Phosphorylation of Annexin II Tetramer by Protein Kinase C Inhibits Aggregation of Lipid Vesicles by the Protein*

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Annexin II tetramer (A-II) is a member of the annexin family of Ca** and phospholipid-binding proteins. The ability of this protein to aggregate both phospholipid vesicles and chromaffin granules has suggested a role for the protein in membrane trafficking events such as exocytosis. A-II is also a major intracellular substrate of both pp60** and protein kinase C; however, the effect of phosphorylation on the activity of this protein is unknown. In the current report we have examined the effect of phosphorylation on the lipid vesicle aggregation activity of the protein. Protein kinase C catalyzed the incorporation of 2.1 ± 0.8 mol of phosphate/mol of A-II. Phosphorylation of A-II caused a dramatic decrease in the rate and extent of lipid vesicle aggregation without significantly effecting Ca**-dependent lipid binding by the phosphorylated protein. Phosphorylation of A-II increased the A_{\text{syn}}(Ca**)* of lipid vesicle aggregation from 0.18 \, \mu \text{m} to 0.65 \, \mu \text{m}. Activation of A-II phosphorylation, concomitant with activation of lipid vesicle aggregation, inhibited both the rate and extent of lipid vesicle aggregation but did not cause disassembly of the aggregated lipid vesicles. These results suggest that protein kinase C-dependent phosphorylation of A-II blocks the ability of the protein to aggregate phospholipid vesicles without affecting the lipid vesicle binding properties of the protein.

In the last few years, sequence analysis has defined a new family of Ca**- and phospholipid-binding proteins variously referred to as annexins, calpactins, or lipocortins (reviewed in Dedman, 1986; Russo-Marie, 1986; Khanna et al., 1987a; Klee, 1988; Glenney, 1988; Schlaepfer and Haugler, 1988; Moss and Crumpton, 1990; Burgoyne, 1990; Johnsson et al., 1990; Geisow et al., 1990). All members of this family bind to biological membranes and anionic phospholipids in a Ca**-dependent manner. The common sequence principle of this family is an array of some 70 residues which, depending on the molecular mass of the protein, is tandemly repeated four or eight times (Weber and Johnsson, 1986; Geisow et al., 1986; Saris et al., 1986; Pepinsky et al., 1988; Sudhof et al., 1988; Kretsinger and Creutz, 1986), and each repeat reveals a 17-residue consensus motif, possibly involved in Ca** or lipid binding (Geisow et al., 1986). Typically the annexins are 30-46-kDa monomeric proteins with the exception of the monomeric A-VI (68 kDa) and A-II, which exists as a 36-kDa monomer and a heterotetrameric protein (A-II).1 A-II consists of two copies of the 36-kDa protein and two copies of an 11-kDa protein. Proteolytic enzymes cleave these proteins into two distinct domains: a large protease-resistant core and a small N-terminal tail. The core domain contains the annexin repeats and displays the Ca** and phospholipid binding sites (Johnsson et al., 1986; Glenney, 1986). The N-terminal tail, which is variable in length and sequence among various annexins, contains the phosphorylation sites (Gould et al., 1986; Glenney and Tack, 1985) and also appears to exert a regulatory function on the core. Furthermore, in the case of A-II, the first 17 residues of the N-terminal tail contain the binding site for the 11-kDa subunit.

