000 × g. Twelve 1-ml fractions were collected from the top to the bottom and analyzed for protein content by the Bradford procedure. The distribution of SNAP-25 (plasma membrane marker) and chromogranin A (chromaffin granule marker) was estimated in fractions 2–12 (40 μg of protein per fraction) by SDS-PAGE and immunoblotting. GM1 was detected in the cell lysate (Ly), the crude membrane fraction (CM), in fractions 2–3 containing plasma membranes (PMs) and in fractions 8–10 containing chromaffin granules (GMs). Two microliters of each of these fractions prepared from resting and nicotine-stimulated cells was dot blotted onto nitrocellulose filter strips, incubated with HRP-conjugated cholera toxin (10 ng/ml; Sigma-Aldrich), and visualized by ECL. In some experiments, cholinergic cells were stimulated with nicotine and then rapidly scraped in 2 ml of 150 mM glucose, potassium salt, 10 mM PIPES, pH 7.2, 5 mM NTA, 0.5 mM EGTA, 0.2% BSA, 5 mM Mg-ATP, 4.5 mM MgCl2, and 1 mM CaCl2, and centrifuged for 15 min at 100 000 × g. The pellet containing the crude membranes was homogenized in 500 μl of the same buffer with or without 30 μM filipin, further incubated for 30 min at 0°C, and then centrifuged for 15 min at 100 000 × g. Pellets and supernatants were solubilized in SDS-sample buffer, and the presence of p36 and p11 proteins was analyzed by electrophoresis and immunoblotting.

Flotation Gradient

Chromaffin cells on 10-cm dishes (5 × 106 cells) were washed in Locke’s solution and stimulated with 10 μM nicotine for 10 min. Cells were lysed in 300 μl of TNE (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1

Vol. 16, March 2005 1109
mM dithiothreitol, and CLAP protease inhibitor cocktail), 10% sucrose, and 1% Triton X-100 at 4°C. The cell pellet was resuspended and further incubated for 30 min on ice. Then, 600 μl of cold 60% Optiprep (Nycomed-Pharma, Oslo, Norway) was added to the extract, and the mix was transferred to a SW60 centrifuge tube (Beckman, Munich, Germany). The sample was overlaid with 900 μl step of each of 35, 30, 25, and 5% Optiprep in TNE, 1% Triton X-100. The gradients were spun for 17 h at 34,000 rpm at 4°C. Ten fractions from the top of the gradient were collected. The fractions were TCA precipitated and analyzed by Western blot with anti-p36, anti-p11, or anti-synaptotagmin antibodies followed by HRP-coupled sec-

Figure 1. Reduction of peripheral annexin 2 alters the exocytotic release of catecholamines in chromaffin cells. Chromaffin cells were infected with the following vectors: pSFV1EGFP-Ax2-S, a control vector containing the sense sequence of p36; pSFV1EGFP-Ax2-AS, a vector containing the antisense sequence of p36; pSFV1EGFP-XM, a vector expressing the dominant negative p36/p11 chimera XM; and pSFV1EGFP-SubX, the empty vector. To facilitate identification of the infected cells, the viruses coexpressed EGFP. Forty-eight hours after infection, cells were stimulated with 10 μM nicotine in Locke’s solution, fixed, and immunostained with anti-p36 antibody. The XM chimeric protein was detected with the H21 monoclonal antibody (note that the anti-p36 antibody does not recognize the XM chimeric protein). (A) Confocal micrographs (bars, 10 μm). (B) Catecholamine release estimated by amperometry. The traces shown are typical responses to a local application of 100 μM nicotine for 5 s (arrow). (C) Amperometric responses were integrated to obtain the total catecholamine secretion expressed in pA per second. Data are the means of 25 cells/group from the same dish ± SEM. Similar results were obtained in three independent experiments performed on two culture preparations and infected with different batches of recombinant virus.
RESULTS

