“In Vitro” Phosphorylation of Annexin 2 Heterotetramer by Protein Kinase C

COMPARATIVE PROPERTIES OF THE UNPHOSPHORYLATED AND PHOSPHORYLATED ANNEXIN 2 ON THE AGGREGATION AND FUSION OF CHROMAFFIN GRANULE MEMBRANES*

(Received for publication, October 28, 1994, and in revised form, July 11, 1995)

Françoise Regnouf, Isabelle Sagot, Bruno Delouche, Ginette Devilliers, Jean Cartaud, Jean-Pierre Henry, and Louise-Anne Pradel§

From the Centre National de la Recherche Scientifique, Unité associée 1112 de Neurobiologie Physico-Chimique, Institut de Biologie Physico-Chimique and the Département de Biologie Supramoléculaire et Cellulaire, Biologie cellulaire des membranes, UMR 9922, Institut Jacques Monod, Université Paris 7, 2 place Jussieu, 75005 Paris, France

Heterotetrameric annexin 2 phosphorylated “in vitro” by rat brain protein kinase C is purified and obtained devoid of unphosphorylated protein; it contains 2 mol of phosphate/mol of heterotetramer. The aggregative and binding properties of the phosphorylated annexin 2 toward purified chromaffin granules are compared with those of the unphosphorylated annexin 2. Annexin 2 binds to chromaffin granules with high affinity. Phosphorylation of annexin 2 decreases the affinity of this binding without affecting the maximum binding capacity. The binding curves are strongly cooperative. It is suggested that a surface oligomerization of the proteins may take place upon binding. Besides, phosphorylation of annexin 2 is followed by a dissociation of the light chains from the heavy chains in the heterotetramer. Whereas annexin 2 induces the aggregation of chromaffin granules at μM calcium concentration, the phosphorylated annexin 2 does not induce aggregation at any concentration of calcium either at pH 6 or 7. The phosphorylation of annexin 2 by protein kinase C, MgATP, and 12-O-tetradecanoylphorbol-13-acetate on chromaffin granules induces a fusion of chromaffin granules membranes observed in electron microscopy. The fusion requires the activation of protein kinase C by 12-O-tetradecanoylphorbol-13-acetate. Given these results and since annexin 2 is phosphorylated by protein kinase C under stimulation of chromaffin cells, it is suggested that phosphorylated annexin 2 may be implicated in the fusion step during exocytosis of chromaffin granules.

Annexin 2 is a calcium-phospholipid-binding protein of the annexin family which has been characterized in the adrenal medulla as chromobindin 8. The heterotetrameric molecule formed of two heavy chains of 36 kDa and two light chains of 11 kDa possesses the unique property among the other annexins to aggregate chromaffin granules at micromolar calcium concentration (1).

It has been shown by immunoelectron microscopy (2) that in chromaffin cells annexin 2 was closely associated with the inner face of the plasma membranes. In cultured chromaffin cells, thin strands were found cross-linking the chromaffin vesicles to the plasma membrane after stimulation with acetylcholine. Similar thin strands were also observed between aggregated chromaffin vesicles when they were mixed with annexin 2 in the presence of calcium. These data strongly suggested that conformational changes were induced in annexin 2 to cross-link the vesicles and the plasma membrane after stimulation of cultured chromaffin cells. When primary cultured chromaffin cells were stimulated by nicotine, annexin 2 was phosphorylated by protein kinase C. The phosphorylation of the protein was concomitant with the catecholamine release. In streptolysin-permeabilized cells, annexin 2 phosphorylated “in vitro” by brain protein kinase C was able to reconstitute secretion of catecholamines in cells depleted of protein kinase C activity (3). Taken together, all these results suggest that annexin 2 could be involved in the exocytotic process. It was, therefore, of interest to reinvestigate the properties of annexin 2 phosphorylated in vitro by protein kinase C.

A study of the effect of phosphorylated annexin 2 tetramer on the aggregation of lipid vesicles (4) demonstrated that phosphorylation of annexin 2 caused a loss of lipid vesicle aggregation. In order to reproduce more closely the physiological conditions found in the chromaffin cells, we focused our study on the properties of the phosphorylated annexin 2 tetramer toward the chromaffin granules in the same experimental conditions as those of Drust and Greutz (1) who observed an aggregation of granules induced by annexin 2 tetramer at micromolar calcium concentration at pH 7 and a fusion of the same granules by lowering the pH to 6 in the presence of arachidonic acid.

