Regulation of annexin I-dependent aggregation of phospholipid vesicles by protein kinase C

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Annexin I is a member of the annexin family of Ca^{2+} - and phospholipid-binding proteins. The ability of this protein to aggregate and to mediate the fusion of various types of vesicles has supported the hypothesis that this protein might be involved in intracellular membrane fusion processes such as exocytosis. Although annexin I has been described as a major *in vitro* substrate of both protein kinase C and the epidermal-growth-factor-receptor protein tyrosine kinase, the functional consequences of these phosphorylation events have not been investigated. In this paper we examine the effect of the phosphorylation of annexin I by protein kinase C on the phospholipid aggregation activity of the protein. The stoichiometry of phosphorylation of the protein was affected by the method of preparation of the phospholipid. Under optimal assay conditions protein kinase C catalysed the incorporation of

2.83 ± 0.23 mol of phosphate/mol of annexin I (mean $\pm$ S.E.M., $n = 21$). Studies with the Ca^{2+} - and phospholipid-independent form of protein kinase C suggested that the phosphorylation of annexin I was stimulated by phospholipid in the absence of Ca^{2+} , although maximal phosphorylation was achieved in the presence of both phospholipid and Ca^{2+} . Phosphorylation of annexin I resulted in a dramatic decrease in the rate and extent of phospholipid vesicle aggregation, without significantly disrupting the binding of the protein to the phospholipid vesicles. The phosphorylation of annexin I increased the EC_{50} (Ca^{2+}) of phospholipid vesicle aggregation from $19 \pm 10 \mu\text{M}$ (mean $\pm$ S.D., $n = 7$) for the native protein to $290 \pm 95 \mu\text{M}$ (mean $\pm$ S.D., $n = 5$) for the phosphorylated protein. These results suggest that protein kinase C may act to inhibit the phospholipid vesicle aggregation activity of annexin I.

INTRODUCTION

Annexin I (A-I; also termed lipocortin I and calpactin II) is member of the annexin family of Ca^{2+} -binding proteins, which, in addition to extensive sequence homology, share the ability to bind to phospholipids in the presence of Ca^{2+} (reviewed in Dedman, 1986; Russo-Marie, 1986; Glenney, 1987; Khanna et al., 1988; Klee, 1988; Schlaepfer and Haigler, 1988; Burgoyne, 1990; Geisow et al., 1990; Haigler and Schlaepfer, 1990; Johnsson et al., 1990; Moss and Crumpton, 1990). A-I was first described as the substrate of epidermal-growth-factor (EGF) receptor/kinase (Fava and Cohen, 1984) and was purified from rat peritoneal lavages as an inhibitor of phospholipase A_2 , and subsequently cloned (Wallner et al., 1986). Limited proteolysis of A-I revealed a two-domain structure for the protein, with a short N-terminal sequence that contains the sites for tyrosine and serine/threonine phosphorylation, and the core or C-terminal domain that contains a 4-fold repeat of a characteristic sequence of 17 amino acids (termed the annexin fold). The C-terminal also contains the Ca^{2+} - and phospholipid-binding region of the protein (Schlaepfer and Haigler, 1987; Ando et al., 1989).

At the subcellular level, A-I has been shown to be present in the cytoplasm and at the cytoplasmic surface of the plasma membrane (Glenney et al., 1987). A-I has also been demonstrated to be present at the cytoplasmic side of the plasma membrane of pituitary cells (Turgeon et al., 1991) and on the matrix vesicles of calcifying tissues (Genge et al., 1989). In detergent-extracted fibroblasts, about 20% of the A-I present has been shown to localize to the cytoskeleton (Zokas and Glenney, 1987).

A-I is a target for several kinases *in vitro* and *in vivo*. Treatment of adrenal chromaffin cells with nicotine has been demonstrated to induce the phosphorylation of A-I at serine (Michener et al.,

1986). Phorbol esters have also been found to stimulate A-I phosphorylation in these cells, presumably by the activation of protein kinase C. Phorbol ester treatment of rat liver cells has also been shown to result in the phosphorylation of membrane-bound A-I (Campos-Gonzalez et al., 1989). In contrast, phorbol ester treatment of A431 cells has been reported not to stimulate the phosphorylation of A-I (William et al., 1989). *In vitro*, A-I has been shown to be phosphorylated by the EGF-receptor tyrosine kinase, protein kinase C, the insulin-receptor tyrosine kinase, recombinant pp60^{src} and recombinant pp50^{abl} (Hirata, 1981; Fava and Cohen, 1984; Michener et al., 1986; Pepinsky and Sinclair, 1986; Karasik et al., 1988; Schlaepfer and Haigler, 1988; Varticovski et al., 1988). The site of tyrosine phosphorylation by the EGF receptor/kinase and by recombinant kinases has been mapped to tyrosine-21 in the N-terminal domain (Haigler et al., 1987; Varticovski et al., 1988). Protein kinase A has been shown to phosphorylate porcine A-I at threonine-216 and at threonine-233 (Pepinsky and Sinclair, 1986; Varticovski et al., 1988). Human A-I has been shown to be phosphorylated by protein kinase C at three sites: serine-27, serine-28 and threonine-24 (Schlaepfer and Haigler, 1988), or at serine-27 and at threonine-41 (Varticovski et al., 1988).

