Annexin 5 as a potential regulator of annexin 1 phosphorylation by protein kinase C

In vitro inhibition compared with quantitative data on annexin distribution in human endothelial cells

Patrick RAYNAL, Françoise HULLIN, Jeannie M. F. RAGAB-THOMAS, Josette FAUVEL and Hugues CHAP*

INSERM Unité 326, Phospholipides Membranaires, Signalisation Cellulaire et Lipoprotéines, Hôpital Purpan, 31059 Toulouse Cédex, France

In vitro phosphorylation of annexin 1 by purified rat brain protein kinase C (PKC) has been studied in the presence of annexin 5, which is not a substrate for PKC. Annexin 5 promoted a dose-dependent inhibition of annexin 1 phosphorylation, which could be overcome by increasing the concentration of phosphatidylserine (PtdSer). In addition, a close relationship was found between the amount of PtdSer uncovered by annexin 5 and the residual phosphorylation of annexin 1. These data fit with the 'surface depletion model' explaining the anti-phospholipase activity of annexins. In order to check the possibility that the *in vitro* effect of annexin 5 could be of some physiological relevance, annexins 1, 2, and 5, as well as the light chain of calpactin 1 (p11), have been quantified in human endothelial cells by measuring the radioactivity bound to the proteins after Western blotting with specific antibodies and ^{125}I-labelled secondary antibody. Our data indicate that annexins 1 and 5, PKC and PtdSer are present in human endothelial cells in relative amounts very similar to those used *in vitro* under conditions permitting the detection of the inhibitory effect of annexin 5. Since annexin 1 remained refractory to PKC-dependent phosphorylation in intact cells, we suggest that annexin 5 might exert its inhibitory effect towards PKC *in vivo*, provided that its binding to phospholipids can occur at physiological (micromolar) concentrations of Ca^{2+}. This was previously shown to occur *in vitro* using phosphatidylethanolamine/phosphatidic acid vesicles [Blackwood and Ernst (1990) Biochem. J. **266**, 195–200]. Using identical assay conditions, which also allowed expression of PKC activity, annexin 5 again inhibited annexin 1 phosphorylation without interfering with PKC autophosphorylation. These data suggest that annexins 1 and 5 might interact with each other on the lipid surface, resulting in a specific inhibition of annexin 1 phosphorylation by PKC. Whether a similar mechanism also occurs *in vivo* remains to be determined.

INTRODUCTION

The newly defined term 'annexin' [1] designates a family of Ca^{2+}- and phospholipid-binding proteins first described as 'lipocortins', since they were thought to mediate the anti-inflammatory effects of glucocorticoids [2]. These proteins, which now include 13 identified members [3], share a similar structure, characterized by the presence of four or eight repeats of a conserved 70-amino-acid-residue sequence including a 17-amino-acid residue consensus sequence and a variable N-terminal domain [4–7]. This structural similarity might account for several common characteristics of these proteins. A main property they share is their ability to bind to negatively charged phospholipids in the presence of Ca^{+}, which is probably responsible for their anti-phospholipase [8–11], anticoagulant [12–14] and anti-inflammatory activities [15]. Their phosphorylation by Ca^{2+}- and phospholipid-dependent protein kinase (PKC) [16–18], receptor and non-receptor protein tyrosine kinases [19,20], and their differential expression during cell growth or following cell differentiation [21–24] indicate a possible role in the control of cell proliferation. Moreover, they interact Ca^{2+}-dependently with actin [25,26] and promote exocytosis in some models of cell secretion [27]. Some of them display collagen-binding properties [28,29], express or modulate Ca^{2+}-channel activities [30,31], hydrolyse inositol 1,2-cyclic phosphate [32] or regulate the activity of DNA polymerase-α involved in lagging strand replication [33]. So numerous properties have obscured their possible physiological significance and, unexpectively, a recent study even reported their active secretion from prostatic cells, despite their cytosolic localization and their lack of signal peptide [34].

Although *in vitro* phosphorylation of annexins 1 and 2 by PKC has been well documented [16–18], a number of attempts to promote PKC-dependent phosphorylation of these proteins in intact cells proved negative [35–39]. For annexin 2, a simple explanation might be that the membrane-bound form of the protein is exclusively the heterotetrameric calpactin 1, whereas monomeric annexin 2 (p36) displays a cytosolic localization [40]. Since association of p36 with p11 renders annexin 2 refractory to phosphorylation by various protein serine threonine kinases [18], a lack of *in vivo* phosphorylation of annexin 2 by PKC is not unexpected, although such phosphorylation occurs in human fibroblasts [41].

With the exception of the covalent dimer identified in placenta [42,43], annexin 1 is normally only present in a monomeric form, at least part of which has been found associated with the plasma membrane [44]. Thus a natural inhibitor could be responsible for the lack of annexin 1 phosphorylation by PKC. When considering available literature data it is clear that annexin 1 is co-expressed with annexin 5 in numerous cells [5], including endothelial cells [45]. Since annexin 5 lacks phosphorylation sites in its N-terminal end, it could be predicted that this protein is a potential inhibitor of PKC under conditions where anionic phospholipids required to activate PKC are present in suboptimal amounts. This point has been checked in the present study, which also showed that the respective amounts of annexins 1 and 5 present in human endothelial cells are actually consistent with a possible inhibitory

Abbreviations used: MEM, (Eagle's) minimal essential medium; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid.

* To whom correspondence and reprint requests should be addressed.

effect of annexin 5 towards annexin 1 phosphorylation by PKC. During the preparation of this manuscript one study described an inhibition of PKC by annexin 5, but apparently by a different mechanism [46].