Reduction of Subplasmalemmal Annexin 2 Inhibits Exocytosis in Chromaffin Cells

To probe the role of annexin 2 (p36) in exocytosis, we modified the expression level of endogenous annexin 2 in chromaffin cells by using the Semliki Forest virus expression system (Knight, 1999). Two types of recombinant virus were constructed: pSFV1EGFP-Ax2-AS, expressing the antisense sequence of p36 (annexin 2) to decrease levels of the endogenous protein, and pSFV1EGFP-XM, which expressed a p36/p11 chimera (XM) that aggregates cytosolic p36 and prevents the formation of the annexin 2 tetramers (Harder and Gerke, 1993; Harder et al., 1993). To identify infected cells, the EGFP marker was included in the viruses under the control of a second promoter. In control cells and cells infected with either an empty vector (subX) or a vector containing the p36 sense sequence (Ax2-S), secretagogue-evoked stimulation triggered the translocation of annexin 2 to the subplasmalemmal region (Figure 1A). Exocytotic capacity, as measured in parallel by using amperometry to resolve the frequency and kinetics of individual secretory granule release events, was unchanged by p36 sense expression (Figure 1B). Amperometric signals were characterized by a rapid increase in the oxidation current for generally 15–20 s and numerous sharp spikes reflecting the release of the contents of single secretory granules. In contrast, cells infected with the p36 antisense virus (Ax2-AS) exhibited an 80% reduction in the level of endogenous p36 as estimated by quantifying the fluorescence intensity (Figure 1A) and a 75% decrease in exocytotic activity (Figure 1, B and C). Similarly, sequestration of endogenous p36 into cytosolic aggregates by infection with pSFV1EGFP-XM strongly reduced the exocytotic response (Figure 1).

A more detailed amperometric analysis was undertaken on clearly defined spikes recorded at a higher resolution. Expression of p36 antisense or XM markedly decreased the number of nicotine-evoked individual exocytotic events (spikes) compared with control cells (Table 1). We examined the spike characteristics that provide information about the kinetics of fusion pore formation, expansion, and closure (Albillos et al., 1997; Burgoyne and Barclay, 2002). The amplitude (average height) and total charge carried by the residual spikes in infected chromaffin cells with reduced subplasmalemmal p36 were not decreased compared with noninfected control cells (Table 1), indicating that the reduction in secretion was not caused by depletion of granule catecholamine. Similarly, the overall shape of the spikes (rise time and fall time) and the mean values for the half-widths of the spikes were not affected (Table 1). Thus, inhibition of p36 translocation to the plasma membrane affects the number but not the kinetics of the single granule release events detected as amperometric spikes. This suggests that annexin 2 at the plasma membrane might be required for the recruitment and/or docking of secretory granules to sites of exocytosis rather than for the fusion event itself.

Peripheral Annexin 2 Is Not Involved in the Actin Depolymerization Preceding Exocytosis

In chromaffin cells, actin filaments are concentrated under the plasma membrane. This actin network forms a barrier that prevents the docking of chromaffin granules to the plasma membrane. Stimulation triggers the reorganization of actin and partial removal of the barrier (Cheek and Burgoyne, 1986; Aunis and Bader, 1988; Sonntag et al., 1988; Vitale et al., 1995), and these actin filament rearrangements are required for exocytosis. Because annexin 2 is an actin-binding protein, we investigated whether the reduction in peripheral p36 might affect this event. Therefore, actin filaments (F-actin) were visualized by rhodamine-phalloidin staining in cells transiently overexpressing the XM mutant. In resting chromaffin cells infected with control or XM-expressing virus, actin filaments localized to the cell periphery, in the form of a continuous cortical ring (Figure 2A). Stimulation with nicotine decreased the amount of F-actin detected in control and pSFV1EGFP-XM–infected cells by 84 and 89%, respectively (Figure 2, A and B), indicating that cortical actin similarly disassembled despite the blockade of p36 translocation in cells expressing XM. Thus, the presence of annexin 2 in the subplasmalemmal region is not a prerequisite for the actin depolymerization that necessarily precedes the recruitment and docking of secretory granules to the plasma membrane.