The N-terminal domains of A-I and A-II appear to play a key role in the regulation of Ca** and phospholipid binding by these proteins. Proteolysis of the N-terminal domain of A-I appears to decrease the A_{\text{syn}}(Ca**)* of binding to phospholipid vesicles (Ando et al., 1989) without decreasing the total Ca*** bound, whereas proteolysis of the N terminus of A-II heavy chain is required for the chromaffin granule aggregation activity of the protein (Drust and Creutz, 1988). Furthermore, an intact N terminus appears to be necessary for reconstitution of secretion by A-I (Ali et al., 1989). The N terminus of A-II heavy chain appears to also play a unique role in the function of A-II since it contains the binding site for the 11-kDa light chain of A-II (p11 subunit). Comparison of the monomeric form of A-II with the tetramer (containing the p11 subunit) suggests that formation of the tetramer results in a molecule with a decreased requirement of Ca** for both phospholipid binding (Powell and Glenney, 1987; Pigault et al., 1990) and that this molecule acquires the ability to aggregate chromaffin granules (Drust and Creutz, 1988). Results from this laboratory have suggested that although both A-I and A-II bind F-actin, only the A-II tetramer can bundle F-actin (Khanna et al., 1990; Ikebuchi and Waisman, 1990). In contrast, both forms of A-II have been suggested to reconstitute secretion (Ali and Burgoyne, 1990; Ali et al., 1989), although these data are controversial (Wu and Wagner, 1991).

The N-terminal domain of A-I and A-II also contains the sites for phosphorylation of the proteins. Several of the annexins have been shown to be in vivo substrates of protein tyrosine kinases; A-II and the A-II are major substrates of p60** (Gerke and Weber, 1985; Glenney, 1985), whereas A-I is a substrate of the epidermal growth factor receptor kinase (Fava and Cohen, 1984; De et al., 1986; Glenney, 1985). A-I, A-II, and A-III are also phosphorylated in vivo by protein kinase C (Michener et al., 1986; Gould et al., 1986). In previous studies of the in vitro phosphorylation of annexins by protein kinase C, we reported a stoichiometry nearing 1 mol of phospho-

* This work was supported by a grant from the Medical Research Council of Canada. 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.

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1 The abbreviations used are: A-II, annexin II tetramer; DAG, 1,2-diolein (diacylglycerol); PAGE, polyacrylamide gel electrophoresis; PS, phosphatidylserine.
 annexin II Tetramer 25977

phosphate/mole of A-I and A-II protein and 0.4 mole of phosphate/mole of A-IIt protein in vitro (Khanna et al., 1986, 1987b). Phosphorylation of A-I appears to involve both serine and threonine residues, whereas A-IIt is phosphorylated only on serine residues (Gould et al., 1986; Khanna et al., 1986; Schlaper and Haigler, 1988; Varticovski et al., 1988).

Phosphorylation of A-II or A-I has previously been shown to influence their lipid binding characteristics. Phosphorylation of A-I by the epidermal growth factor receptor (on tyrosine residues) reduced by 5-fold the Aex(Ca2+) of phospholipid vesicle binding (Schlaper and Haigler 1987), whereas phosphorylation of A-II by p60src decreased the binding of the protein to phospholipid vesicles at low Ca2+ concentrations (Powell and Glenney, 1987).

In the present paper we have investigated the effect of phosphorylation of A-IIt by protein kinase C. Our results suggest that phosphorylation of A-IIt inhibits the ability of the protein to aggregate phospholipid vesicles without affecting the binding of the protein to the phospholipid vesicle. This suggests that protein kinase C may play an important role in the regulation of the function of A-IIt.

Experimental Procedures

Purification of Annexin II—A-IIt was purified from frozen bovine lung (Khanna et al., 1990). All steps were carried out at 4°C. Purified proteins were concentrated to 2-3 mg/ml and stored at -80°C.

Purification of Protein Kinase C—Protein kinase C was purified from rat brain (Pel-Freeze Biologicals) using the procedure established by Wooten et al. (1987), with the exception that a threonine affinity column (Kitano et al., 1986) was substituted for the protamine agarse column. Protein kinase C was stored at -80°C in 5% glycerol and 0.05% Triton X-100.

Lipid Micelle and Vesicle Preparation—Micelles for assaying protein kinase C activity during purification of the enzyme were prepared by the method of Hannun et al. (1986). Two mg of phosphatidylserine (PS) and 0.5 mg of 1,2-diolein (DAG) (Serdary) were dried under nitrogen and then sonicated (three 5-s bursts at 70 watts) in 1 ml of 0.3% Triton X-100.

