Protein kinase C-dependent phosphorylation of annexins I and II in mesangial cells

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In this study we describe the phosphorylation of annexins from cultured rat mesangial cells by protein kinase C (PKC) both *in vitro* and *in vivo*. Annexins I and II were detected either by Western-blot analysis or by immunoprecipitation using specific antibodies. In the presence of [γ - 32 P]ATP, cytosolic annexin I and annexin II were phosphorylated *in vitro* only when Ca^{2+} and phospholipids were added, but not in the presence of phospholipids alone. Annexin I was shown to be a better substrate than annexin II. In experiments *in vivo* performed on 32 P-labelled mesangial cells, the addition of two well-known activators of PKC, namely angiotensin II (AII) and phorbol myristate acetate

(PMA), increased preferentially the phosphorylation of annexin I. Annexin II was phosphorylated to a much lesser extent after AII treatment. Phosphoamino acid analysis of annexins, either by two-dimensional chromatography or by using a specific anti-phosphotyrosine antibody, revealed only phosphoserine in these experiments *in vivo*. The addition of AII to mesangial cells increased serine phosphorylation of annexin I and annexin II, whereas PMA only increased serine phosphorylation of annexin I. V8-protease phosphopeptide mapping of annexin I that was phosphorylated both *in vitro* and *in vivo* by PKC from mesangial cells shows similar phosphopeptides.

INTRODUCTION

Annexins belong to a large family of proteins, at least 12 in number, which share the ability to bind phospholipids in a Ca^{2+} -dependent manner (Crompton et al., 1988). These abundant intracellular proteins have been found in a wide variety of cell types and tissues (Burgoyne and Geisow, 1989) and are located primarily on the cytosolic face of the plasma membrane, in association with the underlying cytoskeleton (Glenney et al., 1987). Although their precise physiological functions have still not been clearly defined, many studies have shown that these proteins are involved in the regulation of important cellular processes such as proliferation (Schlaepfer and Haigler, 1990), differentiation (William et al., 1989; Violette et al., 1990) or membrane traffic, including exocytosis and endocytosis (Drust and Creutz, 1988; Sarafian et al., 1991). They have also been shown to inhibit phospholipase A_2 activity (Pepinsky et al., 1986) and blood coagulation (Tait et al., 1989), and to regulate transmembrane Ca^{2+} -channel activity (Pollard et al., 1990). Annexins appear also to be highly related to mitogenic signal transduction, as they are preferential substrates for both growth-factor receptors (Fava and Cohen, 1984; Pepinsky and Sinclair, 1986) and retrovirally encoded tyrosine kinases (Kristensen et al., 1986; Huang et al., 1986). Changes in the phosphorylation state of annexins can modulate strongly their biological activities by decreasing considerably their anti-phospholipase A_2 activity (Hirata, 1981; Touqui et al., 1986), either by lowering the Ca^{2+} requirements for association with membrane vesicles (Schlaepfer and Haigler, 1987) or by decreasing their affinity for negatively charged phospholipid vesicles (Powell and Glenney, 1987). Protein kinase C (PKC), which plays an important role in the signal-transduction pathway of several agonists (Nishizuka, 1988), has also been shown to phosphorylate annexins I and II both *in vitro* and *in vivo* (Gould et al., 1986; Khanna et al., 1986; Varticovski et al., 1988; Schlaepfer and Haigler, 1988; Antonicelli