Secretagogue-Evoked Stimulation Triggers the Appearance of Lipid Rafts at the Sites of Exocytosis

Annexin 2 has been recently described as a calcium-dependent promoter of lipid raft microdomains in membranes (Babiychuk and Draeger, 2000; Babiychuk et al., 2002; Mayran et al., 2003). Because lipid rafts may be important for exocytosis (Lang et al., 2001; Salaun et al., 2004), we have studied their formation in stimulated chromaffin cells. To

Table 1. Analysis of amperometric spikes from infected chromaffin cells

<table>
<thead>
<tr>
<th></th>
<th>pSFV1EGFP-Ax2S</th>
<th>pSFV1EGFP-Ax2AS</th>
<th>pSFV1EGFP-SubX</th>
<th>pSFV1EGFP-XM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell recorded</td>
<td>36</td>
<td>32</td>
<td>37</td>
<td>30</td>
</tr>
<tr>
<td>Total number of spike</td>
<td>1382</td>
<td>224</td>
<td>1299</td>
<td>182</td>
</tr>
<tr>
<td>No. of spike/cell</td>
<td>38±3</td>
<td>7±1</td>
<td>35±3</td>
<td>6±2</td>
</tr>
<tr>
<td>Rise-time (ms)</td>
<td>7.4±0.5</td>
<td>7.2±0.6</td>
<td>6.9±0.4</td>
<td>7.1±0.4</td>
</tr>
<tr>
<td>Fall-time (ms)</td>
<td>17±1</td>
<td>15.3±1.5</td>
<td>16.2±1.2</td>
<td>14.7±1.6</td>
</tr>
<tr>
<td>Half width (ms)</td>
<td>8.9±0.6</td>
<td>9.1±0.3</td>
<td>9.2±0.5</td>
<td>8.7±0.3</td>
</tr>
<tr>
<td>Ave. height (pA)</td>
<td>1.5±0.16</td>
<td>1.38±21</td>
<td>1.56±19</td>
<td>1.43±14</td>
</tr>
<tr>
<td>Total charge/spike (pC)</td>
<td>1.56±0.16</td>
<td>1.48±0.20</td>
<td>1.68±0.13</td>
<td>1.60±0.18</td>
</tr>
</tbody>
</table>

Amperometric responses were recorded on an expanded time base to avoid overlapping of the spikes. Spikes were only analyzed if they had a base width >10 ms and an amplitude >40 pA. In this way, analyzes were confined to spikes arising immediately beneath the carbon fiber to limit effects on the data of catecholamine diffusion from distant release sites. Spike characteristics were obtained through analysis of amperometric recordings by using Scope version 3.3 software.

...and ordinary antibodies (Bio-Rad, Hercules, CA) and ECL (Amersham Buchler, Braunschweig, Germany). GM1 was detected as described above.

Annexin 2 and Rafts during Regulated Exocytosis

Vol. 16, March 2005 1111

1111
monitor in parallel the exocytic activity and the formation of rafts at the plasma membrane, we used an immunofluorescent approach. Exocytosis was visualized in living cells by adding anti-DBH antibodies into the incubation medium (Chasserot-Golaz et al., 1996). The granule-associated DBH becomes accessible to the antibody only at sites of exocytosis, leading to the appearance of fluorescent patches at the cell surface. In parallel, GM1-containing rafts were visualized using fluorescent cholera toxin (Harder et al., 1998; Janes et al., 1999). As illustrated in Figure 3A, resting chromaffin cells exhibited no DBH patches, confirming the low levels of baseline exocytotic activity in the absence of secretagogue, and displayed only a faint staining with cholera toxin at the cell surface. Stimulation with nicotine for 5 min triggered the appearance of a patchy pattern of DBH surface staining and concomitantly increased the binding of fluorescent cholera toxin (Figure 3A, S1). Note the colocalization between DBH and cholera toxin at the cell surface (Figure 3A, mask), indicating that the GM1-enriched microdomains correspond to the sites of exocytosis. Cholera toxin binding was similarly observed when stimulated cells were fixed before incubation with the toxin, excluding the possibility that GM1 clustering was caused by the toxin itself (Figure 3A, S2). As observed at higher magnification by differential interference contrast (DIC) imaging, the cell membrane remained uniform upon cell stimulation indicating that patches of cholera toxin were not due to morphological heterogeneity of the cell surface (Figure 3B).