Our results extended the previous studies on the properties of phosphorylated annexin 2. We demonstrated that phosphorylated annexin 2 bound to the granules with a lower affinity than the unphosphorylated annexin 2. This binding was strongly cooperative like that of unphosphorylated annexin 2. When annexin 2 was added to a suspension of chromaffin granules in the presence of protein kinase C and TPA, the phosphorylated annexin 2 that was produced induced a spectacular fusion of granules. It was the first time that fusion was induced under phosphorylation of a protein. Besides, this protein was phosphorylated in vivo by protein kinase C under nicotine stimulation of chromaffin cells.

* 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 so to indicate this fact.

§ To whom correspondence should be addressed: Service de Neurobiologie Physico-Chimique, Institut de Biologie Physico-Chimique, 13 rue P. et M. Curie, 75005 Paris, France. Tel.: 33-1-43-25-26-09. Fax: 33-1-40-46-83-31.

1 B. Delouche, J. P. Henry, and L. A. Pradel, manuscript in preparation.

2 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; A-2t, heterotetrameric annexin 2; MES, (2-N-morpholino)ethanesulfonic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
**EXPERIMENTAL PROCEDURES**

Protein Purification—Annexin 2 was purified from bovine lung as described previously (5) with some modifications. Bovine lung was obtained fresh and cleaned at a slaughter house and immediately frozen in liquid nitrogen. It was stored at −80°C until use. All purification steps were carried out at 4°C. Protease inhibitors were present in every buffer used as follows: 0.3 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 5 μM of leupeptin and pepstatin, 20 μM of soybean trypsin inhibitor until the first chromatography, diisopropylfluorophosphate (0.5 mM), added only in the first homogenization. Thawed tissues (250 g) in 2.4 volumes of buffer A (25 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EDTA, 3 mM NaN₃) containing 150 mM NaCl and 10 mM CaCl₂ were homogenized in a Waring blender three times for 15 s each at a low rate and three times for 15 s each at a high rate. The homogenate was centrifuged for 20 min at 16,000 × g. The pellet was washed three times in 1.6 volumes of buffer A containing 1 mM CaCl₂, and for the first washing 300 mM NaCl and for the following washings 150 mM NaCl. The final washed pellet was extracted with 1.2 volumes of buffer A containing 10 mM EGTA and 1% Triton X-100. The homogenate was stirred for 1 h and then centrifuged for 1 h at 100,000 × g. The supernatant was adjusted to 1 mM free Ca²⁺ from stock solution in Me₂SO was diluted in H₂O before use. The homogenate was centrifuged for 20 min at 16,000 × g. The supernatant was applied to a 4.5-ml DE52 column (70 M TPA. Fusion was assessed after 12–15 min of incubation on the other part the stained protein bands were quantified by densitometry with unphosphorylated annexin 2 as the standard. For quantification of ³²P incorporated, protein bands were excised from gel, and the radioactivity was measured with a scintillation counter either directly or after digestion with 30% H₂O₂ (0.3 ml), 5% performic acid (0.2 ml) for one night at 50°C.

Preparation of Chromaffin Granules—Intact bovine chromaffin granules were prepared from crude granule fractions (9) using a Percoll density gradient (10). The resultant granule pellet was washed twice with 10 mM Hepes, pH 7.0, 0.3 M sucrose, resuspended, and stored in the same buffer. The protein concentration was determined by the method of Bradford (11). The actin content of chromaffin granules has been tested by immunoblotting with monoclonal anti actin. The actin content is less than 0.01 μg/50 μg granules protein.

Binding Assays—Binding of ¹²⁵I-labeled A-2t and phosphorylated A-2t to chromaffin granules was measured by incubating increasing concentrations of protein for 20 min at room temperature with 50 μg of granules in a final volume of 0.5 ml of 40 mM Hepes, pH 7.0, or 40 mM MES, pH 6.0, 30 mM KCl, 240 μM sucrose, 10 μM Ca²⁺. Samples were centrifuged for 30 min at 10,000 × g. The granule pellets were first assayed for ¹²⁵I or ³²P in a γ and β counter, respectively, then subjected to SDS-PAGE. The gel was stained with Coomassie Blue, and the labeled 36-kDa bands were quantified using the Phosphorimager with labeled A-2t as a standard.