MATERIALS AND METHODS

Materials

[^{32}P]Orthophosphoric acid, [γ-^{32}P]ATP, and ^{125}I-labelled antibodies directed against mouse or rabbit immunoglobulins were from the Radiochemical Centre (Amersham, Bucks., U.K.). Most reagents were from Sigma (St. Louis, MO, U.S.A.), except phenylmethanesulphonyl fluoride (PMSF), dithiothreitol and *p*-nitrophenyl phosphate (Aldrich; EGA-Chimie, Strasbourg, France). Cell-culture media and antibiotics were from Seromed (Münich, Germany), Protein A–Sepharose from Pharmacia (Uppsala, Sweden), phosphate-free minimal essential medium (MEM) from Flow Laboratories (Les Ulis, France) and okadaic acid from Diagnostic Chemicals (Oxford, CT, U.S.A.). Polyclonal antibodies raised against porcine annexin 1 and bovine annexin 5 were prepared as described previously [45]. As shown by Western blotting, anti-(annexin 5) antiserum recognized annexin 5 from various species, including man, whereas anti-(annexin 1) antiserum displayed a cross-reaction of only 5% against annexin 2. Monoclonal antibodies specific for annexins 1 and 2, and for p11, were obtained from Zymed (San Francisco, CA, U.S.A.). Anti-(annexin 1) monoclonal antibody was monospecific, whereas anti-(annexin 2) monoclonal antibody displayed less than 5% cross-reactivity with annexin 1.

In vitro phosphorylation with purified rat brain PKC

PKC was purified from rat brain in a three-step procedure as described by Wooten et al. [47]. One unit of PKC activity was defined as the amount of enzyme catalysing the incorporation of 1 pmol of phosphate from ATP into histones/min at 30 °C under assay conditions. Phosphorylation assays were performed in a 40 μl incubation volume containing 10 mM Tris/HCl, pH 7.4, 10 mM-$MgCl_2$, 10 μM ATP, 1 μCi of [γ-^{32}P]ATP, phosphatidylserine (PtdSer) and dioleoylglycerol at concentrations indicated in the Figure legends, various amounts of annexins and, alternatively, 0.6 mM EGTA or 1.5 mM $CaCl_2$. The reaction was started by the addition of 10 μl of purified PKC; incubation was allowed to proceed for 30 min at 30 °C and was stopped by the addition of 7 μl of a 6-fold-concentrated Laemmli's sample buffer [49]. Then samples were boiled and subjected to SDS/PAGE. To determine the rate of protein phosphorylation, bands were excised from the dried gel and radioactivity was measured on a Packard Tricarb 4530 spectrometer using the Čerenkov effect.

The same procedure was used for determining annexin 1 phosphorylation by rat brain PKC at μM Ca^{2+} concentrations with the following modifications: purified PKC was from Calbiochem (San Diego, CA, U.S.A.); lipid dispersions contained a mixture of phosphatidylethanolamine (PtdEtn) (22.5 μg/ml), phosphatidic acid (PtdOH) (22.5 μg/ml) and dioleoylglycerol (5 μg/ml); free Ca^{2+} concentration was adjusted using 1 mM Ca/EGTA buffer and taking 6.898×10^6 as the apparent stability constant of EGTA [50].

[^{32}P]Orthophosphoric acid labelling and stimulation of endothelial cells

Confluent cells were incubated for 4 h with 3 ml of phosphate-free MEM containing 0.4% (w/v) human serum albumin and 50–700 μCi of [^{32}P]orthophosphoric acid/ml. In some experiments where ^{32}P labelling was performed overnight, 20% (v/v) dialysed foetal-calf serum was included in the medium instead of albumin. After labelling, PMA (80–160 nM), calcium ionophore A23187 (1–5 μM) or okadaic acid (0.2–1.0 μM) were added, in 6 μl of dimethyl sulphoxide as a vehicle, for 2–60 min. Cells were then washed and scraped into 1 ml homogenization buffer containing phosphatase inhibitors (5 mM *p*-nitrophenyl phosphate, 2 mM-NaF and 100 μM-Na_3VO_4). Cells were disrupted, and 40 μl of homogenate was immediately boiled with Laemmli's buffer [49]. From the remaining homogenate, annexins were prepared either by EGTA extraction or by immunoprecipitation of annexin 1 as described below.

Purification of annexins from endothelial cells

Annexins were isolated by precipitation of membranes in the presence of 6 mM Ca^{2+}, followed by specific extraction with 4 mM EGTA as previously described [45]. In some experiments, pooled EGTA extracts were desalted against buffer A [20 mM Hepes (pH 7.0)/0.5 mM dithiothreitol/0.25 mM PMSF] using a PD-10 column (Pharmacia) and loaded on to an h.p.l.c. anion-exchange chromatography column (Mono-Q, Pharmacia) equilibrated with buffer A. After flow-through collecting and further washing, a linear gradient from 0 to 0.5 M NaCl in buffer A was applied. Fractions were collected and analysed by SDS/PAGE and Western blotting.

Immunoprecipitation of annexin 1

Cells were homogenized as described elsewhere [45] in a buffer containing 4 mM EGTA. Aliquots of homogenate were diluted by addition of an equal volume of a twice-concentrated immunoprecipitation buffer referred to as 'RIPA buffer' [21]: 20 mM Tris/HCl (pH 7.5)/154 mM NaCl/2 mM EDTA/1% (v/v) Triton X-100/0.5% (w/v) sodium deoxycholate/10 mM-NaF/5 mM *p*-nitrophenyl phosphate/100 μM Na_3VO_4. Samples were precleared with 25 μl of a 10% (v/v) suspension of Protein A–Sepharose. Supernatants were then incubated overnight at 4 °C with 5 μl of polyclonal anti-(annexin 1) antiserum. Immunocomplexes were precipitated by addition of 25 μl of the 10% suspension of Protein A–Sepharose after 1 h incubation at 4 °C. Samples were then washed twice in RIPA buffer and once in detergent-free RIPA buffer. Pellets were resuspended in Laemmli's buffer [49], boiled and subjected to SDS/PAGE.

Preparation of partially purified PKC from endothelial cells

A cytosolic extract was prepared and fractionated on a DEAE-cellulose column (DE52, Whatman) as described elsewhere [47], retained proteins being eluted with a linear gradient from 0 to 0.3 M NaCl. Assays were performed as described with purified rat brain PKC.

Protein electrophoresis, Western blotting and autoradiography

SDS/PAGE was performed as described by Laemmli [49] using 12% (w/v) polyacrylamide gels. Proteins were stained with $AgNO_3$ or Coomassie Blue or transferred to nitrocellulose membranes for immunodetection with a 1:1000 dilution of various rabbit polyclonal anti-annexin antibodies or a 1:4000 dilution of monoclonal anti-annexin antibodies as described previously [45]. ^{32}P-labelled proteins were detected by autoradiography using Hyperfilm-MP (Amersham International).