To assess whether the increase in cholera toxin labeling resulted from a de novo synthesis of GM1 during the period of stimulation, we compared the amount of GM1 present in fractions collected from a continuous sucrose density gradient layered with crude membranes prepared from resting or stimulated cells. The amount of GM1 was determined by dot blot by using peroxidase-conjugated cholera toxin, whereas cosegregating proteins were identified by SDS-electrophoresis and immunoblotting. As expected, GM1 gangliosides floated in the 30% Optiprep fraction (Figure 4, B and C). As expected, GM1 gangliosides floated in the 30% Optiprep fraction (Figure 4, B and C). Flotillin was enriched in the same fractions, whereas transferrin receptors, which are not associated to DRMs, were present in the bottom of the tube. Caveolin was not detected (our unpublished data), in agreement with previous studies reporting low levels of caveolae in PC12 cells (Bilderback et al., 1999; Chamberlain et al., 2001). The total amount of GM1 and flotillin was not significantly modified in the Triton X-100 homogenates prepared from resting and stimulated cells (our unpublished data). However, GM1 and flotillin were concentrated in the 30% Optiprep fractions in gradients prepared from stimulated cells (Figure 4, B and C), confirming the formation of detergent-insoluble rafts during the exocytic process.

Both p36 and p11 shifted into the GM1- and flotillin-containing DRMs in stimulated cells, together with synap-
GM1-enriched microdomains colocalize to sites of exocytosis. (A) Chromaffin cells were maintained in the resting state (R) or stimulated 5 min with nicotine in the presence of anti-DBH antibodies (S1, S2). Cells were stimulated in the presence of Alexa-488 conjugated cholera toxin and then fixed (S1) or fixed before incubation with Alexa-488 conjugated cholera toxin and then fixed (S2). DBH staining was revealed with Cy3-conjugated anti-rat antibodies. The higher magnification images of a stimulated chromaffin cell double-labeled with DBH antibodies and cholera toxin and the corresponding DIC image. Bars, 5 μm. (C) Fractions 2–12 (40 μg of protein/fraction) collected from a continuous sucrose density gradient were subjected to gel electrophoresis and immunodetection on nitrocellulose using anti-SNAP-25 (plasma membrane marker) and anti-chromogranin A (chromaffin granule marker) antibodies. To detect the ganglioside GM1, 2 μl of each fraction was dot blotted onto nitrocellulose and incubated with HRP-conjugated cholera toxin.

(D) Resting or nicotine-stimulated chromaffin cells were lysed and processed for subcellular fractionation on sucrose gradients. Fractions corresponding to the Ly, CM, PM, and GM were probed for the presence of GM1 by using HRP-conjugated cholera toxin. Similar results were obtained in two independent fractionation performed with different cell cultures. The histogram represents a semiquantitative analysis of the cholera toxin binding detected in the different fractions under resting (R) and stimulating conditions (S). Data are given as the mean values ± SEM (n = 3).
Results mentioned above indicating that the annexin 2 tetramers localize to cholesterol-dependent microdomains in stimulated cells. Together, these results lead us to conclude that secretagogue-evoked stimulation triggers the formation of cholesterol-dependent rafts in the plasma membrane, which are required to stabilize p11, and most likely the annexin 2 tetramer, near sites of exocytosis in chromaffin cells.

Raft/DRMs Dispersion by Cholesterol Sequestration Is Associated with an Inhibition of Exocytosis

To evaluate the functional importance of the secretagogue-evoked lipid rafts in exocytosis, chromaffin cells were treated with various concentrations of filipin and catecholamine secretion was evoked. Figure 6A shows that filipin induced a dose-dependent inhibition of secretion in response to nicotine or a depolarizing concentration of potassium. Moreover, because cholesterol-dependent DRMs have been suggested to be important for clustering and regulation of neurotransmitter receptors and ion channels (Tsui-Pierchala et al., 2002), we also examined the effect of filipin on secretion from permeabilized chromaffin cells, by-pass the nicotinic receptors and voltage-gated calcium channels. Filipin at 10 μM inhibited to a similar extent calcium-evoked catecholamine release from permeabilized cells (Figure 6B), indicating that the formation of cholesterol-dependent DRMs is required at a step distal to the activation of receptors and mobilization of cytosolic calcium. To confirm that the effect of filipin on exocytosis was due to cholesterol sequestration, we measured the influence of cholesterol addition on catecholamine secretion (Figure 6C). Cholesterol partially reversed the inhibitory effect of filipin, in line with the idea that cholesterol-dependent microdomains are important for exocytosis.

Annexin 2 Participates in the Organization of Lipid Rafts during Exocytosis

To probe the idea that annexin 2 contributes to the formation of lipid rafts in stimulated chromaffin cells, we first compared the time course of p36 translocation, raft formation, and catecholamine secretion in nicotine-stimulated chromaffin cells. As illustrated in Figure 7, the translocation of annexin 2 to the plasma membrane occurred as an early event in the exocytotic pathway that clearly preceded raft aggregation detected by cholera toxin labeling and [3H]norepinephrine secretion.

