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Inhibition of Protein Kinase C by Annexin V†

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ABSTRACT: Annexin V is a protein of unknown biological function that undergoes Ca^{2+}-dependent binding to phospholipids located on the cytosolic face of the plasma membrane. Preliminary results presented herein suggest that a biological function of annexin V is the inhibition of protein kinase C (PKC). In vitro assays showed that annexin V was a specific high-affinity inhibitor of PKC-mediated phosphorylation of annexin I and myosin light chain kinase substrates, with half-maximal inhibition occurring at approximately 0.4 μM. Annexin V did not inhibit epidermal growth factor receptor/kinase phosphorylation of annexin I or cAMP-dependent protein kinase phosphorylation of the Kemptide peptide substrate. Since annexin V purified from both human placenta and recombinant bacteria inhibited protein kinase C activity, it is not likely that the inhibitor activity was associated with a minor contaminant of the preparations. The following results indicated that the mechanism of inhibition did not involve annexin V sequestration of phospholipid that was required for protein kinase C activation: similar inhibition curves were observed as phospholipid concentration was varied from 0 to 800 μg/mL; the extent of inhibition was not significantly affected by the order of addition of phospholipid, substrate, or PKC, and the core domain of annexin I was not a high-affinity inhibitor of PKC even though it had similar Ca^{2+} and phospholipid binding properties as annexin V. These data indirectly indicate that inhibition occurred by direct interaction between annexin V and PKC. Since the concentration of annexin V in many cell types exceeds the amounts required to achieve PKC inhibition in vitro, it is possible that annexin V inhibits PKC in a biologically significant manner in intact cells.

Annexins are a family of Ca^{2+} binding proteins that undergo reversible Ca^{2+}-dependent binding to phospholipids that are located on the cytosolic face of the plasma membrane (Crompton et al., 1988; Crumpton & Dedman, 1990). They are abundant intracellular proteins, and several different annexin gene products are expressed in all mammalian cells examined to date. The exact physiological functions of the annexins are not yet known. Proposed roles include regulation of membrane traffic and exocytosis (Drust & Creutz, 1988; Ali et al., 1989; Nakata et al., 1990; Sarafian et al., 1991), mediation of cytoskeletal–membrane interactions (Gerke & Weber, 1984; Powell & Glenney, 1987; Ikebuchi & Waisman, 1990), mitogenic signal transduction (De et al., 1986; Pepinsky & Sinclair, 1986; Haigler et al., 1987), transmembrane channel activity (Pollard & Rojas, 1988; Rojas et al., 1990), inhibition of blood coagulation (Funakoshi et al., 1987; Iwasaki et al., 1887; Grundmann et al., 1988; Tait et al., 1988; Hauptmann et al., 1989), and inhibition of phospholipase A_2 (Pepinsky et al., 1986, 1988; Wallner et al., 1986). It is not yet known whether different annexins perform different biological functions. It is, of course, also possible that an individual annexin performs different biological functions in different tissues as has been observed for the Ca^{2+} binding protein calmodulin.

It is now important to determine which of the proposed functions of annexins represent actual physiological activities. The inhibition of blood coagulation and phospholipase A_2 activity in vitro appears to occur by Ca^{2+}-dependent sequestration of phospholipid in the reactions, thereby raising doubts about the physiological significance of these observations (Haigler et al., 1987; Davidson et al., 1987; Funakoshi et al., 1987). These inhibitor studies emphasize the importance of clearly distinguishing between phospholipid sequestration and direct affects when investigating new functions of the annexins.

Protein kinase C (PKC) is a key element in the signal transduction pathway by which a number of extracellular effectors modulate intracellular activity [see Nishizuka (1988) for a review]. Cellular PKC is activated by Ca^{2+}-dependent binding of the kinase to phosphatidylserine and diacylglycerol on the cytosolic face of the plasma membrane. Although PKC shares certain Ca^{2+} and phospholipid binding properties with the annexins, there is no structural similarity between the two gene families. Several annexins are good substrates for PKC in in vitro reactions (Gould et al., 1986; Weber et al., 1987; Schlaepfer & Haigler, 1988), and others which have not been studied to date have amino acid sequences that appear to

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constitute PKC substrate recognition sites.