Lipid vesicles for annexin phosphorylation and aggregation reactions were prepared fresh daily (Reeves and Dowbin, 1969). PS (200 μl of 20 mg/ml) in CHCl3, were diluted with 2 volumes of methanol. The solvents were then evaporated under a stream of nitrogen. The lipids were rehydrated in 1 ml of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.5 mM EGTA, 1.5 mM CaCl2, 1.0 μg/ml protein kinase C, and 0.05% Triton X-100. The mixture was then centrifuged for 5 min at 13,000 Xg.

Phosphorylation of Annexin—A-IIt, 35-60 μg/ml, was incubated at 30°C for the indicated times in 25 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.5 mM EGTA, 1.5 mM CaCl2, 1.0 μg/ml protein kinase C, and 200 μl/ml lipid vesicles (400 μg/ml PS and 40 μg/ml DAG). The reaction was initiated by the addition of 25 μM ATP (200-500 cpm/pmol). In time course experiments 25 μl was removed from the reaction mixture and either precipitated with 25% trichloroacetic acid and 2% sodium pyrophosphate and subjected to scintillation counting or, alternatively, boiled with 1 volume of SDS-PAGE sample buffer (0.25 M Tris-HCl (pH 6.8), 10% SDS, 20% glycerol, 2 mM EDTA, 20 mM β-mercaptoethanol) and analyzed by SDS-PAGE.

Phosphoamino Acid Analysis—Phosphorylated annexin was dialyzed in SDS sample buffer and run on 11.5% SDS-PAGE. Protein bands were visualized by staining in Coomassie Blue. The bands were excised from the gels, washed three times in 20% methanol to remove SDS, and then dried by lyophilization. Protein in gel slices was digested by heating at 110°C in 6 M HCl for 2 h. The samples were dried under nitrogen, resuspended in 50 mM ammonium bicarbonate (pH 8.0), and then lyophilized. Phosphoamino acids were reconverted in electrophoresis buffer (buffer D: pyridine/acetic acid/water, 33:1:40 (v/v)) containing a 1 mM concentration each of O-phosphoserine, O- phosphothreonine, and O-phosphotyrosine and spotted on thin layer cellulose plates (Merck). The plates were run in buffer D (pH 3.5), cathode to anode at constant voltage (1,000 volts) for 30 min using phenol red as a marker. Reference amino acids were stained with ninhydrin (0.2% in acetone) and then incubated at 100°C for 5 min. Labeled phosphoamino acids were identified by autoradiography of the cellulose plates.

Peptide Mapping—Tryptic peptide mapping of phosphorylated A-IIt heavy chain was performed as described by Sharma and Wang (1986). Phosphorylated A-IIt (10 μg) was subjected to SDS-PAGE, and protein bands were excised from the gel. Gel pieces containing A-IIt were washed three times in 50% methanol, lyophilized, and then rehydrated in 1.0 ml of 50 mM ammonium bicarbonate (pH 8.0). The gel was digested for 24 h at 37°C with a total of 30 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. Trypsin digestion was added in three equal aliquots at time zero and after 4 and 16 h of digestion. The peptide fragments were lyophilized and redissolved in buffer D, centrifuged, and dried with a gentle stream of nitrogen. The peptides were then rehydrated in 20 μl of buffer D, and 2 μl of sample was spotted onto thin layer cellulose plates (Merck). Electrophoresis in buffer D was carried out a 500 volts for 1.5 h. A standard chromatography in the second dimension was carried out at 1.5 h in butanol/pyridine/acetic acid/water, 50:33:1:40.