et al., 1989). Since the discovery of several closely related PKC isoforms (divided in two groups due to their Ca^{2+} -requirement for activation), it would be of considerable interest to correlate the specific activation of these PKC isoforms by particular agonists with the phosphorylation of one or more annexins. In addition to serving as substrates, these proteins, particularly annexin I, have been recently reported to act as intracellular receptors, on which PKC can bind after activation and translocate to the membrane, in response to a hormonal effector or to the tumour promoter 4β -phorbol 12β -myristate 13α -acetate (PMA) (Mochly-Rosen et al., 1991a,b). Moreover, annexin V, rather than being a substrate for PKC (Kaplan et al., 1988; Coméra et al., 1989), acts more as a potent inhibitor of this kinase (Schlaepfer et al., 1992), suggesting a possible role in the regulation of PKC-dependent phosphorylation *in vivo*. Renal glomerular mesangial cells resemble perivascular smooth-muscle cells, and in the kidney constitute a major site of action of several agonists such as the vasoactive peptide angiotensin II (AII). By its ability to contract mesangial cells, AII induces a decrease in glomerular surface and thereby plays a role in the control of glomerular filtration rate (Blantz et al., 1976). In cultured mesangial cells, the regulation of mitogenesis and phospholipase C-mediated phosphoinositide hydrolysis by various agonists and PMA appears to be highly dependent on PKC activation.

Similar conclusions have been drawn for the hormonal- or PMA-enhanced production of prostaglandins (for review see Pfeilschifter, 1989), although the exact mechanism leading to arachidonic acid release remains to be determined and clarified. It may involve a PKC-dependent phosphorylation of phospholipase A_2 itself, or some phospholipase- A_2 -modulatory proteins such as annexins. We and others have characterized, in mesangial cells, the presence of two kinds of Ca^{2+} -dependent and -independent PKC isoforms (Oudinet et al., 1992a,b; Huwiler et al., 1991, 1992), and in addition we have been able to correlate the activation *in vitro* of each group with the specific phosphoryl-

ation of endogenous proteins. Among these substrates, proteins of 33–35 kDa are phosphorylated in a Ca^{2+} -dependent manner. In the present study, we examine the ability of PKC from rat mesangial cells to phosphorylate annexins I and II both *in vitro* and *in vivo*. In addition, since peptide mitogens such as AII or the tumour promoter PMA enhance protein phosphorylation on tyrosine (Gilmore and Martin, 1983; Force et al., 1991), we analysed the phosphoamino acid content of annexins I and II after activation of PKC *in vivo* by these factors.

EXPERIMENTAL

Materials

Fetal-calf serum and RPMI 1640 medium were purchased from Gibco (Grand Island, NY, U.S.A.) and Flow Laboratories (Irvine, Scotland, U.K.) respectively. Tissue-culture dishes were obtained from Falcon. L- α -Phosphatidyl-L-serine, 1,3-diolein, PMA, DNA from calf thymus and trypsin [treated with tosyl-phenylalanylchloromethane ('TPCK')] were all from Sigma (St. Louis, MO, U.S.A.), and AII was from Ciba-Geigy (Basel, Switzerland). *Staphylococcus aureus* V8 protease was from Boehringer. [γ - ^{32}P]ATP was purchased from Amersham, and X-ray films were from Kodak.

Mesangial-cell cultures

Homogeneous cultures of renal mesangial cells were prepared and characterized as previously described (Baud et al., 1989). Briefly, cells derived from collagenase-treated glomeruli, isolated from Sprague-Dawley rats, were grown in 35 mm culture dishes (Falcon) in RPMI 1640 medium supplemented with 10% calf serum, 10 mM Hepes (pH 7.4), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cultures were maintained at 37 °C under a 5%- CO_2 atmosphere, and the medium was changed every 2 days. Confluent cells (after 30 days of culture) were used in all experiments.