The cellular activities of protein kinases are usually maintained at low basal levels, and this is often accomplished by autoinhibitory domains (Hardie, 1988; Soderling, 1990). A major mechanism by which PKC activity is inhibited is by a high-affinity intramolecular pseudosubstrate site (House & Kemp, 1987). Ca^{2+}-dependent binding of diacylglycerol and phosphatidylserine apparently causes a conformational change in PKC that removes the inhibitor region from the active site and thereby restores activity. In addition to autoinhibitory sites within PKC, other proteins inhibit PKC activity in vitro (Schwantke & Le Peuch, 1984; Pribilla et al., 1988; Pearson et al., 1990) and are being considered as endogenous regulators of this kinase. The best characterized is protein kinase C inhibitor-1, a protein with an apparent molecular weight of 17000 which exhibits half-maximal inhibition at 2.2 μM (Pearson et al., 1990). Unlike the autoinhibitory domain of PKC, protein kinase C inhibitor-1 does not contain a pseudosubstrate site, and the mechanism of inhibition is not known.

We previously reported that annexin V was not a substrate for PKC (Kaplan et al., 1988). We now report that annexin V is a high-affinity inhibitor for PKC in in vitro reactions. Annexin V inhibition of PKC did not occur by sequestration of Ca^{2+} or phospholipid cofactors in the kinase reaction. Inhibition may involve a specific and direct interaction between annexin V and PKC.

Experimental Procedures

Materials. Molecular weight standards for polyacrylamide gel standardization ("high" and "low") were purchased from Bio-Rad (Richmond, CA). [γ-^{32}P]ATP was synthesized from carrier-free [^{32}P]orthophosphate (ICN, Irvine, CA) using Gamma-Prep Synthesis Systems (Promega Biotec, Madison, WI). Epidermal growth factor (EGF) was isolated from mouse submaxillary glands as described (Savage & Cohen, 1972). Annexins I and V were purified from human placenta (Haigler et al., 1987; Schlaepfer et al., 1987). The concentrations of annexins I and V were determined from A_{280} using calculated extinction coefficients of 18600 and 20000 M^{-1}, respectively, which were determined on the basis of known amino acid compositions. Myosin light chain kinase purified from turkey gizzard was a generous gift from the laboratory of Dr. Edwin Krebs (University of Washington, Seattle, WA). The catalytic subunit of cAMP-dependent protein kinase (A-kinase) was a generous gift from Dr. John Scott (Vollum Institute, Portland, OR). The Kemptide peptide substrate and histones type III-S were purchased from Sigma (St. Louis, MO). Recombinant annexin V was expressed in *Escherichia coli* and purified by reversible Ca^{2+}-dependent binding to phospholipid vesicles as described (Kaplan et al., 1988). Des(1–29)annexin I was prepared by plasmin proteolytic digestion of annexin I (Huang et al., 1987) and was purified by reversible Ca^{2+}-dependent binding to phospholipid vesicles (Schlaepfer & Haigler, 1988). PKC was purified from rat brain (Woodget & Hunter, 1987) and stored frozen at −70 °C.