Several lines of evidence have suggested a role for the N-terminus in the regulation of Ca^{2+} binding by the protein. Modification of A-I by phosphorylation with the EGF-receptor kinase (Schlaepfer and Haigler, 1987; Ando et al., 1989), or the removal of the N-terminus by proteolysis (Ando et al., 1989) influence the Ca^{2+} -dependent lipid-binding properties. Proteolysis of the N-terminal domain of A-I decreased the EC_{50} (Ca^{2+}) of binding to phospholipid vesicles from $30 \mu\text{M}$ to $5 \mu\text{M}$ (Ando et al., 1989). Phosphorylation of A-I by the EGF-receptor kinase (on tyrosine residues) reduced by 5-fold the EC_{50} (Ca^{2+}) of

phospholipid-vesicle binding from 20 μM to 4 μM (Schlaepfer and Haigler, 1987). Furthermore, the half-maximal binding of A-I to lipid-coated glass-bead columns was reduced from 30 μM Ca^{2+} to 15 μM Ca^{2+} by the phosphorylation of A-I by the EGF-receptor kinase (Ando et al., 1989).

The physiological function of A-I has not been determined, but the ability of the protein to promote Ca^{2+} -dependent aggregation of phospholipid vesicles (Blackwood and Ernst, 1990) and of chromaffin granules (Drust and Creutz, 1988) *in vitro* has suggested a possible role for the protein in membrane-trafficking events, such as exocytosis.

In previous studies of the *in vitro* phosphorylation of annexins by protein kinase C, we reported a stoichiometry of almost 1 mol of phosphate per mol of A-I and of 0.4 mol of phosphate per mol of the annexin II tetramer (A-II_t; Khanna et al., 1986, 1987). Recently, we examined the effect of protein-kinase-C-dependent phosphorylation of A-II_t on the liposomal-aggregation activity of the protein (Johnstone et al., 1992). It was observed that the stoichiometric phosphorylation of A-II_t resulted in the inhibition of the liposomal-aggregation activity of the protein.

In this paper we report that the phosphorylation of A-I by protein kinase C is stimulated by the presence of phospholipid, independently from the requirement of protein kinase C for phospholipid. Phosphorylation of A-I by protein kinase C was found to increase the Ca^{2+} that is required to promote A-I-mediated lipid-vesicle aggregation. A preliminary report of these data has been published previously in abstract form (Johnstone and Waisman, 1992).

MATERIALS AND METHODS

Preparation of lipid vesicles and micelles

Lipid vesicles were prepared fresh, on the day that they were used. Phosphatidylserine (PS; 400 mg) and 1,2-diolein (DG; 40 mg) in 200 μl of chloroform were diluted with 4 vol. of methanol and then the solvents were evaporated under a stream of nitrogen (Reeves and Dowbin, 1969). The lipids were then sonicated (2×5 s bursts at 70 W with a Braun-probe sonicator) in 1 ml of 25 mM Tris/HCl (pH 7.5). Micelles were prepared using the method of Hannun et al. (1986), which consists of drying under nitrogen 2 mg of PS and 0.5 mg of DG and then sonicating the lipid in 1 ml of 0.3% Triton X-100.

Protein kinase C activity assay

During the purification of rat-brain protein kinase C, the activity of the enzyme was assayed in a final volume of 100 μl (unless otherwise stated) in 25 mM Tris/HCl (pH 7.5), 10 mM MgCl_2 , 0.5 mM EGTA, 0.9 mM CaCl_2 , 10 μl of micelles and 100 $\mu\text{g}/\text{ml}$ histone H1S. The phosphorylation reaction was initiated by the addition of 25 μM [γ - ^{32}P]ATP (200–2000 c.p.m./pmol). The reaction was incubated for 4 min at 30 °C.