Quantitative determination of annexins 1, 2 and 5 in endothelial cells

This was achieved directly on cell homogenates after Western blotting according to Schlaepfer and Haigler [23], using anti-(annexin 1), anti-(annexin 2) or anti-p11 monoclonal antibodies and anti-(annexin 5) polyclonal antibody. Corresponding ^{125}I-labelled secondary antibodies were directed against mouse or rabbit IgGs respectively, and purified human placental annexins were used as calibration standards in the 0.1–2 μg range. Standard curves displayed a linear relationship over the range tested.

Other procedures

Endothelial cells were cultured from human umbilical-cord vein as previously described [51]. All experiments were performed with subcultured cells at passages 2–3, where they displayed typical endothelial-cell characteristics: cobblestone pattern, contact inhibition and staining with anti-(von Willebrand factor) first antibody/fluorescein isothiocyanate-labelled second antibody. Annexins 1, 2, 5 and 6 were purified in large amounts from bovine lung or from human placenta as previously described [14,26,52,53]. Polyclonal anti-annexin antibodies were raised in rabbits immunized with pure proteins [45,53]. Protein was determined as described by Bradford [54], using BSA as a standard. Endothelial-cell phospholipids were extracted by the method of Bligh and Dyer [55] and assayed as previously described [51].

RESULTS

In vitro inhibition by annexin 5 of annexin 1 phosphorylation by PKC

Figure 1 shows that annexin 5 dramatically reduced annexin 1 phosphorylation by PKC. Apparently inhibition was partly overcome by raising the PtdSer concentration. Quantitative data on the effect of annexin 5 at three different PtdSer concentrations are given in Figure 2, which shows a close relationship between the inhibitory concentrations of annexin 5 and the concentrations of PtdSer present in the assay mixture. For instance, half-maximal inhibition of annexin 1 phosphorylation was obtained at annexin 5/annexin 1 molar ratios of 0.4, 1.3 and 2.3, using PtdSer concentrations of 20, 30 and 50 μg/ml respectively.

In order to elucidate further the mechanism of this inhibition, PKC activity towards annexin 1 was represented as a function of the amounts of PtdSer readily available to PKC. As shown by Andree et al. [56] and by Mosser et al. [57], each molecule of annexin 5 is able to coat a surface of 25 nm^2 of phospholipid interface, corresponding to approx. 38 molecules of PtdSer. Taking into account that 50% of PtdSer molecules remain available to external proteins in single walled vesicles, and assuming that all the added annexin 5 was adsorbed on to phospholipids, we calculated the amounts of PtdSer uncovered by annexin 5 at different concentrations of the latter. As shown in Figure 3, annexin 1 phosphorylation was not possible when annexin 5 occupied all the external surface of the vesicles. But while decreasing the annexin 5 concentration, phosphorylation of annexin 1 occurred as soon as some picomolar amounts of PtdSer became available, the intensity of phosphorylation being proportionally related to the amount of PtdSer uncovered by annexin 5 ($r = 0.991$).

Quantitative determination of annexins 1, 2 and 5 in human endothelial cells

Previous studies revealed the presence, in human endothelial cells, of annexins 1, 2, 5 and 6 [13,45,58–60], the 34/35 kDa proteins being the most abundant. As shown in Table 1, endothelial cells were found to contain equal amounts of annexins 1 and 2, whereas the concentration of annexin 5 exceeded by 28% that of annexin 1 plus annexin 2. In addition, p11 was found in molar excess compared with annexin 2. Thus human endothelial cells appeared to be a good model to check for a possible inhibition by annexin 5 of PKC-dependent annexin 1 phosphorylation.

Lack of PKC-dependent phosphorylation of annexin 1 in human endothelial cells

^{32}P-labelled cultured endothelial cells were stimulated with either PMA, A23187 or PMA plus A23187. As shown in Figure 4(a), this resulted in the activation of PKC, as indicated by the significant phosphorylation of a 27 kDa protein previously recognized as an endogenous PKC substrate in the same cells

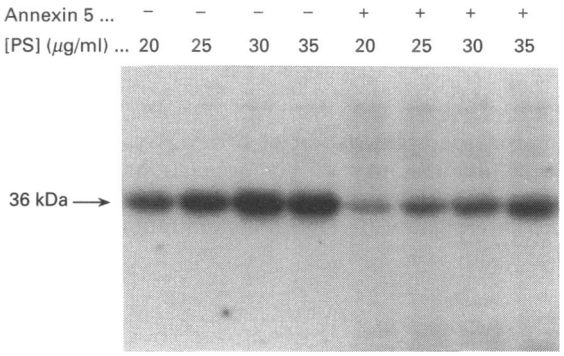

Figure 1 Effect of annexin 5 on *in vitro* phosphorylation of annexin 1 by PKC

Purified bovine annexin 1 (500 ng) was incubated as described in the Materials and methods section with rat brain PKC (1.5 units) in the absence (−) or in the presence (+) of 500 ng annexin 5. Various PtdSer (PS) concentrations (20–35 μg/ml) are indicated on the Figure; the dioleoylglycerol concentration being 10 μg/ml. The Figure shows the autoradiogram obtained after SDS/PAGE.

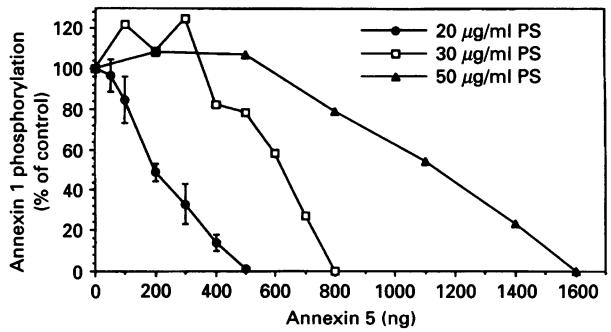

Figure 2 Inhibition by annexin 5 of *in vitro* phosphorylation of annexin 1 by PKC at different PtdSer (PS) concentrations

Purified bovine annexin 1 (500 ng) was incubated as described in the Materials and methods section with rat brain PKC (1.5 units) and increasing amounts of purified bovine annexin 5 at three different PtdSer concentrations: 20 μg/ml (●); 30 μg/ml (□); 50 μg/ml (▲). After 30 min at 30 °C, SDS/PAGE was performed; gels were stained, dried, and the radioactivity of each band was determined after excision by Čerenkov counting. Data are means ± S.E.M. (three determinations) at 20 μg of PtdSer/ml, or means of two determinations at the other PtdSer concentrations.