We then used cells expressing the XM chimera in which the translocation of p36 was impaired. Virus-infected cells identified with EGFP were stimulated with nicotine in the presence of cholera toxin to visualize the formation of rafts, fixed, and then stained with anti-p36 antibodies. As expected, stimulation of cells infected with a control vector triggered the translocation of p36 to the cell periphery and the concomitant appearance of rafts at the plasma membrane (Figure 8). In contrast, in chromaffin cells expressing the XM construct, p36 remained aggregated in the cytosol, and very little cell surface binding of cholera toxin was detected (Figure 8A). Semiquantitative analysis performed on cells expressing XM, and on cells with reduced endogenous p36 due to infection with the p36 antisense virus, confirmed that the amount of cholera toxin associated with the plasma membrane was closely related to the presence of p36 in the cell periphery (Figure 8B). Thus, the formation of rafts in secretagogue-stimulated cells depends on the translocation of p36 to the plasma membrane, suggesting that annexin 2 tetramers actively participate in the formation and/or the stabilization of the GM1-containing microdomains required for exocytosis.
We previously studied the distribution of annexin 2 (p36) and its cellular ligand p11 in chromaffin cells and found that p36 is located throughout the cytoplasm, whereas p11 is present exclusively in the subplasmalemmal region (Chasserot-Golaz et al., 1996). Secretagogue-evoked stimulation triggered the colocalization of p36 and p11 underneath the plasma membrane and the formation of the annexin 2 heterotetramer (p90) near exocytotic sites. Microinjection of a...
peptide that competes for the phosphorylation of annexin 2 by protein kinase C (PKC) prevented the translocation of annexin 2 and inhibited exocytosis (Chasserot-Golaz et al., 1996). This suggested that the presence of annexin 2 at the plasma membrane is required for exocytosis, although we could not exclude a direct inhibition of PKC activity in the microinjected cells. In this study, we attempted to obtain more direct evidence for the involvement of annexin 2 in calcium-regulated exocytosis. Using the Semliki Forest virus expression system, we show that expression of an antisense annexin 2 RNA decreases the synthesis of endogenous annexin 2, most probably by forming double-stranded RNA with the endogenous mRNA. This resulted in a marked inhibition of catecholamine secretion from chromaffin cells. Moreover, expression of the chimeric XM protein corresponding to p11 fused C-terminally to the first 18 residues of annexin 2 also produced a strong inhibition of chromaffin cell secretion. In Madin-Darby canine kidney cells, XM causes the aggregation of endogenous annexin 2 and p11 (Harder and Gerke, 1993; Harder et al., 1993). Similarly, in chromaffin cells, XM formed cytosolic aggregates that prevented the translocation of cytosolic p36 to the plasma membrane upon cell stimulation. Together, these results indicate that exocytosis is strongly inhibited when the formation of the annexin 2 tetramer at the plasma membrane is impaired by a reduction of functional p36. To our knowledge, this is the first direct demonstration of a functional role for annexin 2 in dense-core granule exocytosis, by using molecular tools in living cells. It should be mentioned that our results are in contrast to those of a previous study reporting that XM expression in stably transfected PC12 cell lines does not affect Ca$^{2+}$-dependent secretion (Graham et al., 1997). However, stable XM expression resulted in an increase in the...
expression of endogenous annexin 2, and it cannot be ruled out that the endogenous nonaggregated pool of annexin 2 simply remained sufficient to maintain exocytotic activity in this cell line.

Despite 20 years of extensive study, the precise function of most of the annexins remains to be elucidated. Regulated exocytosis in neuroendocrine cells is a process that requires a specific reorganization of the cortical actin cytoskeleton to allow the recruitment and subsequent docking of secretory granules to the plasma membrane (Cheek and Burgoyne, 1986; Aunis and Bader, 1988; Lang et al., 2001). Because annexin 2 is an actin-binding protein (Gerke and Moss, 2002) that translocates from the cytosol to the plasma membrane in stimulated cells, it was conceivable that the protein, by interacting with cytoskeletal elements, might clear a path for secretory granules to move to the membrane. However, using rhodamine-conjugated phalloidin to visualize actin filaments, we could not correlate the strong inhibition of secretion induced by the XM fusion mutant to a stabilization of the cortical actin barrier. Thus, annexin 2 seems not to play an obvious role in promoting cortical actin depolymerization, although we cannot exclude other subtle modifications of the actin cytoskeleton that might be required in late stages of the exocytotic machinery.