Aggregation and Fusion Assays—Chromaffin granule aggregation was followed by the increase of turbidity at 540 nm as described (12, 13). The reaction mixture in a final volume of 0.5 ml contained 50 μg of chromaffin granules in 40 mM Hepes, pH 7.0, or 40 mM MES, pH 6.0, 240 mM sucrose, 30 mM KCl, and free Ca²⁺ at the determined concentration. Aggregation was initiated by adding unphosphorylated or phosphorylated A-2t and monitored for 5 min at 25°C on a Uvicron 810 spectrophotometer. In the assays with added protein kinase C, the reaction mixture used above contained in addition 50 μM ATP, 5 mM MgCl₂, 50 μM TPA. Fusion was assessed after 12–15 min of incubation at 25°C. It should be noted that the protein kinase C used is collected directly after its elution from the column of phenyl-Sepharose CL-4B, without added 0.05% Triton X-100 and 10% glycerol. The experiments of fusion are made within 24 h.

Electron Microscopy—Samples submitted to the aggregation or fusion assays were fixed for 30 min in glutaraldehyde 2.5% final concentration, in the aggregation buffer, then centrifuged for 15 min at 20,000 × g. The pellets were washed in 0.1 M NaH₂PO₄·Na₂HPO₄ buffer, pH 7.5, then in 0.1 M sodium cacodylate, pH 7.0, were postfixed in a solution of 1% OsO₄ in sodium cacodylate buffer for 30 min, dehydrated, and embedded in Epon-Araldite. Ultrathin sections between 0.06 and 0.1 μm were stained with 5% uranyl acetate and lead citrate and examined with a Philips CM12 electron microscope.

**RESULTS**

Phosphorylation of Annexin 2—The highest level of phosphorylation of heterotetrameric A-2t by purified brain protein kinase C was obtained at pH 6.0, in the presence of 1 mM Ca²⁺ concentration. As shown in Fig. 1, 2.5 ± 0.6 mol of phosphate/mol of protein were incorporated after 30 min; at this time the reaction was complete. At pH 7.0, in the presence of either 10

---

³ Castagna, personal communication.
µM or 1 mM Ca²⁺ no more than 1 mol of phosphate could be incorporated per mol of A-2t. The Coomassie Blue staining and autoradiography of SDS-PAGE of 95% phosphorylated A-2t (Fig. 1, inset) showed the presence of three phosphorylated forms with reduced mobilities quite distinct from that of unphosphorylated A-2t (their apparent molecular masses were, respectively, 38, 39, and 40 kDa for the bands 1, 2, and 3). ³²P phosphorylated A-2t (their apparent molecular masses were, respectively, 38, 39, and 40 kDa for the bands 1, 2, and 3) showed the presence of three phosphorylated forms with reduced mobilities quite distinct from that of unphosphorylated A-2t (their apparent molecular masses were, respectively, 38, 39, and 40 kDa for the bands 1, 2, and 3). ³²P incorporated in the bands 1, 2, and 3 was 1, 2, and 2 mol/mol of 36-kDa heavy chain, respectively. This result suggested that one amino acid residue was phosphorylated per 36 kDa heavy chain in the band 1, whereas two amino acid residues could be phosphorylated per p36 heavy chain in the bands 2 and 3.

The proportions of the phosphorylated forms might vary from one assay to another, but for most of them band 1 represented 85% of the total of the three phosphorylated forms, bands 2 and 3 together accounting for the remaining 15%. Indeed, the intensity of each band on the Coomassie Blue-stained gel and on the autoradiogram decreased from band 1 to 3 and 2, suggesting that the amount of protein phosphorylated on band 1 was greater than on bands 3 and 2. Phosphorylated A-2t was pure and free from unphosphorylated A-2t, protein kinase C, and Triton X-100. The yield of phosphorylated A-2t after purification was 45% with regard to A-2t used for the phosphorylation.

Effect of Phosphorylation of Annexin 2 on Association of Light Chain p11 to the Heavy Chain p36—The succrose gradient sedimentation (Fig. 2A) like the gel filtration analysis on a Superose-12 column (Fig. 2B) shows that about 50% of the monophosphorylated annexin 2 retained its ability to bind p11, the other 50% sedimented or eluted in the same fractions as the diphosphorylated form. On Superose, the elution volume of the heterotetramer was well resolved from that of the monomer, and it was clear that the mono-, like the diphosphorylated forms, were not dissociated in monomers but that p11 light chains were lost.

Binding of Unphosphorylated and Phosphorylated Annexin 2 to Chromaffin Granules—Studies of binding were performed at pH 7.0 and 6.0. At these two pH, ¹²⁵I-labeled A-2t (Fig. 3) and phosphorylated A-2t (Fig. 4) bound to chromaffin granules in a saturable manner.

The concentrations of ¹²⁵I-A-2t used were in a range of 0.17–8 µg for 50 µg of granule protein.