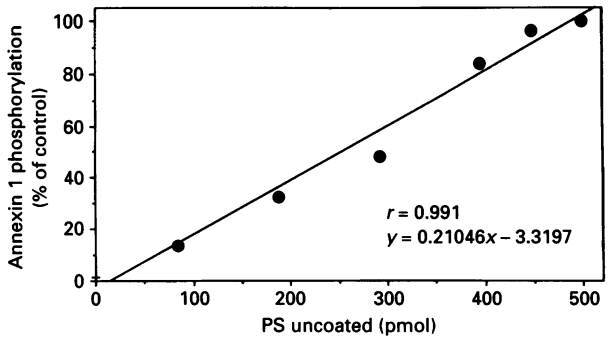

Figure 3 Relationship between *in vitro* phosphorylation of annexin 1 by PKC and the amount of PtdSer (PS) uncoated by annexin 5

The amounts of PtdSer not covered by annexin 5 in phosphorylation assays of annexin 1 were calculated by considering that each molecule of annexin 5 covers a surface of 25 nm^2 of PtdSer, corresponding to 38 molecules of phospholipid, as shown by Andree et al. [56] and Mosser et al. [57], and taking 800 Da as an average molecular mass of PtdSer. PtdSer concentrations were divided by 2 in order to consider only those molecules present in the outer leaflet of monolamellar vesicles. For further details and discussion, see the text. Data are taken from Figure 2 (incubations were performed at 20 μg of PtdSer/ml).

Table 1 Quantitative determination of annexins 1, 2, 5 and of p11 in human endothelial cells

Whole homogenates from human endothelial cells were submitted to SDS/PAGE, followed by electrotransfer and immunodetection using appropriate antibodies as described in the Materials and methods section. Data (means ± S.E.M., *n* being the number of determinations) are expressed as μg or pmol of each protein per 100 μg of total cell protein, taking 38.6, 38.5, 35.8 and 11 kDa as molecular masses of annexins 1, 2 and 5 and of p11 respectively.

	Concentration in endothelial cells		
Protein	(μg/100 μg)	(pmol/100 μg)	*n*
Annexin 1	0.57 ± 0.15	14.8 ± 3.9	4
Annexin 2	0.54 ± 0.12	14.0 ± 3.1	4
Annexin 5	1.43 ± 0.09	39.9 ± 2.5	16
p11	0.25 ± 0.09	22.7 ± 8.2	3

[61,62]. However, phosphorylation of annexins could not be detected by autoradiography of homogenates or of EGTA extracts (Figure 4a). Negative results were also obtained with cells labelled overnight with higher concentrations of [^{32}P]-orthophosphoric acid (700 μCi/ml) and using several agonist concentrations during 2–60 min stimulation (results not shown). In addition, thrombin (1–2 units/ml) and okadaic acid (0.2–1 μM) were unable to promote phosphorylation of annexin 1, while phosphorylation of the 27 kDa protein was enhanced (results not shown). Furthermore, to avoid possible artefacts of the calcium/EGTA extraction procedure, annexin 1 was also isolated by immunoprecipitation with the polyclonal antibody described above. Here again there was no evidence for ^{32}P labelling of the 35 kDa polypeptide band following PMA stimu-

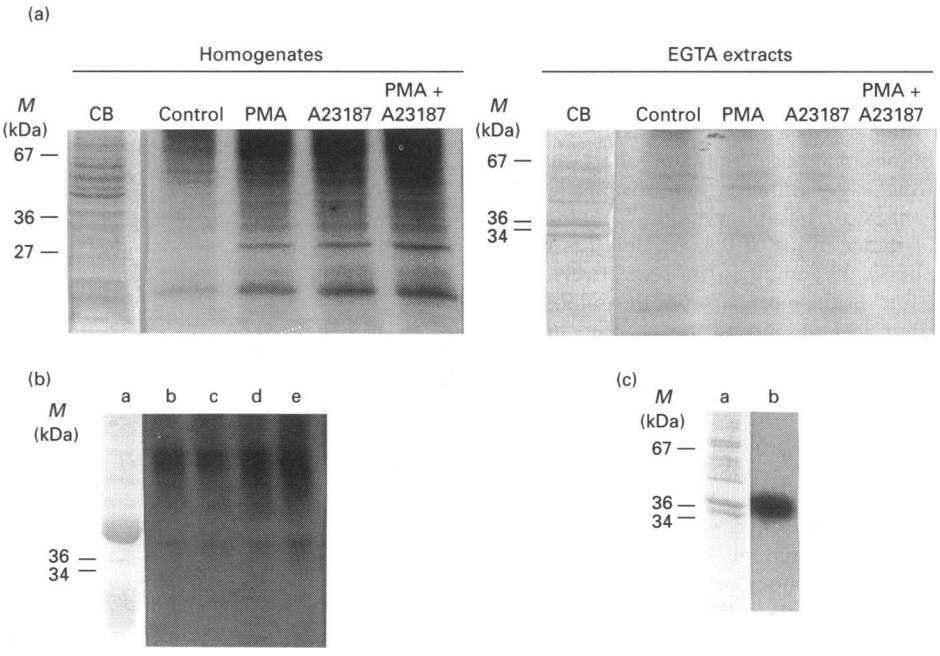

Figure 4 Lack of PKC-dependent phosphorylation of annexin 1 in intact human endothelial cells