Phosphorylation of annexin I and II *in vitro* by PKC

Mesangial cells were first washed with 4 ml of Hepes-buffered RPMI medium, scraped and homogenized in 1 ml of a buffer containing 20 mM Tris/HCl (pH 7.5), 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethanesulphonyl fluoride and 20 $\mu\text{g}/\text{ml}$ leupeptin. The homogenates were centrifuged for 1 h at 105000 g, and the supernatants were designated as the cytosolic fractions. The phosphorylation of annexins by Ca^{2+} -dependent and -independent phospholipid-dependent PKC activity was investigated in samples (100 μl) of cytosols in final volumes of 300 μl of a reaction mixture containing the following: 50 mM glycerol 2-phosphate, 0.3 mM EGTA, 2 mM theophylline, 10 mM magnesium acetate and 1 mM potassium phosphate buffer (pH 7.4), to which an excess of 0.5 mM CaCl_2 and a mixture of phospholipids (26 μg of phosphatidylserine, 0.8 μg of diolein) were added. Phosphorylation of annexins by Ca^{2+} -independent phospholipid-dependent PKC was performed under the same conditions, except that Ca^{2+} was omitted. Basal activity was measured in the absence of both Ca^{2+} and phospholipids. The reaction was initiated by adding [γ - ^{32}P]ATP at a concentration of 0.05 mM (sp. radioactivity 3000–4500 c.p.m./pmol). All incubations were carried out at 30 °C for 3 min. Total endogenous protein phosphorylation was measured on samples (50 μl) of the reaction mixture spotted on 2.1 cm-diameter filter

paper discs (Whatman 3MM) and processed as previously described (Omri et al., 1987). The remaining fraction (250 μl) was mixed with an equal volume in lysis buffer containing the following: 10 mM Tris/HCl, pH 7.4, 50 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 5 mM EGTA, 1 mg/ml BSA, 1 mM phenylmethanesulphonyl fluoride, 100 units/ml aprotinin, 1 $\mu\text{g}/\text{ml}$ each of antipain, leupeptin and pepstatin, and 1 mM orthovanadate, 25 mM NaF, 5 mM *p*-nitrophenyl phosphate and 10 mM sodium pyrophosphate.

Lysates were pre-cleared for 1 h at 4 °C with 1% (v/v) fixed *Staph. aureus* bacteria (Pansorbin; Calbiochem, La Jolla, CA, U.S.A.). Antisera against annexins (see below) were added (1/100 dilution) and allowed to react overnight before collection of the immune complexes with Protein A-Sepharose. Immune complexes were washed three times with lysing buffer and three times with PBS. Immunoprecipitates were analysed by SDS/PAGE (10%-polyacrylamide gel) and labelled bands were detected by autoradiography at -70 °C by using Kodak XAR-5 film. Detection of each annexin by Western blot, using ^{125}I -Protein A or the ECL system (Amersham), was performed on either the same cytosolic fractions or on cells directly lysed in SDS sample buffer as described by Rothhut et al. (1989). Polyclonal antibody against annexin I was raised in a rabbit by popliteal-lymph-node injection (Goudie et al., 1966) of the purified protein from human placenta (Coméra et al., 1990). The specificity of this antibody was tested against purified annexins I, II, III, V and VI from human placenta. Anti-annexin II antibody was kindly provided by J. C. Cavadore (Montpellier, France).

Phosphorylation *in vivo* of annexins from ^{32}P -labelled mesangial cells

After washing twice with phosphate-free RPMI 1640 medium, mesangial cells were incubated at 37 °C in the same medium for 1 h. Then 1 mCi/ml $\text{H}_3^{32}\text{PO}_4$ (8500–9120 Ci/mmol; NEN) was added and the incubation continued for 3 h. Before termination of the incubation, cells were treated for the indicated periods of time with AII (1 μM) or PMA (100 ng/ml). The supernatant was removed, and cells were rapidly rinsed with cold PBS and then scraped in 500 μl of lysing buffer. Annexins I and II were immunoprecipitated with their corresponding antibodies by the protocol described above. The immunoprecipitated phosphoproteins were analysed by SDS/PAGE (10% gels), followed by autoradiography at -70 °C.