For ¹²⁵I-labeled A-2t, the Hill coefficient was 2.0 ± 0.5 at pH 6.0 and 1.7 ± 0.1 at pH 7.0, the apparent Kₐ of these binding at pH 6.0 and 7.0 was 5.7 ± 1.3 and 13.5 ± 1.4 nM, respectively. The Scatchard plot for these bindings showed that the binding of A-2t to granule membranes was strongly cooperative (Fig. 3, A and B), with a Kₐ of 7.8 ± 4 and 34 ± 4 nM, a Hill coefficient of 1.9 ± 0.1 and 1.3 ± 0.1 at pH 6.0 and 7.0, respectively. At saturation, 1.7 ± 0.3 and 2.2 ± 0.6 nmol of A-2t bound per mg of granule proteins at pH 6.0 and 7.0 (n=10) (Table I). The concentration of A-2t necessary to obtain saturation was 8 µg for 50 µg of granule protein.

The phospholipid content determined on the chromaffin granule membranes was 0.48 µmol/mg of proteins. Assuming that 10.4% phosphatidylserine and phosphatidylinositol were found in total lipids of the granule membranes (18–20) and taking these data into account, we obtained 1 mol of A-2t bound per 27 and 33 mol of phosphatidylserine at pH 6.0 and 7.0, respectively (Table I).

For studying binding of ³²P-A-2t, the protein concentrations used were in a range of 0.19–30 µg for 50 µg of granule protein.

The nonlinear regression of the binding curve gave a Hill coefficient of 2.3 ± 0.5 at pH 6.0 and 1.7 ± 0.4 at pH 7.0. The apparent Kₐ for these bindings at pH 6.0 and 7.0 was 54 nM ± 6 and 99 nM ± 23 (Fig. 4, A and B). The Scatchard plot for these bindings demonstrated that the binding of ³²P-A-2t was strongly cooperative (Fig. 4, A and B) with a Kₐ of 70 and 60 nM and a Hill coefficient of 1.6 and 1.3 at pH 6.0 and 7.0, respectively. At saturation, 1.4 and 3.2 nmol of ³²P-A-2t bound per mg of protein i.e. 1 mol of ³²P-A-2t binds 40 and 17 mol of phosphatidylserine at pH 6.0 and 7.0, respectively (n=5) (Table I). The concentration of ³²P-A-2t available to reach the saturation was of the order of 30 µg/50 µg of granule protein.

Binding of phosphorylated A-2t was determined in the presence of protein kinase C and 10% SDS-PAGE. The yield of phosphorylated A-2t after purification was 45% with regard to A-2t used for the phosphorylation.

The phospholipid content determined on the chromaffin granule membranes was 0.48 µmol/mg of proteins. Assuming that 10.4% phosphatidylserine and phosphatidylinositol were found in total lipids of the granule membranes (18–20) and taking these data into account, we obtained 1 mol of A-2t bound per 27 and 33 mol of phosphatidylserine at pH 6.0 and 7.0, respectively (Table I).
Fig. 3. Binding of annexin 2 to chromaffin granules. A, the binding conditions were 40 mM MES, pH 6, 30 mM KCl, 0.24 M saccharose, 10 μM calcium, and 50 μg of granule protein at room temperature. B, the binding conditions were 40 mM Hepes, pH 7, 30 mM KCl, 0.24 M saccharose, 10 μM calcium, and 50 μg of granule protein at room temperature. The binding was sigmoidal (upper part). Scatchard analysis of the A-2t binding data (lower part). The downward concave curve indicated the positively cooperative nature of this interaction. Inset, Hill plot of the binding data. The slope of the regression curve was 1.9 at pH 6 and 1.4 at pH 7. The log(y/(1 − y)) was plotted on the ordinate; log(free (ng)) was plotted on the abscissa. y represented the fractional saturation: bound/maximal binding.

Fig. 4. Binding of phosphorylated annexin 2 to chromaffin granules. The binding conditions were those described in the legend to Fig. 3. A, pH 6. The binding curve was sigmoidal (upper part). The Scatchard analysis gave a downward concave curve (lower part). B, pH 7. The binding curve was sigmoidal. The Scatchard analysis gave a downward concave curve.

Phosphorylated Annexin 2—Drust and Creutz (1) observed an aggregation of chromaffin granule membranes by annexin 2 heterotetramer (calpactin 1) at 3 μM Ca2⁺, pH 7. Moreover, they observed the fusion of granule membranes by lowering the pH to 6.0 in the presence of arachidonic acid.

On the basis of this result we studied the aggregation of granules by A-2t at pH 7.0 and 6.0. Fig. 5 shows that the half-maximum aggregation of A-2t at pH 7.0 occurred at 5 μM Ca2⁺ similar to the previous report (1) and at pH 6.0 at 2.5 μM Ca2⁺. Fig. 5 shows clearly that the phosphorylated A-2t did not promote granule aggregation at any concentration of Ca2⁺ from 0 to 1 mM either at pH 7.0 or 6.0.