Another possible function assigned to annexin 2 relates to the late fusion event. Indeed, it has been reported that phosphorylation by PKC triggers the fusion of purified secretory granules preaggregated by unphosphorylated p36 (Regnouf et al., 1995), suggesting that annexin 2 becomes fusogenic when phosphorylated by PKC. Because secretagogue-evoked stimulation activates PKC to phosphorylate endogenous annexin 2 in chromaffin cells (Delouche et al., 1997), annexin 2 has been proposed to mediate membrane fusion once the granule is brought in proximity to the plasma membrane by SNARE proteins (Regnouf et al., 1995). Our amperometric data do not support this hypothesis. We observed that reduction of annexin 2 expression level in chromaffin cells inhibited the number of exocytotic spikes, but the properties of the remaining spikes remained unchanged with respect to charge and kinetics. This suggests that annexin 2 is involved in the recruitment and/or docking of granules to the exocytotic sites. However, its implication in late events such as the formation of the fusion pore remains to be further investigated.

In many cell types, the association of annexin 2 with the plasma membrane seems to occur preferentially at sites of membrane microdomains, the so-called rafts rich in cholesterol, glycosphingolipids, and glycosylphosphatidylinositol-anchored proteins (Gerke and Moss, 2002). Rafts have been implicated in numerous cellular processes, including signal transduction, molecular sorting, membrane trafficking events, and cell adhesion (Harder et al., 1998; Smart et al., 1999; Dermine et al., 2001). A role for rafts has been recently proposed in regulated exocytosis based on the findings that components of the exocytotic machinery such as syntaxin, SNAP-25, and VAMP2 are associated with rafts (Chamberlain et al., 2001; Lang et al., 2001; Salaun et al., 2004). Lipid rafts are highly dynamic structures that can be very small (a few tens of hundreds of nanometers in diameter) and dispersed but are able to coalesce into large micrometer-sized domains upon cellular stimulation, resulting in the clustering and recruitment of membrane components involved in specific signals or functions (Brown and London, 2000; Abrami et al., 2001; Brown, 2001; Pierini and Maxfield, 2001). Raft dynamics can be influenced by specific proteins, including annexin 2, which has been described as a promoter of lipid microdomain association (Babiychuk and Draeger, 2000). In chromaffin cells, subcellular fractionation experiments have revealed that the translocation of p36 from the cytosol to the cell periphery is accompanied by an increase of the protein in a Triton X-100–insoluble fraction (Chasserot-Golaz et al., 1996; Sagot et al., 1997). Because rafts have been defined by their low density and insolubility in Triton X-100, we examined whether annexin 2 might be involved in the formation of lipid rafts required for exocytosis. We show here that secretagogue-evoked stimulation triggers the Ca2+-dependent formation of GM1-containing domains at the plasma membrane in chromaffin cells. These domains are unlikely to reflect the incorporation of the granule membrane into the plasma membrane and as such be a consequence of the exocytotic process because GM1 cannot be detected in the chromaffin granule membrane. The de novo synthesis of GM1 seems also unlikely considering the small time window in which the cholera-binding sites occur in stimulated cells and the fact that total GM1 was similar in subcellular fractions prepared from resting and stimulated cells. Thus, it is possible that the increase in cholera toxin labeling observed in stimulated cells resulted from the coalescence of small GM1 microdomains into larger units that bound the pentavalent toxin with an increased affinity due to the multivalent display of the aggregated GM1 molecules (Arosio et al., 2004) or that became simply more easy to detect at the light microscopic level. Alternatively, we cannot exclude that GM1 motives are unmasked during stimulation. Centrifugation on density gradients to separate the low-density rafts and analysis of the cosegregating proteins revealed the specific association of the annexin 2-p11 tetramer to the lipid rafts formed in stimulated cells. Moreover, using chromaffin cells expressing the XM chimera or with reduced endogenous p36, we observed a close correlation between the recruitment of annexin 2 to the cell periphery, the enhancement of cholera toxin binding to the cell surface, and the exocytotic response, suggesting that the annexin 2 tetramer actively participates in the coalescence of GM1-containing microdomains and their stabilization into the larger rafts observed in stimulated cells. Finally, we found that cholesterol sequestration by filipin disrupts the annexin 2-containing rafts and in parallel inhibits catecholamine secretion evoked by various secretagogues, suggesting that the lipid microdomains formed in stimulated cells are required for exocytosis. Together, these results support the idea that lipid microdomains formed/stabilized in the plasma membrane by the annexin 2 tetramer play a key role in the organization of the exocytotic machinery in chromaffin cells.