Phosphorylation of A-I

The conditions for the optimal phosphorylation of A-I were determined in a series of experiments in which the concentrations of A-I, Ca^{2+} and phospholipid were systematically varied. A-I was phosphorylated in a final volume of 100 μl in 25 mM Tris/HCl (pH 7.5), 10 mM MgCl_2 , 0.5 mM EGTA and 0.9 mM CaCl_2 in the presence of 1.0 $\mu\text{g}/\text{ml}$ protein kinase C and 100 $\mu\text{l}/\text{ml}$ lipid vesicles (unless otherwise stated, prepared using the method of Reeves and Dowbin, 1969, and containing 400 $\mu\text{g}/\text{ml}$ PS and 40 $\mu\text{g}/\text{ml}$ DG) and at a concentration of 35 $\mu\text{g}/\text{ml}$ (0.946 μM)

A-I. In all assays, phosphorylation was initiated by the addition of 25 μM [γ - ^{32}P]ATP (200–2000 c.p.m./pmol). The reaction was incubated at 30 °C for 30 min (unless otherwise stated). To measure phosphate incorporation, an aliquot was removed from the reaction mixture and precipitated in 25% (w/v) trichloroacetic acid containing 2% (w/v) sodium pyrophosphate in filter-paper funnels. The precipitate was washed three times with 2 ml of 5% (w/v) trichloroacetic acid containing 0.2% sodium pyrophosphate. Radioactivity was determined by liquid-scintillation counting. To obtain samples for SDS/PAGE, the reaction was terminated by the addition of concentrated SDS-sample buffer [0.25 M Tris/HCl, pH 6.8, 10% (w/v) SDS, 20% (v/v) glycerol, 2 mM EGTA, 2 mM EDTA and 20 mM 2-mercaptoethanol] and then incubated in a boiling-water bath for 2 min.

Phosphoamino acid analysis

Phosphorylated annexin was diluted in SDS-sample buffer, and run on SDS/11.5% PAGE. Protein bands were visualized by Coomassie Blue staining. After destaining, the protein bands were excised from the gel, washed three times in 20% (v/v) methanol to remove SDS, and then dried by lyophilization. Protein in the gel slices was digested by heating at 110 °C in 6 M HCl for 2 h. The digested protein samples were dried under nitrogen, resuspended in 50 mM ammonium bicarbonate, pH 8.0, and then lyophilized. Phosphoamino acids were resuspended in electrophoresis buffer (buffer A: pyridine/acetic acid/water, 33:1:40, by vol.) containing 1 mM each of *O*-phosphoserine, *O*-phosphothreonine and *O*-phosphotyrosine. Samples were spotted on thin-layer cellulose plates (Merck), and the plates were run in buffer A, pH 3.5, cathode to anode, at constant voltage (1000 V) for 30 min, using Phenol Red as a marker. Reference amino acids were stained with ninhydrin (0.2% in acetone) and then incubated at 100 °C for 5 min. Labelled phosphoamino acids were identified by autoradiography of the cellulose plates.

Peptide mapping

Tryptic-peptide mapping of phosphorylated A-I was performed using the methods of Sharma and Wang (1986). Phosphorylated A-I (10 μg) was analysed by SDS/PAGE and protein bands were excised from the gel. Gel pieces containing A-I were washed three times in 50% (v/v) methanol, lyophilized and then rehydrated in 1.0 ml of 50 mM ammonium bicarbonate, pH 8.0. The gel was digested for 24 h at 37 °C with a total of 30 μg of 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated trypsin. Trypsin was added in three equal aliquots, at time zero, after 4 h and after 16 h of incubation. The peptide fragments were then lyophilized and suspended in buffer A, centrifuged (16250 *g* for 2 min) and dried with a gentle stream of nitrogen. The peptides were then resuspended in 20 μl of buffer A, and 2 μl of sample was spotted onto thin-layer cellulose plates (Merck). Electrophoresis in buffer A was carried out at 500 V for 1.5 h. Ascending chromatography in the second dimension was carried out for 4.5 h in butanol/pyridine/acetic acid/water (50:33:1:40, by vol.).

Lipid-vesicle aggregation with phosphorylated A-I

Phosphorylated A-I was recovered from the protein kinase C reaction by the dissociation of lipid-vesicle-associated A-I with high concentrations of EGTA. A-I was phosphorylated in a final volume of 2 ml, in 25 mM Tris/HCl (pH 7.5), 10 mM MgCl_2 , 0.5 mM EGTA and 0.9 mM CaCl_2 , in the presence of 1.0 $\mu\text{g}/\text{ml}$

of protein kinase C and 100 μl /ml of lipid vesicles (400 μg /ml PS and 40 μg /ml DG) at a concentration of 35 μg /ml (0.946 μM) A-I, as discussed above. A sample was removed for scintillation counting and SDS/PAGE, and then the reaction mixture was centrifuged in an Eppendorf Microfuge to pellet the lipid vesicles. The lipid vesicles were resuspended in 0.1 ml of 25 mM Tris/HCl (pH 7.5) and 12.5 mM EGTA, sonicated in a bath sonicator for 20 s, and then incubated at room temperature for 10 min. The mixture was then centrifuged (100000 g) and A-I was recovered in the supernatant. The stoichiometry of phosphorylation of A-I was determined by scintillation counting. The phosphorylated A-I (typically about 300 μg /ml) was used in the lipid-vesicle aggregation assay. A-I for the control reactions was incubated in the phosphorylation-reaction system in the absence of ATP. Recovery of A-I was monitored by SDS/PAGE and the Bradford protein assay (Bradford, 1976). Recovery of A-I, as estimated by the recovery of trichloroacetic-acid-precipitated [^{32}P]P_i, was variable, $63 \pm 21\%$ (mean $\pm$ S.D., $n = 10$). Lipid-vesicle aggregation induced by control samples of A-I was indistinguishable from the aggregation induced by the native protein.