Standard PKC Phosphorylation Assay. Unless otherwise indicated, in vitro PKC phosphorylation of annexin I was measured as follows. The annexin I substrate (500 ng, final concentration 0.54 μM) was preincubated at 30 °C for 10 min in a solution containing Tris-HCl buffer (20 mM, pH 7.6), $MgCl_2$ (10 mM), $CaCl_2$ (0.5 mM), purified PKC (2 μg/mL), freshly sonicated vesicles (50 μg/mL), and the indicated concentration of annexin V protein. The vesicles contain 10 parts phosphatidylserine (840032, Avanti Polar Lipids) and 1 part diacylglycerol (1-oleoyl-2-acetyl-*sn*-glycerol, 0-8255, Sigma). The reaction was initiated by the addition of [γ-^{32}P]ATP (20 μM, 10 Ci/mmol) to achieve a final volume of 25 μL. The reaction was allowed to proceed at 30 °C for 10 min and was terminated by the addition of 10 μL of a 4-fold-concentrated solution of Laemmli sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970) followed by heating at 100 °C for 2 min. The reactions were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), and the gels were calibrated by molecular weight standards, stained with Coomassie Blue, destained, dried, and exposed to Kodak XAR-5 film with intensifying screens at −70 °C. Under the standard reaction conditions, incorporation of radioactivity into annexin I was linear with respect to time. Half-maximal phosphorylation occurred at approximately 100 nM annexin I concentration (Schlaepfer & Haigler, 1988). The stoichiometry of annexin I phosphorylation (at 0.5 μM) was approximately 0.85 mol of phosphate incorporated per mole of protein (Schlaepfer & Haigler, 1988).

Phosphorylation of Histones by PKC. Phosphorylation of histones was measured by a modification of a published assay (Woodget & Hunter, 1987). Assays (40 μL) contained Hepes (20 mM, pH 7.4), $MgCl_2$ (10 mM), $CaCl_2$ (1 mM), freshly sonicated vesicles containing phosphatidylserine (50 μg/mL) and diacylglycerol (5 μg/mL), histone type III-S (20 μg), and PKC (5 μL). For annexin V inhibition studies, purified annexin V was added to a final concentration of 0.25–5.0 μM. The reactions were initiated by the addition of [γ-^{32}P]ATP (20 μM, 100 cpm/pmol) and were incubated for 5 min at 30 °C. Assays were terminated by the addition of 0.5 mL of 25% (w/v) trichloroacetic acid. The acid-precipitable material was collected on glass fiber filters (Whatman, 1 cm^2), washed twice with a trichloroacetic acid solution, and then assayed for Cerenkov radiation.

Histones (0.2 μg per reaction) were also phosphorylated by PKC under the Standard PKC Phosphorylation Assay conditions, and the reactions were analyzed by SDS–PAGE and autoradiography. For annexin V inhibition studies, purified annexin V was added to a final concentration of 0.25–5.0 μM.

Preparation of A431 Cell Particulate Fraction. The membrane particulate fraction of EGF-treated A431 cells was prepared (Fava & Cohen, 1984) with the following changes. The homogenization buffer was modified to contain 1 mM EGTA, and the homogenate was spun at 1000*g*. The low-speed particulate pellet was discarded before preparation of the high-speed (100000*g*) membrane particulate fraction. The A431 cell membrane pellet was resuspended by passage through a fine pipet tip into a buffer containing 20 mM Hepes, pH 7.4, 150 mM NaCl, and 2 mM $MgCl_2$. The protein content of the A431 membrane fraction was determined by the BCA micro protocol method (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. The membranes contained EGF-stimulated tyrosine kinase activity and were stored frozen at −70 °C.

A431 EGF-Stimulated Tyrosine Kinase Phosphorylation Assay. In vitro phosphorylation of annexin I on tyrosine was performed as follows. The reaction mixture contained the isolated A431 cell membranes (2.0 μg total protein), Hepes buffer (20 mM, pH 7.4), glycerol (5% w/v), $MgCl_2$ (20 mM), $CaCl_2$ (0.5 mM), $ZnCl_2$ (25 μM), $NaVO_3$ (50 μM), *p*-nitrophenyl phosphate (2.5 mM), EGF (0.5 μM), [γ-^{32}P]ATP (20 μM, 5 Ci/mmol), annexin I (500 ng), and the indicated concentration of annexin V in a final reaction volume of 30 μL. Reaction tubes containing all components except [γ-^{32}P]ATP were preincubated for 10 min on ice. The phosphorylation reaction was initiated by the addition of [γ-