In order to test the dynamic of the phosphorylation of annexin 2 on granules, we studied directly the phosphorylation by adding protein kinase C to a suspension of granules containing in addition to the components of the aggregation medium,
Following the 5 min of aggregation, glutaraldehyde was submitted to aggregation in the presence of A-2t or TPA without protein kinase C (Fig. 8). In these conditions (Fig. 8), we observed in the presence of unphosphorylated annexin 2 with MgATP, TPA, or protein kinase C and TPA, most of the aggregated granules were induced by adding 4–5 μg of protein kinase C, 50 μM TPA, alone or mixed. Aggregation and fusion were expressed after 12 min of incubation in the absence (lanes 1) or presence (lanes 2) of protein kinase C. a, Comassie Blue; b, PhosphorImager. The two 32P-labeled bands were respectively mono (38 kDa) - and di (40 kDa) - phosphorylated proteins.

The values given in this table are the average of ten individual assays for A-2t and five assays for 32P-A-2t. The decrease of absorbance observed was indicative of an aggregation of granules, then, a slow decrease of the absorbance. After 12–15 min, the starting base line was reached a plateau after 5 min. If we suppressed protein kinase C in the reaction mixture, we observed only an increase of absorbance at 540 nm that reached a plateau after 5 min. If we suppressed TPA or MgATP instead of protein kinase C, we observed the same effect. These results suggested that MgATP, TPA, or protein kinase C alone were unable to induce the decrease of the aggregation (Table II). The decrease of absorbance observed with all the components cited above might reflect either a reversibility of the aggregation, a lysis, or a fusion of granules. Given these results, we fixed and examined the different assays for electron microscopy.

**FIG. 6. Effect of protein kinase C on chromaffin granules aggregated by annexin 2.** The reaction mixture contained a granule suspension of 0.1 mg of protein/ml, 100 μM Ca2+, 50 μM ATP or [γ-32P]ATP, 5 mM MgCl2, 50 μM TPA in buffer, pH 6, as described under “Experimental Procedures.” The aggregation and the phosphorylation reactions were simultaneously initiated by 0.4 μg of protein kinase C and 5 μg of A-2t added together in the mixture. The change in absorbance was monitored for 12 min at 540 nm in the absence (●) or presence (○) of protein kinase C. Inset: 10% SDS-PAGE of granules pelleted after 12 min of aggregation in the absence (lanes 1) or presence (lanes 2) of protein kinase C. a, Comassie Blue; b, PhosphorImager. The two 32P-labeled bands were respectively mono (38 kDa) and di (40 kDa) phosphorylated proteins.

In these conditions (Fig. 6), we observed in the first 2 min of the incubation an increase of absorbance indicative of an aggregation of granules, then, a slow decrease of the absorbance. After 12–15 min, the starting base line was attained. If we suppressed protein kinase C in the reaction mixture, we observed only an increase of absorbance at 540 nm that reached a plateau after 5 min. If we suppressed TPA or MgATP instead of protein kinase C, we observed the same effect. These results suggested that MgATP, TPA, or protein kinase C alone were unable to induce the decrease of the aggregation (Table II). The decrease of absorbance observed with all the components cited above might reflect either a reversibility of the aggregation, a lysis, or a fusion of granules. Given these results, we fixed and examined the different assays for electron microscopy.

**TABLE I**

| Kd Adjustment | Scatchard values | Kd Adjustment | Scatchard values |
|---------------|------------------|---------------|------------------|
| pH 7          | 13               | 2             | 2               |
| pH 6          | 6                | 1.7           | 1.4             |
| 32P-A-2t pH 7 | 99               | 1.7           | 1.3             |
| pH 6          | 54               | 2             | 2               |

**TABLE II**

Fusogenic effect of protein kinase C and TPA on membranes of chromaffin granules cross-linked by unphosphorylated annexin 2.

| Added | Aggregation | Fusion |
|-------|-------------|--------|
| Protein kinase C | - | - |
| TPA | - | - |
| Protein kinase C + TPA | - | - |
| Annexin 2 | + | - |
| Annexin 2 + TPA | + | - |
| Protein kinase C + annexin 2 | + | - |
| Protein kinase C + TPA + annexin 2 | + | - |