The lipid-vesicle aggregation reaction was carried out in a final volume of 1 ml and at a final concentration of A-I of 2 μg /ml and in 25 mM Tris/HCl (pH 7.5), 1 mM MgCl_2 , 1.5 mM EGTA and 0.025 ml of lipid vesicles (400 μg /ml PS and 40 μg /ml DG). Phosphorylated or control A-I that had been extracted into 100 μl of 25 mM Tris/HCl (pH 7.5) and 12.5 mM EGTA, as discussed above, was adjusted to a final concentration of 26.3 mM Tris/HCl (pH 7.5), 1.05 mM MgCl_2 , 1.579 mM EGTA, 10.5 μg /ml PS and 1.05 μg /ml DG, in a final volume of 9.5 ml. The reaction mixture was divided into 0.950 ml aliquots and aggregation was initiated by the addition of CaCl_2 (50 μl) to the required concentration, and monitored by the increase in absorbance at 540 nm at 5 min intervals for a total period of 30 min. The Ca^{2+} concentration of CaCl_2 stock solutions was determined by atomic absorption. The final Ca^{2+} concentrations were estimated by the method of Fabiato and Fabiato (1979) and verified by Ca^{2+} electrode and fura-2 measurement. To measure the Ca^{2+} -dependence of binding of A-I to the phospholipid vesicles, aliquots of the aggregation reaction were removed and pelleted by centrifugation at 100000 g for 15 min and the binding of A-I to the lipid vesicles was assessed by SDS/PAGE of the pellet.

Miscellaneous techniques

Bovine lung obtained from a local slaughterhouse was used to purify A-I (Khanna et al., 1990) and the concentration was determined from the previously determined A_{280} of 0.49 for a solution of A-I of concentration 1 mg/ml (Schlaepfer and Haigler, 1987). A-I was stored at -80°C . Protein kinase C was purified from rat brain (Pel Freeze) using the method of Wooten et al. (1987), except that a threonine-Sepharose column (Kitano et al., 1986) was substituted for the protamine-agarose column. Protein kinase M was obtained after prolonged storage of protein kinase C, such that proteolytic fragmentation of the kinase occurred. Protein kinase C was stored at -80°C in 5% (v/v) glycerol and 0.5% Triton X-100. SDS/PAGE was done using the method of Laemmli (1970). For autoradiography, the gels were exposed to Kodak X-Omat AR film with an intensifying screen at -80°C for 1 h.

Materials

PS and DG were obtained from Serdary; histone H1S, *O*-phosphoserine, *O*-phosphothreonine and *O*-phosphotyrosine

were obtained from Sigma. [γ - ^{32}P]ATP was from Amersham. SDS, Tris and EGTA were purchased from Boehringer Mannheim. Triton X-100 was purchased from Fluka. Acrylamide, bisacrylamide and *NNN'*-tetramethylethylenediamine were obtained from BioRad. All other chemicals were purchased from CanLab.

RESULTS AND DISCUSSION

Stoichiometry of phosphorylation of A-I by protein kinase C

As a first step towards an understanding of the potential role of phosphorylation in the regulation of A-I function, we tested the effects of various assay components on the stoichiometry of phosphorylation of A-I by rat-brain protein kinase C, to define the experimental conditions that would optimize the rate and stoichiometry of the phosphorylation of A-I. Hannun et al. (1986) designed an assay system for protein kinase C using mixed micelles to satisfy the phospholipid requirement of protein kinase C. The micelles were generated by sonicating PS and DG (in a ratio of 4:1) in the presence of 0.3% Triton X-100 (Table 1). However, the stoichiometry of phosphorylation of A-I by protein kinase C using these phospholipid micelles was low (0.49 ± 0.16 mol of phosphate per mol of A-I; mean $\pm$ S.E.M., $n = 6$). Furthermore, the stoichiometry of phosphorylation was not increased by increasing the concentration either of micelles or of protein kinase C (results not shown). We therefore tested phospholipid vesicles prepared by two other methods. Firstly, lipid vesicles were prepared in the absence of Triton X-100, by drying chloroform-solubilized PS and DG and directly sonicating the dried lipids into 25 mM Tris (pH 7.5). Secondly, the lipid vesicles were prepared using the method of Reeves and Dowbin (1969), which involved the dilution of identical concentrations of chloroform-solubilized PS and DG as in the first method, except that the phospholipid was diluted in 4 vol. of methanol before drying under nitrogen. The second method promotes the formation of a lipid emulsion, which results in a more even drying of the lipid. Subsequently these dried lipids can be more easily rehydrated by the addition of the 25 mM Tris (pH 7.5) and are therefore more uniformly resuspended by sonication (Reeves and Dowbin, 1969). We found that the Reeves and Dowbin (1969) procedure generated phospholipid vesicles that supported a higher stoichiometry of phosphorylation (2.83 ± 0.23 mol of phosphate per mol of A-I; mean $\pm$ S.E.M., $n = 21$) than vesicles prepared by the sonication of lipid that had been dried in