Cultured endothelial cells were labelled with 50 μCi of [^{32}P]orthophosphoric acid/ml during 4 h. (**a**) Cells were incubated for 20 min at 37 °C with the following additions: 3 μl of dimethyl sulphoxide (control); 0.16 μM PMA; 5 μM A23187; 0.16 μM PMA plus 5 μM A23187. In the 'Homogenates' panel, 40 μl aliquots of cell homogenate from control or agonist-treated cells were subjected to SDS/PAGE, stained with Coomassie Blue (left lane, 'CB'; representative of the various samples) and autoradiographied overnight (other lanes). In the 'EGTA extracts' panel, the EGTA extracts from control or treated cells were subjected to SDS/PAGE, stained ('CB'; representative of other EGTA extracts) and autoradiographied for 3 days. (**b**) Cells were stimulated with 0.16 μM PMA for various times and annexin 1 was immunoprecipitated with polyclonal anti-(annexin 1) antibody. Immunoprecipitates were analysed by SDS/PAGE: lane a, Coomassie Blue staining of control cells, representative of other immunoprecipitation patterns; lane b–e, autoradiograms (48 h) from control (b) or PMA-treated cells (c, 5 min; d, 15 min; e, 30 min). (**c**) Bovine annexin 1 (1 μg) was phosphorylated *in vitro* with rat brain PKC as described in the legend to Figure 5, then added to an endothelial-cell homogenate, which was submitted to the EGTA extraction procedure. Lane a, Coomassie Blue staining of the EGTA extract; lane b, overnight autoradiography. Values on the left refer to molecular masses (*M*) in kDa.

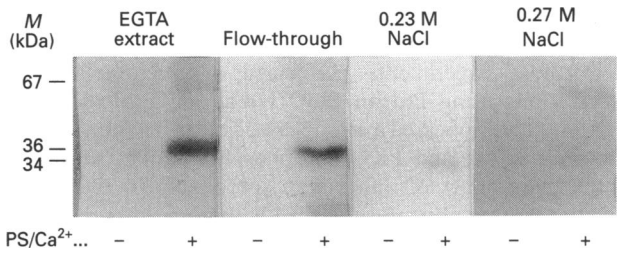

Figure 5 *In vitro* phosphorylation of endothelial-cell annexins with purified rat brain PKC

EGTA extract and fractions collected from the anion-exchange Mono-Q column (EGTA extract, flow-through, i.e. annexin 1 plus annexin 2, fractions eluted at 0.23 and 0.27 M NaCl, i.e. annexins 5 and 6 respectively; 0.5 μg of each) were desalted and incubated with purified rat brain PKC (1.5 units) and [γ-^{32}P]ATP in the presence of EGTA (lanes −) or Ca^{2+} and 150 μg of PtdSer (PS)/ml plus 10 μg of dioleoylglycerol/ml (lanes +). ^{32}P-labelled proteins were then separated by SDS/PAGE and analysed by overnight autoradiography. Abbreviation: *M*, molecular mass.

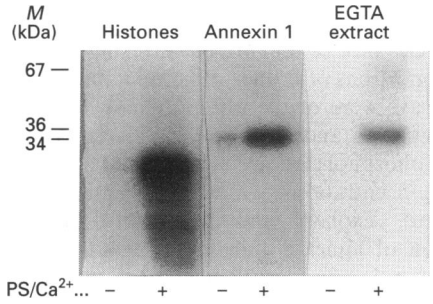

Figure 6 *In vitro* phosphorylation of histones and annexin 1 by PKC partially purified from human endothelial cells

PKC was isolated from cultured human endothelial cells and incubations were performed as described in the Materials and methods section in the presence of 0.5 unit of PKC, 2 μg of histones or 0.5 μg of purified bovine lung annexin 1 or 0.5 μg of an EGTA extract from human endothelial cells. Assays were performed in the presence of 0.6 mM EGTA (−) or upon addition of 1.5 mM $CaCl_2$ plus 150 μg of PtdSer(PS)/ml plus 10 μg of dioleoylglycerol/ml (+). Phosphorylated proteins were analysed by SDS/PAGE and overnight autoradiography.

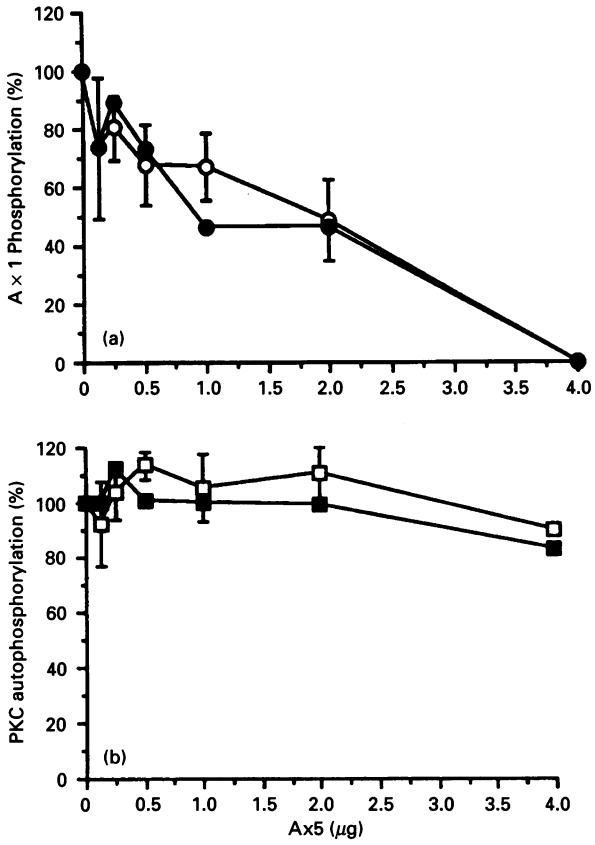

Figure 7 Effects of annexin 5 (AxV) on PKC-dependent annexin 1 (Ax1) phosphorylation (a) and on PKC autophosphorylation (b) at micromolar Ca^{2+} concentrations

Purified human annexin 1 (Ax1) (500 ng) was incubated as described in the Materials and methods section with rat brain PKC (1.5 units) and increasing amounts of purified human annexin 5 (Ax5). Lipid dispersions contained 22.5 μg of PtdEtn/ml, 22.5 μg of PtdOH/ml and 5 μg of dioleoylglycerol/ml. The free Ca^{2+} concentration was adjusted to 1 μM (○) or 10 μM (●). After autoradiography, the 35 kDa (annexin 1) and 80 kDa (PKC) radioactive bands were determined for radioactivity as described in the Materials and methods section. Data are means ± S.E.M. for three or four different experiments (1 μM Ca^{2+}) or from a typical experiment (10 μM Ca^{2+}).

lation of endothelial cells (Figure 4b). Finally, the possibility that annexin 1 was dephosphorylated by endogenous phosphatases or N-terminally cleaved by proteinases, despite the presence of specific enzyme inhibitors throughout the calcium/EGTA extraction procedure, was definitely ruled out. As shown in Figure 4(c), when annexin 1 was previously phosphorylated *in vitro* by PKC and added to homogenates from non-labelled endothelial cells, it was isolated in the EGTA extract without any loss of radioactivity.