50 μg of chromaffin granules were suspended in the phosphorylating medium: 40 mM MES buffer, pH 6, 240 mM sucrose, 30 mM KCl, 100 μM free calcium, 50 μM ATP, 5 mM MgCl2. Initial absorbance of this granule suspension was approximately 0.35 at 540 nm. Aggregation or fusion were induced by adding 4–5 μg of annexin 2, 0.4–0.5 μg of protein kinase C, 50 μM TPA, alone or mixed. Aggregation and fusion were expressed after 12 min incubation at 25 °C. Each assay represents three experiments.
surface and multiple fusion events were observed (Fig. 8b). 80–90% of the granules were fused. These results demonstrated that unphosphorylated annexin 2 was not a fusogenic protein but that phosphorylation of this protein rendered it fusogenic. The phosphorylation and the fusion appeared to be dependent of the activation of protein kinase C by TPA.

**DISCUSSION**

In order to understand how phosphorylation could regulate secretion, we carried out a comparative study of the respective biochemical properties of unphosphorylated and phosphorylated annexins 2.

For the first time, annexin 2 phosphorylated in vitro by brain protein kinase C has been purified devoid of protein kinase C and unphosphorylated annexin 2. ³²P labeling of the protein has been quantified. The phosphorylated protein has been obtained in amounts (0.5 mg) that allowed a careful study of its properties.

The purified phosphorylated protein contains up to 2.5 mol of phosphate/mol of heterotetramer. During the phosphorylation process we observe that the rate of the phosphorylation of one serine residue is faster than the phosphorylation of one another, since, in most cases, the phosphorylation experiments give 85% of monophosphorylated and 15% of diphosphorylated heavy chain. If we refer to the in vitro phosphorylation of annexin 2 (calpactin 1) by protein kinase C described by Johnsson et al. (21) and Gould et al. (22), the major phosphorylation site involves serine 25, located near tyrosine 23, a relatively short region of the p36 heavy chain known to provide contact with the p11 light chain but likely accessible to protein kinase C and tyrosine kinase v-Src (21, 22). The second phosphorylation site must involve another serine residue of the amino-terminal domain, likely residue 11 which is close to a lysine residue (21). The fact that the diphosphorylated form appears concomitantly with the monophosphorylated form suggests that the phosphorylation on serine 25 could induce the exposure of serine 11. Powell and Glenney (23) have reported that phosphorylation of tyrosine 23 by tyrosine kinase v-Src decreased the affinity of light chain to the heavy chain. This is in accord with our data which show that a fraction of monophosphorylated form and the totality of the diphosphorylated form of annexin 2 have lost their light chains. However, the two phosphorylated heavy chains do not seem to be dissociated in monomers but rather to exist as homodimers. This is surprising, since until now, it was admitted that the small subunits p11 induced p36 dimerization and that annexin 2 existed either as a heterotetramer or as a monomer (24). Johnsson et al. (21), however, noticed that the minor phosphorylated p36 resulting from kinase C treatment, following reconstitution with p11 presented a small shift in the elution profile from Superose S12. They attributed this shift to a weak remaining affinity between p36 and p11. The formation of an homodimer devoid of p11 would suggest that the phosphorylation of the heavy chains could induce a head-to-head association. Such a dimerization has been observed on a monomeric annexin, annexin 1, in placenta (25) and in brain (26).

When chromaffin cells in primary culture labeled with ³²P are stimulated by nicotine, the immunoprecipitated p36 heavy chain shows a labeled band which migrates slower than p36, at the same distance as monophosphorylated p36 obtained by the in vitro phosphorylation by protein kinase C. Hence, in stimulated cells, protein kinase C phosphorylates preferentially one site on the 36-kDa heavy chain.

Phosphorylation of annexin 2 by protein kinase C reduces its affinity to granule membranes with no change in its binding capacity. An average $B_{\text{max}}$ values of 2.2 nmol of annexin 2/mg of granule protein (19%, w/w) is obtained either with unphosphorylated or phosphorylated annexin 2. If one assumes that 1 mg of granule protein correspond to $5 \times 10^{13}$ granules, 2.5 $\times 10^3$ mol of annexin 2 bind to one granule. These values are in agreement with a binding of annexin 2 to phospholipids, since the amount of the more represented integral membrane protein, cytochrome b$_{561}$ is 1.75 $\times 10^3$ mol in the granule membrane (18).

It is likely that annexin 2 binds to phosphatidylserine heads, since it has been demonstrated that it binds in a Ca$^{2+}$-depend-
ent manner specifically to phosphatidylserine. If we calculate the binding of 1 mol of unphosphorylated or phosphorylated annexin 2 in relation to the content of phosphatidylserine/mg of granule protein, we find that 1 mol of annexin 2 would bind 28–30 mol of phosphatidylserine, i.e. 14–15 mol/36-kDa heavy chain. This number is close to that found for protein kinase C which binds 12 mol of phosphatidylserine/mol (27) but far less than the fairly low stoichiometry of the binding of synapsin I, 1 mol/900 mol of acidic phospholipids (28). Finally, this protein-phospholipid interaction could be a major factor in the high affinity binding.