Table 1 Effect of various phospholipid preparations on A-I phosphorylation

The phosphorylation reaction used 25 mM Tris/HCl (pH 7.5), 10 mM MgCl_2 , 0.5 mM EGTA and 0.9 mM CaCl_2 , in the presence of 1.0 μg /ml of protein kinase C and 200 μl /ml of lipid vesicles, until steady-state incorporation of phosphate was achieved, as detailed in the Materials and methods section. The ratios of phospholipid in the various mixtures are expressed as relative concentration ratios. Abbreviations used: C, choline; PC, phosphatidylcholine.

Preparation (ratio, by vol.)	Stoichiometry (mean $\pm$ S.D.)
PS/DG (4:1) in 0.3% Triton X-100	0.49 ± 0.2 ($n = 6$)
PS/DG (10:1)	1.31 ± 0.13 ($n = 34$)
PS/DG (40:1)	0.9 ± 0.06 ($n = 4$)
PS/PC/DG (10:10:1)	0.9 ± 0.3 ($n = 4$)
PS/PC/DG (6:3:1)	0.9 ± 0.3 ($n = 4$)
PS/PC/DG (3:6:1)	0.85 ± 0.5 ($n = 4$)
PS/PC/C/DG (10:10:10:1)	0.7 ± 0.5 ($n = 3$)
PS/DG* (10:1)	2.83 ± 0.23 ($n = 21$)

* Prepared using the method of Reeves and Dowbin (1969).

Table 2 Phosphorylation of A-I by protein kinase M

The phosphorylation reaction was allowed to occur until steady-state incorporation of phosphate into A-I was achieved, as detailed in the Materials and methods section. The Ca^{2+} concentration was 0.250 mM and the phospholipid vesicles were added at a volume of 100 $\mu\text{l}/\text{ml}$ (prepared according to Reeves and Dowbin, 1969, at an initial concentration of 400 $\mu\text{g}/\text{ml}$ PS and 40 $\mu\text{g}/\text{ml}$ DG). In the absence of additions, the assay contained 25 mM Tris/HCl (pH 7.5), 10 mM MgCl_2 and 0.5 mM EGTA. Results are expressed as the mean $\pm$ S.D., and are representative of the data from two protein kinase M preparations performed in duplicate. The maximum phosphorylation of A-I, expressed as 100%, was 2.2 mol of phosphate per mol of A-I.

Addition	Phosphorylation (% of maximum)	
	A-I	Histone H1S
None	0	45 $\pm$ 7
Phospholipid	68 $\pm$ 10	73 $\pm$ 10
Ca^{2+} , phospholipid	100	100

chloroform directly into buffer (1.31 ± 0.13 mol of phosphate per mol of A-I; mean $\pm$ S.E.M., $n = 34$) (Table 1). In contrast, the protein kinase C activity, measured with histone as a substrate, varied by only 10% among the three different phospholipid preparations. These results suggest that the method of preparation of the phospholipid is an important determinant of the stoichiometry of phosphorylation of A-I.

We also examined the effect of varying the composition of the phospholipid preparations on their ability to support phosphorylation of A-I. As shown in Table 1, regardless of the phospholipid composition, the direct sonication of phospholipid into buffer results in a phospholipid preparation that supports a stoichiometry of phosphorylation of about 1 mol of phosphate per mol of A-I. Accordingly, all subsequent phosphorylation reactions used phospholipid vesicles that had been prepared by the Reeves and Dowbin (1969) procedure.

The observation that the method of preparation of the phospholipid could influence the stoichiometry of phosphorylation of A-I by protein kinase C suggested that the interaction of phospholipid with A-I might be an important factor in controlling the extent of phosphorylation of A-I by protein kinase C. We therefore tested this possibility by isolating protein kinase M, the Ca^{2+} - and phospholipid-independent form of protein kinase C, and examining the phospholipid requirement of the phosphorylation reaction. As shown in Table 2, the phosphorylation of A-I by protein kinase M was inhibited in the absence of both Ca^{2+} and phospholipid vesicles. Although the extent of phosphorylation of A-I was stimulated slightly by the presence of both phospholipid vesicles and Ca^{2+} , it was interesting that, in the absence of Ca^{2+} but in the presence of phospholipid vesicles, the extent of phosphorylation of A-I was about 68% of maximal.