Extraction of Phosphorylated Annexin-A-IIt was phosphorylated in a final volume of 4 ml as discussed above except the MgCl2 concentration was reduced to 2 mM. After 20 min, 10 μl was removed to determine the stoichiometry of phosphorylation. The reaction mixture was centrifuged at 13,000 Xg, and the pellet, containing lipid and lipid-associated A-IIt, was reconstituted in 25 mM Tris-HCl (pH 7.5) and 12.5 mM EGTA. The lipase and protein mixture were sonicated in a bath type sonicator for 15 s and then incubated for 10 min at room temperature. The mixture was then centrifuged for 5 min at 13,000 Xg. The supernatant, containing A-IIt, was removed and centrifuged twice to remove residual lipid vesicles. Recovery of phosphorylated protein was determined by scintillation counting of trifloroacetic acid-precipitated protein. Control samples were incubated in the absence of ATP and recovered the same way. Recovery of phosphorylated A-IIt, which bound to and was pelleted with lipid vesicles in the presence of Ca2+, was 83 ± 6 (n = 5) as confirmed by SDS-PAGE analysis of the supernatant and pellet. The recovery of A-IIt in the EGTA-extracted and pelleted lipid vesicles, as determined by recovery of trifloroacetic acid-precipitable radioactive protein, averaged 45% ± 1 (n = 5).

Lipid Vesicle Aggregation—Lipid vesicle aggregation reaction was carried out in a final volume of 1 ml, and the final A-IIt concentration was 23 pmol/ml. Phosphorylated or control A-IIt was extracted as discussed above into 100 μl of 25 mM Tris-HCl (pH 7.5) and 12.5 mM EGTA. This was diluted into 9.6 ml of 25 mM Tris-HCl (pH 7.5), 1.5 mM MgCl2, and 1.5 mM EGTA. Lipid vesicles (250 μl) were added, and the mixture was divided into 0.950-ml aliquots. Aggregation was initiated by the addition of CaCl2 (50 μl of CaCl2 stocks typically ranging from 5 to 50 μM) to the required concentration and monitored by the increase in absorbance at 540 nm at 5-min intervals for a total period of 30 min. The final absorbance change in the presence of A-IIt-induced aggregation varied between 0.140 and 0.160 absorbance units. Free Ca2+ concentrations were determined by the estimation of Fabiato and Fabiato (1979).

Phosphorylation and Aggregation Reaction—The aggregation reaction was carried out with 23 pmol of A-IIt and 0.5 μg of protein kinase C in 25 mM Tris-HCl (pH 7.5), 2 mM MgCl2, ATP (50 μM), and 1.5 mM EGTA at room temperature. Controls were carried out in the absence of ATP. Aggregation and phosphorylation were simultaneously initiated by the addition of CaCl2. The increase in absorbance at 540 nm was monitored at 5-min intervals for 30 min. To measure the phosphorylation of A-IIt which occurred during aggregation, the concentrations of free Ca2+ were carried out, and the stoichiometry of phosphorylation was measured as described above.

Miscellaneous Techniques—Protein concentration was measured using the Bradford (1976) Coomassie blue dye binding assay using bovine serum albumin as a standard. The A-IIt concentration was also determined spectrophotometrically using an extinction coefficient of As280 of 0.65 for 1 mg/ml (Gerke and Weber, 1986). SDS-
polyacrylamide gel electrophoresis was performed using the method of Laemmli (1970). The concentration of all CaCl2 stock solutions was determined by atomic absorption spectroscopy.

Materials—All reagents were of analytical grade. Coomassie Blue dye reagent was purchased from Bio-Rad. SDS, bisacrylamide, acrylamide, dithiothreitol, NaCl, Tris-HCl, and trypsin were purchased from Boehringer Mannheim. Triton X-100 was purchased from Fluka. [γ-32P]ATP was purchased from Amersham. Acetic acid, acetamide, dithiothreitol, NaCl, Tris-HCl, and trypsin were purchased from BDH. Trichloroacetic acid, HCl, ammonium bicarbonate, EDTA, and EGTA were purchased from CanLab. All other chemicals were purchased from Sigma.