Two-dimensional phosphoamino acid analysis

The phosphoamino acid content of immunoprecipitated annexins I and II from ^{32}P -labelled mesangial cells was determined as described by Kamps and Sefton (1989). Briefly, immunoprecipitates separated by SDS/PAGE on a 10% gel were electrotransferred on to a poly(vinylidene difluoride) membrane (Immobilon-P; Millipore) by using a semi-dry apparatus (Pharmacia), the transfer buffer consisting of the following: 192 mM glycine, 25 mM Tris/HCl, pH 8.3, 0.01% SDS, 20% methanol. Labelled proteins were detected after autoradiography of the filter at -70 °C (Kodak XAR-5 film). Bands of interest were then excised, washed, resuspended in 5.7 M HCl, and acidic hydrolysis was performed at 110 °C for 1 h. Phosphoamino acid analysis was performed by high-voltage two-dimensional t.l.c. as described by Cooper et al. (1983), using a HTLE-7000 chromatography apparatus (C.B.S. Scientific Co., Del Mar, CA, U.S.A.). ^{32}P -labelled amino acids were detected and aligned with standards by autoradiography at -70 °C (Hyper Film βmax ; Amersham). Phosphoamino acid standards were located by spraying the

sheets with 0.25% ninhydrin in acetone. In addition, the phosphotyrosine content of immunoprecipitated annexins from non-labelled cells was analysed by Western blotting using a monoclonal anti-phosphotyrosine antibody (UBI, Lake Placid, NY, U.S.A.).

Phosphopeptide mapping analysis

Two-dimensional tryptic peptide mapping of annexin I phosphorylated *in vitro* (upper and lower bands) was performed as described by Luo et al. (1990). Annexin I immunoprecipitates were subjected to SDS/PAGE and transferred to nitrocellulose. Bands of interest were then excised, washed in water, resuspended in 200 μ l of 10 mM $(\text{NH}_4)_2\text{CO}_3$ buffer, pH 8.8, and digested with 50 μ g of TPCK-treated trypsin at 37 °C overnight. After freeze-drying the resulting phosphopeptides were resolved on cellulose thin-layer plates by electrophoresis at pH 1.9 for 20 min at 1 kV in the first dimension, followed by ascending chromatography in the second dimension. ^{32}P -labelled peptides were detected by autoradiography after 15 days of exposure at -70 °C.

Phosphopeptide mapping of annexin I phosphorylated both *in vitro* and *in vivo* was also performed by the protocol described by Cleveland et al. (1977), by using *Staph. aureus* V8 protease. Gel slices containing immunoprecipitated ^{32}P -labelled annexin I were digested with 1 μ g of the protease in the stacking gel of an SDS/15%-polyacrylamide gel. After electrophoresis, the radioactive phosphopeptides were detected by autoradiography.

RESULTS

In a first set of experiments, we analysed the presence of annexins I and II in mesangial cells by Western blotting using specific polyclonal antibodies raised against the corresponding proteins. As shown in Figure 1, annexins I and II are detected both in total cellular extracts (lanes 1 and 3) and in cytosolic preparations (lanes 2 and 4) of mesangial cells. Annexin I appears as a main protein band of 35 kDa, which is the well-characterized molecular mass of this protein found in many cell types. However, in cytosols slower-migrating forms, around 32 kDa, are also shown. In contrast, annexin II was found as a single form in both preparations, with an apparent molecular mass of 34 kDa. The specificity of the anti-annexin I serum, tested against different annexins (I, II, III, V and VI), shows (Figure 2b) that only annexin I was recognized, suggesting that the lower band shown in Figure 1, lane 2, is not the result of cross-reactivity with other annexins.

We then tested the phosphorylation *in vitro* of cytosolic annexins in a PKC-dependent manner under the following conditions: without Ca^{2+} and phospholipids (Figure 3, lanes 1 and 4), with phospholipids (lanes 2 and 5), and with Ca^{2+} and phospholipids (lanes 3 and 6). The addition of phospholipids to cytosols increased 2-fold the phosphorylation of endogenous proteins from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (basal activity 8.24 ± 1.59 , + phospholipids 17.66 ± 4.97 pmol of $^{32}\text{P}/100 \mu\text{g}$ of DNA; $n = 6$), whereas the phosphorylation of immunoprecipitated annexins I and II under these conditions appeared hardly detectable or totally absent, as shown in Figure 3, lanes 2 and 5 respectively. The lack of phosphorylation of annexin I and II (lanes 1, 2, 4, 5) was not due to the absence of immunoprecipitated proteins, because a Western-blot analysis of the same samples revealed the presence of both annexins (results not shown). When Ca^{2+} was added to phospholipids, the 3-fold increase in endogenous phosphoproteins observed (basal activity 8.24 ± 1.59 , + Ca^{2+} and