Figure 1 shows the time-course of phosphorylation of A-I by protein kinase C under optimal conditions. In the presence of Ca^{2+} and phospholipid, A-I was phosphorylated to a ratio of 2.9 ± 0.9 mol of phosphate per mol of A-I (mean $\pm$ S.D., $n = 21$). The phosphorylation of A-I by protein kinase C was rapid, with half-maximal incorporation occurring in less than 5 min. Human A-I has previously been shown to be an *in vitro* substrate of protein kinase C, but the stoichiometry was reported to be 0.85 mol of phosphate per mol of protein (Schlaepfer and Haigler, 1988). Previous work from our laboratory suggested the in-

Figure 1 Phosphorylation of A-I by protein kinase C

A-I (4.6 μg) was phosphorylated by protein kinase C in a reaction mixture containing 25 mM Tris/HCl (pH 7.5), 10 mM MgCl_2 , 0.5 mM EGTA, 0.9 mM CaCl_2 , 10 μl of lipid vesicles (400 $\mu\text{g}/\text{ml}$ PS and 40 $\mu\text{g}/\text{ml}$ DG, prepared using the methods of Reeves and Dowbin, 1969) and 0.1 μg of protein kinase C at 30 $^{\circ}\text{C}$, in a final volume of 100 μl . The reaction was initiated by the addition of 25 μM ATP (440 c.p.m./pmol [γ - ^{32}P]ATP). Aliquots were removed at various time intervals and the incorporation of phosphate into A-I was determined by liquid-scintillation counting. Aliquots were also analysed by SDS/PAGE (a) and autoradiography (b). Phosphorylation of A-I (band a) generates phosphoforms of the protein with reduced mobility on SDS/PAGE (bands b and c).

corporation of 1 mol of phosphate per mol of A-I (Khanna et al., 1986, 1987). Since the phospholipid vesicles used in those studies were prepared by direct sonication of phospholipid into buffer, those results would be consistent with our current findings that the method of preparation of the phospholipid vesicles affects the stoichiometry of phosphorylation (Table 1).

When aliquots were removed at various times during the phosphorylation of A-I and analysed by SDS/PAGE, it was observed that multiple Coomassie-Blue-stained bands were generated. As shown in Fig. 1(a), two new bands (referred to as band b and band c) with reduced mobility on SDS/PAGE were generated compared with the native A-I (band a). When the phosphorylation reaction was complete, the Coomassie-Blue-stained A-I was about equally distributed between band a and band b, with only a small amount of stained protein found in band c (Figure 1a). Analysis of the Coomassie-Blue-stained bands by autoradiography suggested that all three Coomassie-Blue-stained bands, resolved by SDS/PAGE, were phosphorylated by protein kinase C (Figure 1b). A time-dependent pattern in the appearance of phosphate in these bands was observed. After the reaction had progressed for about 1 min, about 0.8 mol of phosphate per mol of A-I had been incorporated, the only band to be phosphorylated was band a (Figure 1b), which corresponded to the major Coomassie-Blue-stained band (Figure 1a) and so the native protein was phosphorylated but did not shift in mobility on SDS/PAGE. In contrast, after the phosphorylation reaction had proceeded for about 10 min and about 2 mol of phosphate per mol of A-I had been incorporated, two additional Coomassie-Blue-stained bands appeared (Figure 1a, bands b and c) and autoradiographic analysis suggested that all three Coomassie-Blue-stained bands contained phosphate (Figure 1b).

The apparent resolution of three Coomassie-Blue-staining and phosphate-labelled forms of A-I by SDS/PAGE (Figure 1)

Figure 2 Tryptic phosphopeptide maps of A-I

A-I was phosphorylated by protein kinase C and resolved into three phosphoforms by SDS/PAGE. Each band was excised from the gel and subjected to tryptic cleavage. Phosphopeptides were resolved on thin-layer cellulose plates by electrophoresis (500 V for 1.5 h) at pH 3.5 in the first dimension, followed by chromatography in the second dimension. The origin is indicated by ○. Electrophoresis was carried out in the horizontal dimension as indicated. (a) Phosphopeptide map of band a; (b) phosphopeptide map of band b; (c) phosphopeptide map of band c. The figure is representative of three separate tryptic digests.

suggested that each of the three phosphoforms of A-I contained a distinct pattern of phosphate incorporation into the phosphorylation sites of the protein. However, the protein bands resolved by SDS/PAGE could also have been artifactual. To distinguish between these two possibilities, A-I was phosphorylated by protein kinase C, resolved by SDS/PAGE and the Coomassie-Blue-stained bands removed and subjected to peptide mapping. The phosphopeptide maps are shown in Figure 2. The phosphopeptide maps that were generated from the three Coomassie-Blue-stained bands were distinct from each other (Figure 2), suggesting that the phosphorylation of A-I produced three distinct phosphoforms of the protein.