RESULTS

Phosphorylation of A-II1—A time course of the phosphorylation of A-II1 by rat brain protein kinase C is presented in Fig. 1. A-II1 was phosphorylated to 2.1 ± 1.2 mol of phosphate/mol of A-II1 (S.D., n = 19). Phosphorylation of A-II1 by protein kinase C was rapid, with half-maximal incorporation occurring within 2 min, and the reaction was essentially complete by 30 min. Phosphorylation of A-II1 by protein kinase C generated multiple forms of the heavy chain, which could be distinguished by reduced mobility on SDS-PAGE (Fig. 1, inset A). As phosphorylation progressed, Coomassie Blue staining of the protein band with identical mobility to band a decreased in intensity, and there was a concurrent increase in the Coomassie Blue staining intensity of bands b and c. The autoradiogram of the gel (Fig. 1, inset B) shows that all of the Coomassie Blue-stained bands were radioactive, suggesting that the time-dependent increase in phosphorylation of annexin II heavy chain results in the generation of bands b and c. Comparison of the Coomassie Blue-stained bands a and b at 30 min (Fig. 1, inset A) suggests that although the staining intensity of each band is comparable, the incorporation of 32P into band b is greater (Fig. 1, inset B), inferring that the stoichiometry of phosphorylation of band b is greater than band a.

Phosphoamino Acid and Phosphopeptides—Previous studies have suggested that protein kinase C phosphorylates A-II1 exclusively on serine residues in vivo and in vitro (Gould et al., 1986; Khanna et al., 1986); however, the in vitro studies have demonstrated low stoichiometry of phosphorylation (typically about 0.5 mol of P/mmol of A-II1). Considering the higher stoichiometry observed under optimal assay conditions (Fig. 1), the possibility existed that additional amino acid residues might be phosphorylated. The phosphoamino acids were analyzed, and the presence of only phosphoserine was detected (not shown). Phosphopeptide maps of SDS-PAGE bands a and b were generated (Fig. 2). In each digest, the major phosphopeptide was the same, but the relative proportions of the secondary and minor phosphopeptides were different. In digests of band a (Fig. 2A), phosphopeptide 8 was the major phosphopeptide, whereas phosphopeptides 4, 7, and 11 were secondary in intensity on autoradiograms. In digests of band b (Fig. 2B), phosphopeptide 8 was again the major phosphopeptide, whereas the relative proportion of phosphopeptide 4 to phosphopeptide 7 was increased, and a new phosphopeptide, 5, appeared. These results suggest that the altered mobility of phosphorylated A-II1 on SDS-PAGE was caused by distinct phosphoforms of the protein.

Lipid Vesicle Aggregation by Phosphorylated A-II1—To study the effect of phosphorylation on an in vitro activity of A-II1, we chose to examine lipid vesicle aggregation. The conditions of the aggregation assay and the composition of the vesicles used for aggregation were chosen not only for their aggregation properties but also for their ability to support protein kinase C activity. Aggregation of these vesicles, composed of PS and DAG, under our assay conditions typically resulted in an increase in light scattering (540 nm) of 5–7-fold over initial values. A typical experiment showing the effect of phosphorylation of A-II1 on the rate of lipid vesicle aggregation is shown in Fig. 3. Phosphorylation of A-II1 reduced the rate and extent of lipid vesicle aggregation. Conversely, in control experiments in which A-II1 was incubated in the phosphorylation reaction in the absence of added ATP, there was no apparent difference between it and untreated A-II1 in terms of the Ca2+ concentration required to induce lipid vesicle aggregation or in the rate or extent of lipid vesicle aggregation induced.

Ca2+ Dependence of Lipid Vesicle Aggregation by A-II1—The Ca2+ dependence of lipid vesicle aggregation by phos-
The addition of protein after the aggregation reaction was complete, and the lipid-associated phosphorylated and nonphosphorylated A-II is shown in Fig. 4. Phosphorylation was monitored by measuring the absolute change in absorbance at 540 nm in the presence of nonphosphorylated (C—O), or phosphorylated (●—●) A-II (2.7 mol of phosphate/mol).