Figure 1 Western-blot analysis of different extracts of mesangial cells, probed with antibodies against annexins I and II

Cells were either lysed directly in SDS sample buffer (lanes 1 and 3), or cytosolic fractions were prepared from them (lanes 2 and 4), as described in the Experimental section. Blots were incubated with polyclonal antibodies raised against annexin I (lanes 1 and 2) or annexin II (lanes 3 and 4) and revealed with ^{125}I -Protein A. Molecular-mass standards are shown on the right.

Figure 2 Specificity of anti-annexin I serum

Purified annexins from human placenta were subjected to SDS/PAGE, transferred to nitrocellulose, tested with anti-annexin I serum and revealed with the ECL system. Panel (a) shows the Coomassie-Blue-stained gel and panel (b) the result of the Western blot. Lanes 1 and 6, molecular-mass standards (in kDa) on the left; lanes 2 and 3, annexin I, 1 and 2 μg respectively; lane 4, annexin II (2 μg); lane 5, annexin III (1 μg); lane 7, annexin V (2 μg); lanes 8 and 9, annexin VI, 1 and 2 μg respectively.

phospholipids 24.27 ± 5.93 pmol of $^{32}\text{P}/100 \mu\text{g}$ of DNA; $n = 6$) was also associated with a strong phosphorylation of annexins I and II (Figure 3, lanes 3 and 6). Under these conditions annexin I appears as two main phosphorylated species (lane 3) migrating

Figure 3 Immunoprecipitation of annexins I and II that had been phosphorylated *in vitro*

Cytosolic proteins were subjected to phosphorylation *in vitro* in the absence of Ca^{2+} and phospholipids (lanes 1 and 4), with phospholipids (lanes 2 and 5), or with Ca^{2+} and phospholipids (lanes 3 and 6). Immunoprecipitations were performed with anti-annexin I (lanes 1–3) and anti-annexin II (lanes 4–6) antibodies. Phosphoproteins were analysed by SDS/PAGE (10% gels), followed by autoradiography at -70°C . Molecular-mass standards are shown on the right.

Figure 4 Two-dimensional phosphotryptic peptide map of the forms of annexin I phosphorylated *in vitro*

The phosphotryptic peptide maps of the upper and lower bands of annexin I from Figure 3, lane 3, are shown respectively in panel (a) and (b). The origin of the migration is indicated by 'o'. Arrow 1 corresponds to the first dimension (electrophoresis); the second dimension is ascending chromatography, shown by arrow 2. Phosphopeptides of interest are indicated by small arrows.

at 35 kDa (upper band) and 32 kDa (lower band). Annexin II phosphorylation (lane 6) shows a single phosphoprotein migrating at 34 kDa.

Bands of annexin I phosphorylated *in vitro* were compared by two-dimensional phosphotryptic peptide mapping. Figure 4 shows that the phosphopeptide derived from the upper band (panel a) co-migrates with the peptide from the lower band (panel b), reinforcing the fact that they are related and share common phosphorylation site(s).

In vivo, the phosphorylation of both annexins was studied by incubating ^{32}P -labelled cells in the presence of either AII or PMA, two well-known activators of PKC in this cell system.

Figure 5 Immunoprecipitation of annexins I and II from mesangial cells ^{32}P -labelled *in vivo*

Before immunoprecipitation, ^{32}P -labelled cells were treated either with AII for 1 min (lanes 2 and 10) or 5 min (lanes 1 and 11), or with PMA for 1 min (lanes 4 and 8) or 5 min (lanes 5 and 7). Control cells are shown in lanes 3, 6 and 9. Phosphoproteins were immunoprecipitated with anti-annexin I (lanes 1–5), anti-annexin II (lanes 7–11) or a non-immune serum (lane 6) and analysed by SDS/PAGE (10% gels), followed by autoradiography at -70°C . Annexins I and II are respectively shown as 'a' and 'b'. Molecular-mass standards are shown on the right.