Additional controls were performed in order to verify that endothelial cells contained annexins whose phosphorylation could be achieved under appropriate *in vitro* conditions. This is illustrated in Figure 5, which shows that a polypeptide band from EGTA extracts running at 35–36 kDa upon SDS/PAGE was phosphorylated by PKC. The same protein(s), probably annexin 1 plus annexin 2, was (were) recovered in the flow-through fractions obtained after Mono-Q chromatography, whereas annexins 5 and 6, which were eluted at 0.23 and 0.27 M NaCl respectively, proved to be very poor substrates for PKC.

Finally, it was verified that PKC partially purified from human endothelial cells was also able to phosphorylate annexin 1 *in vitro*. For this purpose, a cytosolic extract from endothelial cells was fractionated on a DEAE-cellulose column as described in the Materials and methods section. Fractions displaying the highest Ca^{2+}- and phospholipid-dependent kinase activities were eluted at about 0.05–0.1 M NaCl (results not shown) the total yield being more than 90%. This indicated the absence of endogenous inhibitors of PKC in the cytosolic extract, using the standard assay with histones as a substrate, and allowed us to estimate the specific activity of PKC in whole endothelial cells as 37.7 ± 2.3 units/mg (mean ± S.E.M., four determinations). The purified PKC preparation was able to phosphorylate histones, purified bovine annexin 1, as well as annexin 1 present in EGTA extract from endothelial cells (Figure 6) to high levels. Thus endothelial cells contain PKC isoforms able to phosphorylate annexin 1 *in vitro* but not *in vivo*.

Comparison of the relative amounts of annexins, PKC and phospholipids present in intact cells and *in vitro* assays

Quantitative determination of PKC (37.7 ± 2.3 units/mg of total cell protein; mean ± S.E.M.; four determinations) allowed us to

calculate that 676 pmol of PtdSer, 3.9 pmol of annexin 1 and 10.6 pmol of annexin 5 were present in intact endothelial cells per unit of PKC activity. For comparison, the relative proportions of PtdSer used during *in vitro* experiments described in Figure 2 were 333, 500 and 833 pmol per unit of PKC activity. Total inhibition of the phosphorylation of 8.6 pmol of annexin 1 by 1 unit PKC activity was obtained in the presence of 9.3, 14.9 and 29.8 pmol of annexin 5 respectively, depending on the PtdSer concentration. It is thus noteworthy that the relative proportions of annexins, PtdSer and PKC used in our *in vitro* assays were in the same range of magnitude as those present in intact endothelial cells.

In vitro inhibition by annexin 5 of annexin 1 phosphorylation by PKC at micromolar Ca^{2+} concentrations

The previous data on PKC inhibition by annexin 5 were obtained under standard *in vitro* conditions, i.e. at millimolar Ca^{2+} concentrations. Further attempts to demonstrate that same effect in the presence of 1–10 μM Ca^{2+} were unsuccessful (results not shown), probably owing to the failure of annexin 5 to interact with PtdSer at such low Ca^{2+} concentrations [63]. However, further experiments were performed by replacing PtdSer with a mixture of PtdEtn/PtdOH, which were previously shown to bind annexin 5 with a half-maximal effect of Ca^{2+} occurring at 1.2 μM [63]. In addition PtdOH was also shown to activate PKC under similar conditions [64,65]. As shown in Figure 7(a), annexin 5 promoted a dose-dependent inhibition of annexin 1 phosphorylation by PKC at 1 or 10 μM Ca^{2+}. However, since the ratio of PKC autophosphorylation/annexin-1 phosphorylation was higher under those particular conditions, it was also possible to determine the phosphorylation of the 80 kDa polypeptide band corresponding to PKC. This revealed that annexin 5 was without effect on PKC autophosphorylation.

DISCUSSION

A main finding of the present study was the observation that annexin 5 exerts an antagonistic effect against PKC-dependent phosphorylation of annexin 1. From our present data the effect of annexin 5 is strictly dependent on the amount of available PtdSer and can be overcome by raising PtdSer concentration. A very recent study of Schlaepfer et al. [46] reported a similar observation, but for unknown reasons inhibition of PKC by identical concentrations of annexin 5 (of the order of 1 μM) still occurred at much higher PtdSer concentrations (up to 800 μg/ml), suggesting, in this case, a direct inhibition of PKC by annexin 5. The main difference between the two assays is that PKC activity was not entirely dependent on the presence of phospholipids in the latter study. In contrast, our data could be easily explained by the 'surface depletion model' proposed by Davidson et al. [8] in the case of the anti-(phospholipase A_2) activity of annexins in general. Such a model permits an understanding of various inhibitory actions of annexins towards phospholipases with different specificities [9–11] or towards the phospholipid-dependent enzyme complexes of plasma coagulation [12–14]. In the case of PKC, the inhibitory effect of annexin 5 would be enabled by the lack of potential phosphorylation sites in the short N-terminal end of the protein, in contrast with annexin 1. A competition for a limited amount of PtdSer between annexin 5 on the one hand, and PKC and annexin 1 on the other hand, is particularly well indicated by data of Figure 3.