A striking difference between the phosphopeptide map of band a (Figure 2a) and band b (Figure 2b) is the appearance of an enhanced phosphopeptide at position 8 (Figure 2b) of the phosphopeptide map of band b. Furthermore, the phosphopeptide map of band c (Figure 2c) contains two enhanced phosphopeptides compared with band a (Figure 2a), one at position 8 and one at position 2. Therefore, the most significant difference between the phosphopeptide maps of band b and band c is the enhancement of the phosphopeptide at position 2. It is also interesting that the phosphopeptide maps of bands b and c have phosphopeptides of diminished intensity at positions 6, 10 and 11 compared with the phosphopeptide map of band a. Since we have been unable to observe any contaminating phosphatases in the A-I or in the protein kinase C preparations, it is unlikely

Figure 3 Phosphoamino acid analysis of phosphorylated A-I

(a) A-I was phosphorylated by protein kinase C (see the Materials and methods section) and aliquots were removed at the times indicated and incubated with SDS-sample buffer. The samples were resolved by SDS/PAGE and the A-I phosphoforms were excised and subjected to partial acid hydrolysis. The phosphoamino acids were separated by high-voltage electrophoresis on thin-layer plates. Phosphotyrosine (Y), phosphoserine (S) and phosphothreonine (T) were added as internal standards and detected with ninhydrin. The autoradiograph of ^{32}P -labelled amino acids was produced by overnight exposure at -80°C . A-I was phosphorylated for 30 min and then resolved by SDS/PAGE. (b) Coomassie-Blue-stained bands, corresponding with the phosphoforms of A-I bands a, b and c, were excised and the protein was digested by acid hydrolysis. The samples were separated by high-voltage electrophoresis on thin-layer plates. Phosphotyrosine (Y), phosphoserine (S) and phosphothreonine (T) were added as internal standards and detected with ninhydrin. The autoradiograph of the ^{32}P -labelled amino acids was produced by overnight exposure.

that the diminished intensity of those phosphopeptides is due to dephosphorylation. It would appear more likely that the proteolytic sites of the phosphoforms of A-I may not be uniformly accessible to trypsin.

The presence of multiple phosphorylation sites in A-I has been suggested by other laboratories. Varticovski et al. (1988) proposed that serine-27 and threonine-41 of porcine A-I were phosphorylated by protein kinase C. Schlaepfer and Haigler (1988) suggested that human A-I was phosphorylated on serine-27, serine-28 and threonine-24. Unfortunately, since there is no published sequence for bovine A-I, prospective phosphorylation sites in bovine A-I cannot be compared with those of the A-I sequence. Since phosphorylated A-I has been reported to contain both phosphoserine and phosphothreonine, we analysed the phosphoamino acid content of A-I during the phosphorylation reaction. As shown in Figure 3(a), at the earliest time in the phosphorylation reaction, the predominant phosphoamino acid is phosphothreonine. As the phosphorylation reaction approached completion, both phosphoserine and phosphothreonine were observed. A-I was phosphorylated by protein kinase C, resolved into three phosphoforms on SDS/PAGE, and each phosphoform removed from the gel and subjected to phosphoamino acid analysis (Figure 3b). The

Figure 4 A-I-dependent vesicle aggregation as a function of Ca^{2+} concentration

Phosphorylated (filled symbols) or native annexin I (open symbols) at a concentration of $2 \mu\text{g/ml}$, and in a final volume of 1.0 ml , were each combined with $25 \mu\text{l}$ of lipid vesicles and 25 mM Tris/HCl (pH 7.5), 2 mM MgCl_2 and 0.5 mM EGTA. Aggregation was initiated by the addition of Ca^{2+} . Vesicle aggregation was monitored spectrophotometrically by the increase in light scattering at 540 nm . The final Ca^{2+} concentrations were determined using the method of Fabiato and Fabiato (1979), and were 0.115 mM (squares) and 0.50 mM (circles).

phosphoform of A-I corresponding in mobility with the native A-I (band a) contains predominantly phosphothreonine. This result is consistent with our observation that band a is the first phosphoform of A-I to be phosphorylated (Figure 1b), and that the first phosphoamino acid to appear during the phosphorylation of A-I is phosphothreonine (Figure 3a). In contrast, the phosphoform with reduced mobility on SDS/PAGE, band b, (Figure 1b) contains predominantly phosphoserine (Figure 3b) and this phosphoform appears later in the phosphorylation reaction, as does phosphoserine (Figure 3a). Similarly, the third phosphoform of A-I, band c (Figure 3b), contains predominantly phosphoserine. These results therefore confirm that different phosphoforms of A-I are produced upon phosphorylation by protein kinase C. The physiological significance of this multisite phosphorylation is unclear.