Figure 6 Two-dimensional phosphoamino acid analysis

The phosphoamino acid content of immunoprecipitated annexins I (a, b, c) and II (d, e, f) was analysed from control cells (a and d) or after treatment for 1 min with AII (b and e) or PMA (c and f). The positions of the standard phosphoamino acids are indicated as follows: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. The origin of the migration is indicated by 'o'. Exposure time was 21 days at -70°C with intensifying screens.

After incubation for 1 or 5 min, AII and PMA increased preferentially the phosphorylation of annexin I (Figure 5, lanes 1, 2 and 4, 5) as compared with control (lane 3). In contrast, annexin II phosphorylation was only slightly increased by AII (Figure 5, lanes 10, 11), whereas PMA had no effect on annexin II phosphorylation (lanes 7, 8) as compared with control (lane 9). A non-immune serum tested in the same conditions failed to detect proteins corresponding to these molecular masses (lane 6).

To analyse further phosphorylation of annexins I and II, the corresponding bands were excised and subjected to two-dimensional phosphoamino acid determination. Under these conditions, only phosphoserine was detected for both proteins in

Figure 7 Comparison by V8-protease mapping of annexin I phosphorylated *in vitro* and *in vivo*

Lanes 1 and 2 correspond respectively to phosphopeptides from annexin I phosphorylated *in vivo* after treatment of cells with AII (lane 1) or PMA (lane 2). Lanes 3 and 4 are the phosphopeptides derived from the upper form (lane 4) and lower form (lane 3) of annexin I phosphorylated *in vitro*. Owing to differences in the labelling, lanes 1–3 and lane 4 were respectively obtained after 15 days and 4 days of exposure. Positions of molecular-mass standards are shown on the right.

the absence of any agonist (Figures 6a and 6d). The incorporation of ^{32}P into this amino acid increased mostly for annexin I when cells were incubated for 5 min with either AII or PMA (Figures 6b and 6c). Annexin II phosphorylation showed only a slight increase in serine phosphorylation stimulation for 5 min with AII (Figure 6e). No difference was observed in the phosphorylation of annexin II by PMA (Figure 6f) as compared with control, confirming the results from Figure 3 (lanes 7–9). The lack of tyrosine phosphorylation was confirmed by using a highly specific antibody against phosphotyrosine tested on immunoprecipitated annexins I and II from non-labelled cells and treated with AII and PMA (results not shown). In order to investigate a possible PKC-dependent phosphorylation of annexin I *in vivo*, peptide maps were compared. As shown in Figure 7, V8-protease-treated annexin I that had been phosphorylated *in vivo* after the addition of AII (lane 1) or PMA (lanes 2), and *in vitro* (lanes 3 or 4), generated identical phosphopeptides. These results strongly suggest that PKC is responsible for the phosphorylated form of annexin I detected *in vivo*.

DISCUSSION

Mesangial cells contain both annexins I and II, two members of a family of Ca^{2+} -dependent phospholipid-binding proteins which share considerable sequence similarity. By using a combination of Western blotting and immunoprecipitation, we describe the presence and the phosphorylation of annexins I and II in mesangial cells. When cytosolic preparations were analysed by Western blotting for the presence of annexin I, a major 35 kDa form was found, and also additional bands migrating at 32 kDa were detected. According to the specificity of the annexin I serum shown in Figure 2, these latter forms probably represent proteolytic fragments of the migrating 35 kDa form, since annexin I has been shown to be sensitive to the Ca^{2+} -activated neutral protease calpain I (Ando et al., 1989), and to an endogenous Ca^{2+} -dependent membrane protease (Chuah and Pallen, 1989). Phosphorylated annexins have been widely studied, and shown

to be substrates *in vitro* and *in vivo* for various kinases (Crompton et al., 1988). Mesangial-cell cytosolic preparations were shown to contain two groups of PKC isoforms: one which is Ca^{2+} -sensitive and phospholipid-dependent, and one which is Ca^{2+} -insensitive but phospholipid-dependent. We therefore tested the ability of these annexins to undergo phosphorylation under different conditions of PKC activation.