How might annexin 2 influence the formation of lipid rafts in stimulated cells? Considering the binding domains of the annexin 2-p11 heterotetramer, different scenarios may be evoked. Oligomerization of annexin 2 can occur at the cytoplasmic leaflet of the plasma membrane. Because one molecule of annexin 2 can bind four molecules of phosphatidylinositol-4,5-biphosphate (PIP2)-binding protein (Hayes et al., 2004; Rescher et al., 2004), a major plasma membrane phosphoinositol required in exocytosis for the ATP-dependent priming reactions preceding fusion (Hay et al., 1995; Holz et al., 2000). Thus, another attractive hypothesis is that annexin 2 stabilizes PIP2 microdomains in the plasma membrane, which in turn recruit specific PIP2-binding proteins acting in the subsequent stages of exocytosis (Grishanin et al., 2004). In addition, the cortical actin cytoskeleton may provide constraints for the lateral mobility of rafts and increase their stability. Hence, the annexin 2 tetramer formed...
at the plasma membrane may participate in the formation of membrane-cytoskeleton complexes that could control raft assembly. Although additional experimental evidence is now required to explore these possibilities, it is of interest to mention that we found actin in the lipid rafts formed in stimulated cells (our unpublished data), suggesting that the cortical actin cytoskeleton may partner with annexin 2 to stabilize lipid raft domains and organize them into functional exocytotic sites.

The functional characteristics of the sites of exocytosis that ensure tethering of vesicles/granules to the appropriate active zones at the plasma membrane, as well as organization of the exocytotic machinery for rapid and efficient release, remain poorly understood, especially in neuroendocrine cells. Elements with an ability to compartmentalize the plasma membrane and thereby spatially and temporally organize the proteins required for docking and fusion may be crucial for speed and accuracy of the exocytic process. As such, cholesterol-dependent lipid microdomains are ideally suited to bring together and efficiently assemble components of the exocytic pathway, and the observations that SNAREs form cholesterol-dependent clusters in the plasma membrane are in line with this idea (Chamberlain et al., 2001; Lang et al., 2001). The present results provide for the first time a molecular support for the de novo formation of lipid rafts at the granule docking sites in stimulated neuroendocrine cells. Rise in intracellular calcium triggers the recruitment of cytosolic annexin 2 to the plasma membrane. We propose that p11 is the prime anchor for annexin 2 at plasma membrane. By engaging homophilic lateral interactions and binding to negatively charged phospholipids and phospholipid-depletion, annexin 2 tetramers could then induce raft clustering. Once formed, raft structures and the associated cholesterol may further stabilize the lipid–annexin 2 interactions, resulting in annexin 2-membrane scaffolds that may be required to assemble components of the exocytotic machinery. It is of interest to note that the calcium-dependent recruitment of annexin 2 to the plasma membrane offers the cell a mechanism to link spatial control of regulated exocytosis to cell surface receptor activation and calcium signaling.

ACKNOWLEDGMENTS

We thank Dr. Michael A. Frohman for helpful comments and critically reading the manuscript and S. Groux and T. Thabouly for technical assistance. We are grateful to F. Conon (Centre National de la Recherche Scientifique Unité Mixte Recherche-5541, Bordeaux, France) for generously providing carbon fiber electrodes. We acknowledge the confocal microscopy facilities of Plateforme Imagerie In Vitro of IFR 37. This work was supported by the Association de la Recherche sur le Cancer (no. 3208).

REFERENCES


Molecular Biology of the Cell

1118
Annexin 2 and Rafts during Regulated Exocytosis

PtdIns-4,5-P2 as being important for exocytosis. J. Biol. Chem. 275, 17878–17885.