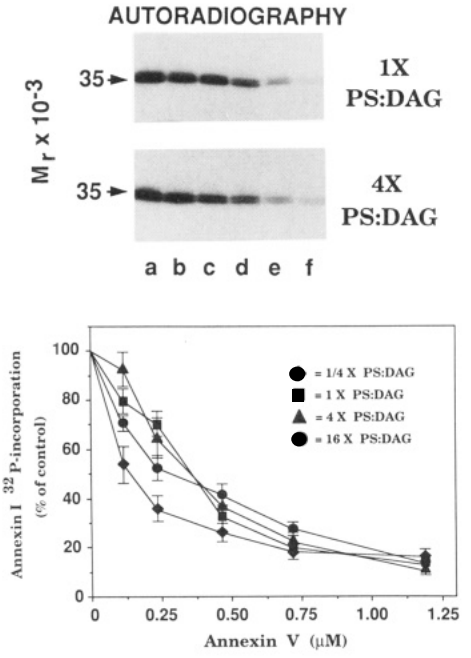

FIGURE 1: Annexin V inhibition of protein kinase C activity as a function of phospholipid concentration. Annexin I (500 ng per reaction) was used as an in vitro substrate for purified rat brain PKC as described under Experimental Procedures. The standard phosphorylation reactions contained 50 μg/mL phosphatidylserine/diacylglycerol vesicles (1X PS:DAG). Other reactions contained either 12.5 μg/mL (1/4X PS:DAG), 200 μg/mL (4X PS:DAG), or 800 μg/mL (16X PS:DAG), respectively. Increasing amounts of purified annexin V protein were added to the incubation mixtures prior to reaction initiation. Upon termination of the reactions, the phosphorylated products were fractionated by SDS–PAGE and visualized by autoradiography (exposure time was 30 min). (Top) Autoradiography of the phosphorylated M_r 35 000 annexin I region of interest for the IX and 4X PS:DAG reactions is shown. Lanes a–f contained 0, 100, 200, 400, 600, and 1000 ng of annexin V added to each phosphorylation reaction, respectively. (Bottom) Phosphorylated annexin I was excised from each gel lane and digested with 90% Protosol (NEN Research Products, Boston, MA), and the incorporated radioactivity was determined by liquid scintillation counting. Background radioactivity, estimated as the amount present in the absence of added substrate (not shown), was subtracted from each experimental point. The average amount of ^{32}P radioactivity incorporated into annexin I is expressed as a percentage of the control reaction (0 ng of annexin V added). The error bars represent the standard deviation of duplicate or triplicate experimental points.

^{32}P]ATP and incubated on ice for 10 min. The reactions were terminated by the addition of 13 μL of a 4-fold-concentrated stock of Laemmli sodium dodecyl sulfate sample buffer (Laemmli, 1970) followed by heating at 100 °C for 2 min. The reactions were analyzed by SDS–PAGE followed by autoradiography as described for the PKC reactions.

Results

Annexin V Inhibits PKC Phosphorylation of Annexin I. Previous studies have shown that annexin I is an excellent in vitro substrate for PKC purified from rat brain (Schlaepfer & Haigler, 1988). Figure 1 shows that annexin V inhibited this phosphorylation reaction under standard conditions (curve labeled 1X PS:DAG) by up to 90% at 1.2 μM with half-maximal inhibition occurring at about 0.4 μM. The concentration of the substrate annexin I in the reaction was 500 nM, approximately 5-fold higher than its apparent K_m. The same inhibition curves were observed using three different preparations of PKC purified by the method of Woodget and Hunter (1987) and in a commercial preparation (Calbiochem, San Diego, CA) purified by the method of Kikkawa et al. (1982) (data not shown).