However, it cannot be concluded from the experiments depicted in Figures 1–3 whether the critical event resulting in the loss of annexin 1 phosphorylation by PKC involves a competition between annexin 5 and PKC, annexin 5 and annexin 1, or both. But in those experiments performed at micromolar Ca^{2+} concentrations using PtdEtn/PtdOH/diacylglycerol as a lipid dispersion, there appeared a strong dissociation between annexin 1 phosphorylation and PKC autophosphorylation, suggesting that the main effect of annexin 5 is probably to impair the association of the substrate annexin 1 with the membrane lipids. An identical conclusion was recently reached by Edashige et al. [66,67] using the PKC inhibitor cepharantine. As an alternative explanation, the two annexins could interact with each other on the lipid surface, as recently shown for annexins 4, 6 and 7 [68]. Such an interaction might impair accessibility of PKC to annexin 1 without interfering with PKC autophosphorylation. In those experiments performed with PtdEtn/PtdOH/dioleoylglycerol vesicles, annexin concentrations were also in the same range as those of Ca^{2+}, that is, micromolar. However, an inhibitory effect of annexin 5 due to Ca^{2+} sequestration can be excluded, since incubations were performed in the presence of 1 mM Ca/EGTA buffer, which should have kept the free Ca^{2+} concentration almost constant.

A difficult question raised by these *in vitro* studies concerns the physiological relevance of our present investigations. Although the relative proportions of PtdSer, PKC and annexins present in the *in vitro* assays were of the same order of magnitude as in intact endothelial cells, and despite the fact that *in vitro* inhibition of annexin 1 phosphorylation was detected at physiological (micromolar) concentrations of Ca^{2+}, one cannot exclude other mechanisms to explain the lack of PKC-dependent phosphorylation of annexin 1 in intact endothelial cells. For instance, annexin 1 was recently shown to display a partial nuclear localization which differs from the cytoskeletal distribution of annexin 5 [44]. Similar differences in the subcellular localization of PKC and its substrate annexin 1 might also contribute to the lack of annexin 1 phosphorylation.

In conclusion, our data demonstrate that annexin 5 could act as an inhibitor of PKC by competing for phospholipid binding. Definite evidence that this effect is physiologically relevant still awaits further study, although it occurs *in vitro* at physiological (micromolar range) Ca^{2+} concentrations. Finally, during the preparation of this manuscript, another study reported similar *in vitro* effects of annexin 5 on PKC activity using histone III-S as substrate [69].

This work was supported by grants from Association pour la Recherche contre le Cancer, Fédération Nationale des Centres de Lutte contre le Cancer and Conseil Régional Midi-Pyrénées. Thanks are due to Dr. Isabelle Vilgrain and Dr. Jean-Marc Pellosin for pertinent advice during *in vitro* studies of PKC as well as to Mr Michel Nauze for his help with SDS/PAGE analyses. We are also gratefully indebted to Mrs. Yvette Jonquière for correcting the English manuscript.

REFERENCES

1 Crumpton, M. J. and Dedman, J. R. (1990) Nature (London) **345**, 212
2 Browning, J., Pepinsky, B., Wallner, B. P., Flower, R. J. and Peers, S. H. (1990) Nature (London) **346**, 324
3 Wice, B. M. and Gordon, J. I. (1992) J. Cell Biol. **116**, 405–422
4 Geisow, M. J., Fritsche, U., Hexham, J. M., Dash, B. and Johnson, T. (1986) Nature (London) **320**, 636–638
5 Klee, C. B. (1988) Biochemistry **27**, 6646–6653
6 Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, F., Chow, E. P., Burne, C., Huang, K. S., Pratt, D., Wachter, L., Hession, C., Frey, A. Z. and Wallner, B. P. (1988) J. Biol. Chem. **263**, 10799–10811
7 Hauptmann, R., Maurer-Fogy, I., Krystek, E., Bodo, G., Andree, H. and Reutelingsperger, C. (1989) Eur. J. Biochem. **185**, 63–71
8 Davidson, F. F., Dennis, E. A., Powell, M. and Glenne, J. R., Jr (1987) J. Biol. Chem. **262**, 1698–1705