**Fig. 4. Ca**

**phosphorylated and nonphosphorylated A-II is shown in Fig. 4. The phosphorylated protein aggregated the lipid vesicles to the same extent as nonphosphorylated protein, but higher concentrations of Ca**

**2** were required to induce the same level of aggregation. The Ca**

**2** concentration required to induce half-maximal aggregation increased from 0.18 μM ± 0.6 (n = 3) for nonphosphorylated A-II to 0.65 mM ± 0.4 (n = 3) for phosphorylated protein. Aggregation experiments performed at a variety of Ca**

**2** concentrations, in the absence of A-II, suggested that A-II-independent lipid vesicle aggregation only occurred at Ca**

**2** concentrations greater than 5 mM Ca**

**2**.

To determine if the reduction of aggregation by phosphorylated A-II was because of a reduction of lipid binding activity, the lipid vesicle binding properties of phosphorylated and nonphosphorylated A-II were examined. Following the 30-min aggregation reaction, the reaction mixture was centrifuged at 100,000 x g, and the protein bound to lipid vesicles was assessed on SDS-PAGE. The inset to Fig. 4 shows the recovery of phosphorylated protein and nonphosphorylated protein, recovered in the pellet, from lipid vesicles incubated at 0.6, 200, and 400 μM Ca**

**2**. In the absence of Ca**

**2**, no protein was recovered in the pellet. Phosphorylation did not reduce the amount of protein recovered with lipid vesicles in the high speed pellet, indicating that the A-II bound to lipid vesicles at concentrations of Ca**

**2** that were insufficient to support lipid vesicle aggregation.

**Phosphorylation during Aggregation**—To examine the effect on the A-II-mediated aggregation of lipid vesicles during simultaneous activation of both the phosphorylation of A-II by protein kinase and A-II-induced aggregation of lipid vesicles, A-II was incubated in the presence of lipid vesicles and protein kinase C, with or without added ATP (Fig. 5). The phosphorylation and aggregation reactions were both initiated by the addition of CaCl₂ (0.6 μM). The aggregation reaction was monitored spectrophotometrically. Initially, in both samples, aggregation was rapid, but as phosphorylation progressed, the relative rate of the reaction containing ATP was reduced. In parallel control experiments, in which the protein kinase C was omitted from the reaction mixture, the addition of ATP did not affect the rate or extent of vesicle aggregation.

**Phosphorylation and A-II Subunit Structure**—Johnson et al. (1986) reported that monomeric A-II phosphorylated by protein kinase C resulted in multiple phosphoforms of the protein on SDS-PAGE, but phosphorylated A-II produced only a single band on SDS-PAGE. This presents the possibility that disruption of the A-II tetramer upon phosphorylation by protein kinase C is responsible for the effect of phosphorylation on lipid vesicle aggregation. Therefore, the mobility of A-II phosphorylated to 4 mol of phosphate/mol of protein was determined by gel permeation chromatography (Fig. 6). Phosphorylated A-II displayed a mobility indistinguishable from nonphosphorylated A-II, suggesting that the tetramer is intact following phosphorylation.

**DISCUSSION**

Annexin-IIt has been shown to mediate the Ca**

**2**-dependent aggregation of phospholipid vesicles and chromaffin granules. These activities have been interpreted as evidence for a regulatory role for the protein in membrane trafficking events such as exocytosis. Although A-II has been identified as an in vivo substrate of protein kinase C, the effect of phosphorylation of the protein on the activity of the protein has not been determined. In the current report we examine the effect of protein kinase C-dependent phosphorylation of A-II on the lipid vesicle aggregation activity of the protein.

The stoichiometry of phosphorylation of A-II by protein kinase C is much higher than our previous report (Khanna et al., 1987a, 1987b). This is because of our observation that the

**Fig. 5. Effect of protein kinase C on A-II-induced lipid vesicle aggregation.** 23 pmol of A-II and 0.5 μg of protein kinase C were incubated at a final volume of 1 ml in 25 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1.5 mM EGTA, and 25 μl of lipid vesicles at room temperature. The phosphorylation and aggregation reactions were simultaneously initiated by addition of Ca**