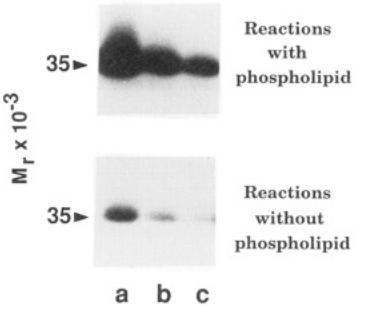

FIGURE 2: Annexin V inhibition of phospholipid-independent protein kinase C phosphorylation of annexin I. Annexin I (500 ng per reaction) was used as an in vitro substrate for purified rat brain PKC as described under Experimental Procedures. The phosphorylation reactions were performed in the presence of 50 μg/mL phosphatidylserine/diacylglycerol vesicles ("Reactions with phospholipid") or in the absence of added vesicles ("Reactions without phospholipid"). Increasing amounts of purified annexin V protein were added to the incubation mixtures prior to reaction initiation. Upon termination of the reactions, the phosphorylated products were fractionated by SDS–PAGE and visualized by autoradiography (exposure time was 3 h). The M_r 35 000 annexin I region of interest of the autoradiogram is shown: lanes a, b, and c contained 0, 500, and 1500 ng of annexin V added to each phosphorylation reaction, respectively.

The phosphorylation reaction used in this study is a complex mixture of Ca^{2+}, phospholipid, substrate, inhibitor, and kinase. Interpretation of the mechanism of annexin V inhibition is complicated because all three proteins bind both Ca^{2+} and phospholipid; annexins do not bind to diacyglycerol. The possibility that annexin V inhibition occurs because it sequesters the Ca^{2+} cofactor of the kinase can be excluded on the grounds that the molar concentration of Ca^{2+} exceeded that of protein by 1000-fold. However, it was not possible to a priori exclude phospholipid sequestration. The molar ratio of phospholipid to annexin V in the standard reaction at half-maximal inhibition was approximately 150:1, and previous studies estimated that under optimal conditions annexin V has a binding surface area of 42–59 phospholipid molecules per protein (Andree et al., 1990; Meers et al., 1991). Since these calculations do not permit a conclusive determination of whether inhibition occurred by sequestering the phosphatidylserine allosteric activator, the following studies were designed to experimentally address this issue.

Annexin V Inhibition of PKC Is Not Due to Phospholipid Sequestration. The dose response of annexin V inhibition of annexin I phosphorylation did not change significantly when the amount of phospholipid was increased 4-fold or 16-fold above the amount in the standard reaction (Figure 1). When the phospholipid in the reaction was reduced 4-fold, the amount of annexin V required for half-maximal inhibition was reduced approximately 2-fold (Figure 1, 1/4X PS:DAG). Taken together, these studies show that the phospholipid concentration can be changed 64-fold with only relatively minor changes in the inhibition curves. If inhibition occurred by sequestration of the phospholipid vesicles, the inhibition curves should have been shifted dramatically by these changes. We also found that the inhibition curves did not change to a detectable extent by varying the order of addition of substrate, inhibitor, or kinase (data not shown).

Furthermore, although the PKC activity in our preparation was greatly stimulated (over 20-fold) by the addition of phospholipid, residual activity could be measured even in the absence of phospholipid (Figure 2). Using densitometeric analysis of the data presented in Figure 2 and other experiments, we estimate that half-maximal inhibition of PKC-dependent phosphorylation of annexin 1 occurred at approxi-

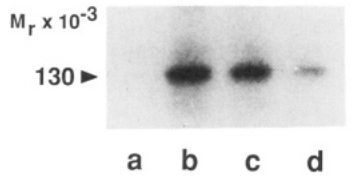

FIGURE 3: Annexin V inhibition of protein kinase C phosphorylation of purified myosin light chain kinase. Purified myosin light chain kinase (MLCK, 2 μg per reaction) was used as an in vitro substrate for purified rat brain PKC in the standard phosphorylation reaction as described under Experimental Procedures. Increasing amounts of purified annexin V protein were added to the incubation mixtures prior to reaction initiation. Upon termination of the reactions, the phosphorylated products were fractionated by SDS–PAGE and visualized by autoradiography (exposure time was 9 h). Only the phosphorylated M_r 130000 MLCK region of interest is shown. Lane a was the control phosphorylation reaction without added PKC or annexin V. Lanes b, c, and d contained 0, 300, and 1000 ng of annexin V added to each phosphorylation reaction, respectively.

mately 0.5 μM in the absence of phospholipid. This value is not significantly different from the value obtained in the presence of phospholipid (Figure 1).