9 Aarsman, A. J., Mynbeck, G., van den Bosch, H., Rothhut, B., Prieur, B., Comera, C., Jordan, L. and Russo-Marie, F. (1987) FEBS Lett. **219**, 176–180
10 Machoczek, K., Fischer, M. and Söling, H. D. (1989) FEBS Lett. **251**, 207–212
11 Gassama-Diagne, A., Fauvel, J. and Chap, H. (1990) J. Biol. Chem. **265**, 4309–4314
12 Funakoshi, T., Hendrickson, L. E., McMullen, B. A. and Fuzikawa, K. (1987) Biochemistry **26**, 8087–8092
13 Maurer-Fogy, I., Reutelingsperger, C. P., Pieters, J., Bodo, G., Stratowa, C. and Hauptmann, R. (1988) Eur. J. Biochem. **174**, 585–592
14 Chap, H., Comfurius, P., Bevers, E. M., Fauvel, J., Vicendo, P., Douste-Blazy, L. and Zwaal, R. F. A. (1988) Biochem. Biophys. Res. Commun. **150**, 972–978
15 Miele, L., Cordella-Miele, E., Facchiano, A. and Mukherjee, A. B. (1988) Nature (London) **335**, 726–730
16 Varticovski, L., Chahwala, S. B., Whitman, M., Cantley, L., Schindler, D., Chow, E. P., Sinclair, L. K. and Pepinsky, R. B. (1988) Biochemistry **27**, 3682–3690
17 Schlaepfer, D. D. and Haigler, H. T. (1988) Biochemistry **27**, 4253–4258
18 Johnsson, N., Van, P. N., Söling, H. D. and Weber, K. (1986) EMBO J. **5**, 3455–3460
19 Fava, R. A. and Cohen, S. (1984) J. Biol. Chem. **259**, 2636–2645
20 Glenney, J. R., Jr. and Tack, B. F. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 7884–7888
21 William, F., Mroczkowski, B., Cohen, S. and Kraft, A. S. (1988) J. Cell. Physiol. **137**, 402–410
22 Isacke, C. M., Lindberg, R. A. and Hunter, T. (1989) Mol. Cell. Biol. **9**, 232–240
23 Schlaepfer, D. D. and Haigler, H. T. (1990) J. Cell Biol. **111**, 229–238
24 Keutzer, J. C. and Hirschhorn, R. R. (1990) Exp. Cell Res. **188**, 153–159
25 Gerke, V. and Weber, K. (1984) EMBO J. **3**, 227–233
26 Glenney, J. R., Jr., Tack, B. and Powell, M. A. (1987) J. Cell Biol. **104**, 503–511
27 Ali, S. M., Geisow, M. J. and Burgoyne, R. D. (1989) Nature (London) **340**, 313–315
28 Pfäffle, M., Ruggiero, F., Hofmann, H., Fernandez, M. P., Selmin, O., Yamada, Y. Garrone, R. and von der Mark, K. (1988) EMBO J. **7**, 2335–2342
29 Wirl, G. and Schwartz-Albiez, R. (1990) J. Cell. Physiol. **144**, 511–522
30 Burns, A. L., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Alijani, M. R. and Pollard, H. B. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3798–3802
31 Diaz-Munoz, M., Hamilton, S. L., Kaetzel, M. A., Hazarika, P. and Dedman, J. R. (1990) J. Biol. Chem. **265**, 15894–15899
32 Ross, T. S., Tait, J. F. and Majerus, P. W. (1990) Science **248**, 605–607
33 Jindal, H. K., Chaney, W. G., Anderson, C. W., Davis, R. G. and Vishwanatha, J. K. (1991) J. Biol. Chem. **266**, 5169–5176
34 Christmas, P., Callaway, J., Fallon, J., Jones, J. and Haigler, H. T. (1991) J. Biol. Chem. **266**, 2499–2507
35 Wijkander, J. and Sundler, R. (1989) Biochim. Biophys. Acta **1010**, 78–87
36 Stratton, K. R., Worley, P. F., Huganir, R. L. and Baraban, J. M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2498–2501
37 Bienkowski, M. J., Petro, M. A. and Robinson, L. J. (1989) J. Biol. Chem. **264**, 6536–6544
38 William, F., Haigler, H. T. and Kraft, A. S. (1989) Biochem. Biophys. Res. Commun. **160**, 474–479
39 Michener, M. L., Dawson, W. B. and Creutz, C. E. (1986) J. Biol. Chem. **261**, 6548–6555
40 Zokas, L. and Glenney, J. R., Jr. (1987) J. Cell Biol. **105**, 2111–2121
41 Gould, K. L., Woodgett, J. R., Isacke, C. M. and Hunter, T. (1986) Mol. Cell. Biol **6**, 2738–2744
42 Hayashi, H., Owada, M. K., Sonobe, S. and Kakunaga, T. (1989) J. Biol. Chem. **264**, 17222–17230
43 Pepinsky, R. B., Sinclair, L. K., Chow, E. P. and Obrineco, B. (1989) Biochem. J. **263**, 97–103
44 Raynal, P., van Bergen en Henegouwen, P. M. P., Hullin, F., Ragab-Thomas, J. M. F., Fauvel, J., Verkleij, A. and Chap, H. (1992) Biochem. Biophys. Res. Commun. **186**, 432–439
45 Hullin, F., Raynal, P., Ragab-Thomas, J., Fauvel, J. and Chap, H. (1989) J. Biol. Chem. **264**, 3506–3513
46 Schlaepfer, D. D., Jones, J. and Haigler, H. T. (1992) Biochemistry **31**, 1886–1891
47 Wooten, M. W., Vandenplas, M. and Nel, A. E. (1987) Eur. J. Biochem. **164**, 461–467
48 Sahal, D. and Fujita-Yamaguchi, Y. (1987) Anal. Biochem. **167**, 23–30
49 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
50 Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. and Putney, J. W., Jr. (1983) J. Biol. Chem. **258**, 15336–15345
51 Thomas, J. M. F., Hullin, F., Chap, H. and Douste–Blazy, L. (1984) Thromb. Res. **34**, 117–123
52 De, B. K., Misono, K. S., Lukas, T. J., Mroczkowski, B. and Cohen, S. (1986) J. Biol. Chem. **261**, 13784–13792
53 Fauvel, J., Vicendo, P., Roques, V., Ragab-Thomas, J., Granier, C., Vilgrain, I., Chambaz, E., Rochat, H., Chap, H. and Douste-Blazy, L. (1987) FEBS Lett. **221**, 397–402
54 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
55 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–918
56 Andree, H. A. M., Reutelingsperger, C., Hauptmann, R., Hemker, H. C., Hermens, W. T. and Willems, G. M. (1990) J. Biol. Chem. **265**, 4923–4928
57 Mosser, G., Ravanat, C., Freyssinet, J. M. and Brisson, A. (1991) J. Mol. Biol. **217**, 241–245
58 Patte, C., Rothhut, B., Russo-Marie, F. and Blanquet, P. R. (1991) Exp. Cell Res. **197**, 12–20
59 Flaherty, M. J., West, S., Heimark, R. L., Fujikawa, K. and Tait, J. F. (1990) J. Lab. Clin. Med. **115**, 174–181
60 Reutelingsperger, C. P. M., Hornstra, G. and Hemker, C. (1985) Eur. J. Biochem. **151**, 625–629
61 Darbon, J. M., Tournier, J. F., Tauber, J. P. and Bayard, F. (1986) J. Biol. Chem. **261**, 8002–8008
62 Demolle, D., Lecomte, M. and Boeynaems, J. M. (1988) J. Biol. Chem. **263**, 18459–18465
63 Blackwood, R. A. and Ernst, J. D. (1990) Biochem. J. **266**, 195–200
64 Lee, M.-H. and Bell, R. M. (1989) J. Biol. Chem. **264**, 14797–14805
65 Lee, M.-H. and Bell, R. M. (1992) Biochemistry **31**, 5176–5182
66 Edashige, K., Utsumi, T. and Utsumi, K. (1991) Biochem. Pharmacol. **41**, 71–78
67 Edashige, K., Utsumi, T., Sato, F., Ide, A., Kasai, M. and Utsumi, K. (1991) Arch. Biochem. Biophys. **296**, 296–301
68 Zaks, W. J. and Creutz, C. E. (1991) Biochemistry **30**, 9607–9615
69 Shibata, S., Sato, H. and Maki, M. (1992) J. Biochem. (Tokyo) **112**, 552–556

Received 5 November 1992/12 January 1993; accepted 15 January 1993