The binding of annexin 2 to granules is strongly cooperative. It is unknown whether this positive cooperativity of unphosphorylated annexin 2 is a consequence of enhanced ligand presentation once membrane aggregation begins (zipper mechanism) or if it arises from a surface oligomerization along the membrane, since phosphorylated annexin 2 does not aggregate granules.

We have observed that annexin 2 induces the aggregation of chromaffin granules at micromolar calcium concentration either at pH 7 or 6. The electron microscopy data corroborate these results. The presence of numerous aggregated granules observed at pH 6 and at micromolar Ca$^{2+}$ concentration demonstrates that annexin 2 joins tightly the granule membranes at micromolar Ca$^{2+}$ concentration, pH 6, but is unable to fusion the membranes at this pH. The phosphorylated annexin 2, tested at the same protein concentration as unphosphorylated protein, does not aggregate chromaffin granules from 0.1 μM to

![Electron micrographs of chromaffin granules incubated with unphosphorylated annexin 2 and protein kinase C. Granules were incubated at 100 μM calcium, pH 6.0, for 12 min with MgATP, TPA, and A-2t (a) or with MgATP, TPA, A-2t, and protein kinase C (b) Bar: 1 μm.](image)
1 μM Ca²⁺ concentration. These results are in accord with those obtained by J ohnstone et al. (4) who obtained no aggregation of phosphatidylserine liposomes with phosphorylated annexin 2. Electron microscopy shows that the granules brought together with phosphorylated annexin 2 are not aggregated or fused; however, their membranes appear fluffy. This appearance does not result from some traces of detergent, since Triton X-100 used for the preservation of purified protein kinase C has been eliminated on a detergent removing gel.

This effect on the morphology of the membrane may result from a conformational change of the phosphorylated protein which perturbs the phospholipid bilayer upon binding of the protein. Indeed, when protein kinase C is added in the presence of its activator TPA and annexin 2 in a suspension of granules, phosphorylation of annexin 2 induces the fusion of granules. The fusion is dependent on the activation of protein kinase C, since in the absence of TPA, the enzyme is unable to decrease the aggregation induced by unphosphorylated annexin 2. These results demonstrate that if unphosphorylated annexin 2 promotes the formation of contacts between membranes, the fusion effect is obtained only when the membrane structure is perturbed by a change of conformation likely induced by the phosphorylation of the molecule of annexin 2. This fusion effect is comparable with that described by Drust and Creutz (1) on chromaffin granules and by Mayorga et al. (29) on endosomes. In these two cases, annexin 2 is able to fuse the membranes unless arachidonic acid is added. In our hands, phosphorylated annexin 2 is able by itself to fuse the aggregated membranes, but only if the phosphorylation by protein kinase C is effected on annexin 2 cross-linked membranes.

In conclusion, we have demonstrated that the binding of unphosphorylated as phosphorylated annexin 2 to the granule membranes is a positive cooperative mechanism that expresses likely an oligomerization of the annexin molecules at the surface of the membranes. This oligomerization may encircle a small patch of phospholipids, since each mole of annexin 2 may bind 28–30 mol of phosphatidylserine. This notion of cluster is in agreement with the lipidic fusion pore model of Monck and Fernandez (30). If we refer to the electron microscopic studies from Nakata et al. (2) which show that annexin 2 forms fine strands associating closely the granule membrane to the plasma membrane under stimulation of the cell, the close interaction of unphosphorylated annexin 2 we have observed with chromaffin granule membranes alone may be similar with that of granule membrane and plasma membrane, since the cytoplasmic face of the plasma membrane of chromaffin cells may have a similar lipid composition and one could predict a similar interaction (31).