We also tested annexin V as an inhibitor of PKC phosphorylation of myosin light chain kinase, a PKC substrate (Ikebe et al., 1985) that does not bind phospholipid. These experiments were performed using a preparation of myosin light chain kinase that did not retain kinase activity. Annexin V also was a good inhibitor in this reaction with half-maximal inhibition occurring between 0.36 and 1.2 μM annexin V (Figure 3), i.e., at approximately the same concentration required to inhibit phosphorylation of annexin I.

To further test whether inhibition was due to sequestration of phospholipid, we tested des(1–29)annexin I as an inhibitor of PKC-dependent phosphorylation of annexin 1. Des(1–29)annexin I does not contain the PKC phosphorylation sites because it is missing the amino-terminal domain, but it retains the core domain and its associated Ca^{2+} and phospholipid binding activities. These binding activities of des(1–29)annexin I are very similar to those of the native protein and of annexin V (Schlaepfer & Haigler, 1988). Figure 4 shows that des(1–29)annexin I was a very poor inhibitor of PKC-catalyzed phosphorylation of annexin I. Although a small amount of inhibition was observed at the highest concentration of des(1–29)annexin I tested, it is clear it was at least an order of magnitude less potent than annexin V in inhibition of PKC. Since annexin V and the core domain of annexin I have similar phospholipid binding properties, and since only annexin V is a high-affinity inhibitor of PKC, it is likely that inhibition occurs by a specific interaction between PKC and annexin V.

Annexin V Interaction with PKC in Other Reactions. Figure 4 also shows that recombinant annexin V inhibited PKC-dependent phosphorylation of annexin I to the same extent as did placental annexin V. These results indicate that inhibition was due to annexin V and not a minor contaminant of the protein preparation. This conclusion was supported by the observation that inhibitor activity from both sources quantitatively and reversibly bound to phospholipid vesicles in a Ca^{2+}-dependent manner as one would expect for an annexin (data not shown).

In contrast to the above reactions, annexin V (0.25–5.0 μM) did not inhibit phosphorylation of histone III-S in either the presence or the absence of phospholipid in a standard reaction described under Experimental Procedures. Although this reaction was not extensively investigated to determine why inhibition was not observed, it is worth noting a previous report showing that a potent pseudosubstrate inhibitor of PKC was

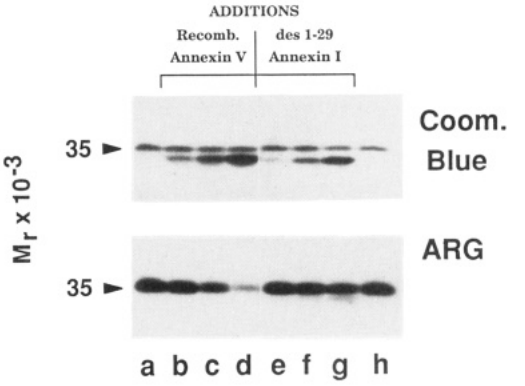

FIGURE 4: Comparison of recombinant annexin V and des(1–29)-annexin I inhibition of protein kinase C activity. Annexin I (500 ng per reaction) was used as an in vitro substrate for rat brain purified PKC in the standard phosphorylation reaction as described under Experimental Procedures. Increasing amounts of recombinant annexin V or des(1–29)annexin I (M_r 32000) were added to the incubation mixtures prior to reaction initiation by the addition of [γ-^{32}P]ATP (2.0 Ci/mmol). Upon termination of the reactions, the phosphorylated products were fractionated by SDS-PAGE, stained with Coomassie Blue (see "Coom. Blue" panel), and visualized by autoradiography (see "ARG" panel, exposure time was 2.5 h). The M_r 35000 region of interest is shown. Lanes a and h were control annexin I phosphorylation reactions. Lanes b, c, and d contained 400, 800, and 1500 ng of added recombinant annexin V protein, respectively. Lanes e, f, and g contain 400, 800, and 1500 ng of added des(1–29)annexin I protein, respectively.