The results *in vitro* show that annexins I and II can be phosphorylated and specifically immunoprecipitated from these preparations and may therefore serve as substrates for Ca^{2+} - and phospholipid-dependent PKC, annexin I being a better substrate than annexin II. In these cytosolic preparations annexin I appears as two highly phosphorylated species of 35 and 32 kDa. Both bands were compared by two-dimensional phosphotryptic peptide mapping. The results show that they share the same phosphorylation site(s), suggesting that the 35 kDa phosphorylated form is more sensitive to proteases, as described previously (Haigler et al., 1987). This supports the data obtained with the Western-blot analysis presented in Figure 1 (lane 2), where the 32 kDa species was only found in the cytosolic preparations where Ca^{2+} was added to achieve PKC activation. Under these conditions, Ca^{2+} -dependent proteases are likely to be activated.

Our results are in agreement with phosphorylations of annexins I and II *in vitro* reported by several authors (Khanna et al., 1986; Varticovski et al., 1988; Schlaepfer and Haigler, 1988). It should be noticed that Ca^{2+} is absolutely required for annexin phosphorylation, suggesting either that Ca^{2+} is needed to activate only the Ca^{2+} - and phospholipid-dependent PKC, namely PKC α , in mesangial cells, or that Ca^{2+} binding to annexins is a prerequisite for PKC-dependent phosphorylation. This Ca^{2+} -dependent phosphorylation of annexins by PKC isoforms has also been reported (William et al., 1989).

In addition to the results shown above, annexins are also substrates for PKC *in vivo*, since we have shown that activation of the kinase by AII increased the serine phosphorylation of these proteins. This has been clearly established for annexin I, since peptide maps from annexin I phosphorylated *in vitro* and *in vivo* are identical. As with experiments *in vitro*, we found that annexin I represents a better substrate for PKC than annexin II. These results are closely related to those described by Antonicelli et al. (1989) in thyroid cells.

Activation of PKC by PMA increased only serine phosphorylation of annexin I, whereas annexin II phosphorylation was not detected. In contrast with results reported by others, which showed that annexin II is a good substrate for PKC *in vivo* (Gould et al., 1986), we could not detect such a PMA effect on mesangial cells. Nevertheless a similar result has been described (William et al., 1989), showing a lack of annexin I phosphorylation in A431 cells treated with phorbol esters, whereas this protein was found to be a good substrate *in vitro* for PKC.

In mesangial cells, the lesser degree or the lack of phosphorylation of annexin II *in vivo* by AII and PMA, respectively, compared with annexin I, is probably due to the presence of a smaller amount of annexin II protein present, as suggested by Western-blot experiments (results not shown). Alternatively, it could be the result of differential localization of annexin II and the PKC isoforms specifically activated by AII or PMA.

It is apparent that under our conditions tyrosine phosphorylation, secondary to the activation of the phospholipase C–PKC pathway by vasoactive peptides such as AII, could not be detected for annexins I and II. These results show that annexins I and II do not participate in the tyrosine-

phosphorylating events induced by AII and PMA, as has been shown for other high-molecular-mass proteins (Force et al., 1991). On the other hand, protein tyrosine phosphatases are probably activated and could mask the tyrosine phosphorylation of annexins. Tyrosine phosphorylation is also a very transient phenomenon and is possibly undetectable under these conditions (non-quiescent cells). However, experiments performed in the presence of sodium orthovanadate, a well known inhibitor of cellular tyrosine phosphatases, also failed to reveal tyrosine phosphorylation of annexins. In this study, the phosphorylation of annexins by PKC both *in vitro* and *in vivo* in mesangial cells suggest that this process may represent an important step in transducing the biological effect of various agonists. This phosphorylation process could modulate the interaction between these annexins and cytoskeleton proteins or phospholipids in regulating membrane processes, for example phospholipase A₂ activity.

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