In addition, in response to stimulation of primary chromaffin cells annexin 2 is phosphorylated by protein kinase C. The translocation of protein kinase C at sites adjacent to those of annexin 2 on the membrane and the activation of the enzyme by diacylglycerol, a second messenger mediated by the activation of phospholipase C, are absolutely required for the phosphorylation of annexin 2. Upon phosphorylation, a conformational change of annexin 2 may occur in the NH₂-terminal domain of the molecule that leads to a decrease in affinity of the phosphoprotein affecting calcium and phospholipid binding on the two heavy chains at the opposite face of the molecule. This conformational change would explain the perturbation of the phospholipid bilayer and the fusion induced by phosphorylation of annexin 2. The results obtained strongly support our study on phosphorylation of annexin 2 by protein kinase C in primary cultures of streptolysin permeabilized chromaffin cells stimulated by 4 μM calcium, where the activators of protein kinase C, TPA and diacylglycerol, activate in the same time the phosphorylation of annexin 2 and the secretion of catecholamines. This activation of secretion concomitant with an increase of phosphorylation of annexin 2 is not in accord with the suggestion of Creutz that phosphorylation of annexin in stimulated chromaffin cells might play a role in down-regulating exocytosis, since an inhibitory effect of the phosphorylated protein was observed on the granule aggregating activity (7). In fact, in vivo, the fusogenic effect of the phosphorylated protein may be the major effect, the inability of the phosphorylated protein to aggregate granules being effective only when the protein dissociates from the fused membranes.

Acknowledgments—We thank Dr. Stetzkowski-Marden for useful comments and revising the manuscript; we express our gratitude to Dr. J. F. Chiche for his help in FPLC chromatography.

REFERENCES

1. Drust, D. S., and Creutz, C. E. (1988) Nature 331, 88–91
2. Nakata, T., Sobue, K., and Hirokawa, N. (1990) J. Cell Biol. 110, 13–25
3. Sarafian, T., Prade, L. A., Henry, J. P., Aunis, D., and Bader, M. F. (1991) J. Cell Biol. 114, 1135–1147
4. Johnstone, S. A., Habushy, I., and Waisman, D. M. (1991) J. Biol. Chem. 266, 25976–25981
5. Rignouf, F., Rendon, A., and Pradel, L. A. (1991) J. Neurochem. 56, 1985–1996
6. Kitano, T., Go, M., Kikkawa, U., and Nishizuka, Y. (1986) Methods Enzymol. 124, 349–359
7. Deleted in proof
8. Pelech, S. L., Meier, K. F., and Krebs, E. G. (1986) Biochemistry 25, 8348–8353
9. Smith, A. D., and Winkler, H. (1967) Biochem. J. 103, 480–482
10. Carty, S. E., Johnsson, R. G., and Scarpa, A. (1980) Anal. Biochem. 106, 338–345
11. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
12. Creutz, C. E., Pasolts, J. C., and Pollard, H. B. (1978) J. Biol. Chem. 253, 2898–2906
13. Wang, W., and Creutz, C. E. (1991) Biochemistry 31, 9934–9939
14. Frappier, T., Rignouf, F., and Pradel, L. A. (1987) Eur. J. Biochem. 169, 651–657
15. Rignouf, F., Nguyen, E., Cassoly, R., and Pradel, L. A. (1985) Eur. J. Biochem. 153, 313–319
16. Laemmli, U. K. (1970) Nature 227, 680–685
17. Fabiato, A., and Fabiato, F. (1979) J. Physiol. (Paris) 75, 463–505
18. Winkler, H. (1976) Neuroscience 1, 65–80
19. Blaschko, H., Firemark, H., Smith, A. D., and Winkler, H. (1967) Biochem. J. 104, 545–549
20. Phillips, J. H. (1973) Biochem. J. 136, 579–587
21. Johnson, N., Nguyen Van P., Sołing, H. D., and Weber, K. (1986) EMBO J. 5, 3455–3460
22. Gould, K., Woolfetti, J. R., Isakke, C. M., and Hunter, T. (1986) Mol. Cel. Biol. 6, 2738–2744
23. Powell, A. M., and Glenney, J. R. (1987) Biochem. J. 247, 321–328
24. Glenney, J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 4258–4262
25. Pepinsky, R. B., Sinclair, L. K., Chow E. P., and O’Brine-Greco, B. (1989) Biochem. J. 263, 97–103
26. Pradel, L. A., and Rendon, A. (1993) FEBS Lett. 327, 41–44
27. Newton, A. C., and Koshland, Jr., D. (1989) J. Biol. Chem. 264, 14099–14115
28. Benfenati, F., Greengard, P., Brunner, J., and Bähler, M. (1989) J. Biol. Chem. 264, 1851–1862
29. Mayorga, L. S., Beron, W., Sarrouf, M. N., Colombo, M. I., Creutz, C., and Stahl, P. D. (1994) J. Biol. Chem. 269, 30927–30934
30. Monck, J. R., and Fernandez, J. M. (1992) J. Biol. Chem. 267, 119, 31115–31123
31. Damer, C. K., and Creutz, C. E. (1994) J. Biol. Chem. 269, 31115–31123