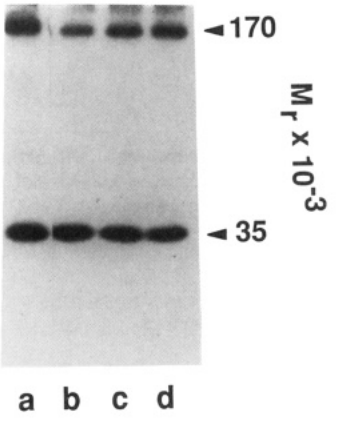

FIGURE 5: A431 membrane phosphorylation of annexin I in the presence of annexin V. Annexin I (500 ng per reaction) was used as an in vitro substrate for the EGF-stimulated tyrosine kinase activity present in the isolated A431 cell membrane fraction as described under Experimental Procedures. Increasing amounts of purified annexin V protein were added to the incubation mixtures prior to reaction initiation. Upon termination of the reactions, the phosphorylated products were fractionated by SDS–PAGE and visualized by autoradiography (exposure time was 4 h). Lane a was the control reaction without added annexin V protein. Lanes b, c, and d contained 500, 1000, and 2000 ng of annexin V added to each phosphorylation reaction, respectively.

100-fold less effective in inhibition of histone III-S phosphorylation (substrate concentration = 0.1 μM) than other substrates (House & Kemp, 1987). In our standard assay, it was not technically feasible to test annexin V at 100-fold higher levels.

We also noted that annexin V did not appear to inhibit autophosphorylation of PKC (data not shown). However, in the reactions used in this study, autophosphorylation occurred to a much lower extent than the phosphorylation of exogenous substrates.

Annexin V Did Not Inhibit Phosphorylation by Other Kinases. Annexin I also is a high-affinity substrate for the membrane-embedded epidermal growth factor receptor/kinase

Table I: Annexin V Did Not Inhibit cAMP-Dependent Protein Kinase[a]

annexin V concn (μM)	kinase act. (cpm incorporated)
0	101 857
0.58	103 550
1.16	108 808
2.32	102 681
3.48	98 493

[a] The activity of cAMP-dependent protein kinase was assayed as described by Scott et al. (1985) using Kemptide as substrate in the presence of the indicated concentration of annexin V. The values for incorporated radioactivity represent the averages of duplicate reactions. Duplicates differed by less than 15%.

in an in vitro reaction that requires Ca^{2+} (Fava & Cohen, 1984; Pepensky & Sinclair, 1986; Haigler et al., 1987). Figure 5 shows that annexin V did not inhibit autophosphorylation either of the receptor or of the added annexin I in this reaction. Likewise, annexin V was without effect in inhibition of phosphorylation of a synthetic peptide substrate by cAMP-dependent protein kinase (Table I).

Discussion

Annexin V was shown to inhibit PKC-catalyzed phosphorylation of annexin I in an in vitro reaction. Inhibition was nearly complete and of high affinity with half-maximal inhibition occurring at approximately 0.4 μM annexin V (Figure 1). Similar annexin V inhibition was observed when myosin light chain kinase was the substrate for PKC (Figure 3). However, annexin V did not inhibit Ca^{2+}-dependent phosphorylation of annexin I by the membrane-embedded EGF receptor/kinase (Figure 5) nor did it inhibit cyclic AMP dependent protein kinase phosphorylation of a synthetic peptide (Table I). The lack of inhibition in the latter two reactions indicates that the inhibition of the PKC reaction was not by an indirect mechanism such as annexin V having ATPase or phosphatase activity. The observation that annexin V did not hydrolyze *p*-nitrophenyl phosphate in a sensitive spectrophotometric assay provides additional evidence that it is not a phosphatase (data not shown).