**2** to a final concentration of 0.6 μM. Lipid vesicle aggregation was measured by the absolute change in absorbance (540 nm) in the presence of protein kinase C in the absence (C—O) or presence (●—●) of 50 μM ATP. Error bars indicate the S.E. of five individual assays.
phosphorylation reaction is dependent on protein concentration; increasing the protein concentration above 0.7 nmol/ml resulted in a decrease in the stoichiometry of phosphorylation. The decline in stoichiometry of phosphorylation of A-II$\alpha$ with increased protein concentration could not be reversed by an increase in the amount of lipid vesicles or by increasing the amount of protein kinase C (not shown). A-II$\alpha$ was optimally phosphorylated by protein kinase C at protein concentrations below 0.7 nmol/ml. The average level of phosphorylation under our assay conditions was 2.1 \pm 1.2 mol of phosphate/mol of protein (n = 19). The light chain was not visualized on autoradiograms, suggesting that the phosphorylation sites on A-II$\alpha$ were restricted to the heavy chain. Electrophoresis of phosphorylated A-II$\alpha$ on SDS-polyacrylamide gels revealed two new forms of A-II$\alpha$ heavy chain, with reduced electrophoretic mobility relative to nonphosphorylated heavy chain. All three forms of the heavy chain were detected under our assay conditions. Phosphorylation of A-II$\alpha$ by protein kinase C did not induce subunit dissociation, as demonstrated by gel permeation chromatography on a Superose 12 (Pharmacia LKB Biotechnology Inc.) column. The column was developed with 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100, and the radioactivity of column fractions was determined by scintillation counting. The profiles of the radioactivity of column fractions of phosphorylated A-II$\alpha$ and the absorbance at 280 nm nonphosphorylated A-II$\alpha$ (left peak) and A-II$\alpha$ monomer (right peak) are shown.

The Ca$^{2+}$ concentrations required to induce lipid vesicle binding and lipid vesicle aggregation by A-II$\alpha$ are quite similar (Powell and Glenney, 1987; Blackwood and Ernst, 1990). However, the fact that phosphorylation of A-II$\alpha$ inhibits the lipid vesicle aggregating activity at Ca$^{2+}$ concentrations at which the A-II$\alpha$ is found to bind to the lipid vesicles suggests that phosphorylation by protein kinase C differentially affects the two phenomena. A-II$\alpha$-induced lipid vesicle aggregation may be mediated by a single A-II$\alpha$ protein interacting with two lipid vesicles. Alternatively a protein-protein bridge, formed by the interaction of two A-II$\alpha$ molecules associated with distinct lipid vesicles, may be required to aggregate lipid vesicles. Zaks and Creutz (1991) found evidence that annexin IV, VI, or VII aggregates chromaffin granules by both mechanisms; protein-protein interaction was observed during chromaffin granule aggregation at low Ca$^{2+}$ concentrations, whereas aggregation at high Ca$^{2+}$ concentrations occurred in the absence of protein-protein interaction. The membrane, or lipid vesicle aggregation induced by A-II$\alpha$, like other annexins, may operate through distinct mechanisms at low Ca$^{2+}$ and high Ca$^{2+}$ concentrations. Phosphorylation of A-II$\alpha$ by protein kinase C may therefore inhibit only the A-II$\alpha$-induced lipid vesicle interaction which occurs at low Ca$^{2+}$ concentrations, thus requiring increased Ca$^{2+}$ concentrations to aggregate lipid vesicles with phosphorylated A-II$\alpha$. The second possibility is that a single annexin tetramer forms a bridge between two lipid vesicles. The lipid vesicle binding sites is then reduced from two to one by protein kinase C-induced phosphorylation of A-II$\alpha$.

A-II$\alpha$ and protein kinase C are both potentially involved in stimulus secretion coupling (Burgoyne et al., 1990). The roles for protein kinase C in A-II$\alpha$-mediated secretion, however, are controversial. Ali and Burgoyne (1990) suggest that A-II$\alpha$ can reconstitute secretion in protein-depleted adrenal cells in the presence of the protein kinase C inhibitor, staurosporin. Sarafian et al. (1991) maintain that to reconstitute secretion in permeabilized adrenal chromaffin cells, A-II$\alpha$ must be phosphorylated by protein kinase C. With the highly phosphory-
ated A-IIt, the effect of protein kinase C phosphorylation on membrane aggregation can be further investigated.

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