Since PKC is activated by Ca^{2+}-dependent binding to phosphatidylserine- and diacylglycerol-containing vesicles (Nishizuka, 1988), and since annexin V also binds to these vesicles in a Ca^{2+}-dependent manner (Schlaepfer et al., 1987), it was important to determine whether annexin V inhibited PKC by preventing binding to these activators. Several different lines of evidence indicate that this was not the case. First, the core domain of annexin I which has Ca^{2+} and phospholipid binding properties similar to annexin V was not an effective inhibitor of PKC (Figure 4). Second, the amount of inhibition was not affected by the order of addition of phospholipid, substrate, or PKC. Third, the inhibition of PKC by annexin V was affected to only a small extent by the amount of phospholipid in the reaction (Figures 1 and 2). In the extreme case, the residual activity of PKC observed even in the absence of phospholipid was inhibited by annexin V (Figure 2), and the inhibition curve was indistinguishable from that observed with as high as 800 μg/mL phospholipid in the reaction (Figure 1). It is worth noting that over this range of phospholipid concentrations the annexin V changed from essentially all free in solution to essentially all bound to vesicles. Since the inhibition properties did not change, these results suggests that annexin V was an equally effective inhibitor whether free or bound to phospholipid.

Taken together, the above studies suggest that the mechanism by which annexin V inhibits PKC is by direct interaction between the two proteins. Considering the mechanism by which other proteins inhibit kinases in general (Soderling, 1990), it is reasonable to propose that the inhibitor activity of annexin V resides within a short linear amino acid sequence. Annexins contain a well-defined two-domain structure consisting of a structurally conserved core domain which contains the Ca^{2+} and phospholipid binding sites and small amino-terminal domains that have no sequence similarity except at conserved phosphorylation sites. It will be informative to determine which domain contains the putative PKC inhibitor activity. We are currently focusing our attention on a potential pseudosubstrate site, Arg^{122}-Ala-Ile-Lys^{125}, in the core domain of annexin V. The X-ray crystal structure of annexin V shows that this segment is exposed to solvent and is located on the convex surface of the protein opposite the face that binds Ca^{2+} and, presumedly, phospholipid (Huber et al., 1990). A potential pseudosubstrate sequence is not found at an analogous location in the other annexins.

A few other proteins have been identified that inhibit PKC in vitro and are being considered as endogenous regulators of PKC activity (Schwantke & Le Peuch, 1984; Pribilla et al., 1988; Pearson et al. 1990; Toker et al., 1990). All of these proteins are clearly distinct from annexin V, but one deserves a closer comparison. Toker et al. (1990) obtained a partially purified preparation of proteins from sheep brain that contained three proteins with apparent molecular weights ranging from 29 000 to 33 000 and showed that the preparation inhibited PKC in vitro. Partial sequence analysis of these proteins revealed that they had some sequence similarity with the annexins, particularly annexin II. Additional studies of the structures and function of these proteins are required to determine whether they actually are members of the annexin family. It is noteworthy that their preparation inhibited PKC-catalyzed phosphorylation of histones while annexin V did not in our studies. Other recent studies raised the possibility that annexins may interact directly with PKC and participate in translocation of the activated enzyme to the plasma membrane (Mochly-Rosen et al., 1991a,b).

Since these studies clearly show that annexin V is a high-affinity inhibitor of PKC in vitro, it is of interest to speculate on whether this reflects a physiological function of the protein in intact cells. Annexin V is a major cellular protein (Haigler et al., 1987; Pepinsky et al., 1988), and its concentration in intact cells clearly exceeds the amounts required to achieve PKC inhibition in vitro (Schlaepfer & Haigler, 1990). The nearly ubiquitous nature of the tissue expression of PKC and annexin V makes it impossible to draw clear insights into their relationship, but it is of interest to note that both are present in high concentrations in brain (Pepinsky et al., 1988; Nikkawa & Nishizuka, 1982) and that there is a 5-fold increase in annexin V when PC12 cells are stimulated to differentiate into neurites by NGF additions (Schlaepfer & Haigler, 1990). However, it is not reasonable to propose that the amount of cellular annexin V exerts an acute regulatory role on PKC because annexin V has a relatively long cellular half-life (Schlaepfer & Haigler, 1990). In summary, the proposal that annexin V inhibits PKC in a biologically significant manner in intact cells is a viable hypothesis that deserves further study.

Registry No. Protein kinase C, 9026-43-1.

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