Regulation of A-I function by phosphorylation

Although A-I has been shown to be a substrate of protein kinase C both *in vitro* and *in vivo*, the functional consequences of the phosphorylation event are not known. Since A-I has been shown to bind to and aggregate phospholipid vesicles, we investigated the effect of the phosphorylation of A-I on the ability of the protein to aggregate phospholipid vesicles. A-I was phosphorylated by protein kinase C in the presence or in the absence of ATP. The phosphorylated or the control protein was isolated (see the Materials and methods section) and the phospholipid-aggregation activity of the protein examined. Figure 4 presents a time-course of the aggregation of phospholipid vesicles by phosphorylated and by native A-I. When the aggregation reaction was initiated with 0.115 mM Ca^{2+} , the rate and the extent of phospholipid-vesicle aggregation by the phosphorylated protein was attenuated compared with that induced by the native protein. In contrast, when the phospholipid aggregation reaction was initiated with 0.5 mM Ca^{2+} , the rate and the extent of phospholipid aggregation by the phosphorylated and by the native proteins were similar. This result suggested that the phosphorylation of A-I by protein

Figure 5 Ca^{2+} -dependent aggregation of lipid vesicles with phosphorylated A-I

Native (open symbols) A-I or A-I phosphorylated by protein kinase C (filled symbols; $2 \mu\text{g}$) was incubated with $25 \mu\text{l}$ of lipid vesicles ($400 \mu\text{g/ml}$ PS and $40 \mu\text{g/ml}$ DG) in 25 mM Tris/HCl (pH 7.5), 2 mM MgCl_2 and 0.5 mM EGTA, in a final volume of 1.0 ml . Phospholipid-vesicle aggregation was initiated by the addition of Ca^{2+} and monitored spectrophotometrically at 540 nm after 30 min . The final Ca^{2+} concentration was determined by the method of Fabiato and Fabiato (1979). To measure lipid binding by A-I, the lipid vesicles were centrifuged after the aggregation reaction was complete, and the lipid-associated protein was analysed by SDS/PAGE. The inset shows native protein (a) and phosphorylated protein (b) that co-sedimented with the vesicles at Ca^{2+} concentrations of 47 , 97 and $188 \mu\text{M}$. In experiments in which A-I was incubated in the absence of lipid vesicles, aggregation of native or phosphorylated A-I was not observed at Ca^{2+} concentrations from $1 \mu\text{M}$ to 1 mM .

kinase C regulated the phospholipid-aggregation activity of the protein by increasing the Ca^{2+} requirement of the phospholipid-vesicle aggregation activity of the protein.

To test the possibility that phosphorylation of A-I by protein kinase C increased the Ca^{2+} requirement for the phospholipid-vesicle-aggregation activity of the protein directly, we examined the Ca^{2+} dependency of the phospholipid-vesicle-aggregation activity of the phosphorylated and of the native protein (Figure 5). The Ca^{2+} concentration required to induce half-maximal aggregation was $19 \pm 10 \mu\text{M}$ (mean $\pm$ S.D., $n = 7$) for the native protein, and $290 \pm 95 \mu\text{M}$ (mean $\pm$ S.D., $n = 5$) for the phosphorylated protein. In contrast, the incubation of native or of phosphorylated protein in the absence of phospholipid vesicles or the incubation of phospholipid vesicles in the absence of A-I did not produce significant increases in turbidity (results not shown). The higher Ca^{2+} requirement of phosphorylated A-I suggested that the phosphorylated protein might be unable to bind to the phospholipid vesicles at Ca^{2+} concentrations that support the binding of the native protein to the vesicles. However, as shown in Figures 5(a) and 5(b), the binding of the phosphorylated protein to the phospholipid vesicles occurs at Ca^{2+} concentrations at which vesicle aggregation is absent. This suggests that the primary effect of phosphorylation of A-I by protein kinase C is to inhibit the phospholipid-aggregation activity of the protein.

Phosphorylation of annexins represents a general inhibitory signal

A-I and A-II have been shown to possess several *in vitro* activities including binding to and aggregation both of chromaffin granules and of phospholipid liposomes, as well as binding to F-actin (reviewed in Klee, 1988). The dissimilar subcellular

localization of these proteins and the demonstration, in permeabilized adrenal-medulla cells, that A-II_t but not A-I can reconstitute secretion (Ali and Burgoyne, 1990; Sarafian et al., 1991), suggest that these proteins have different physiological functions. In contrast, the presence of an N-terminal regulatory domain on both proteins that contains multiple phosphorylation sites both for tyrosine and for serine/threonine protein kinases, suggests that the phosphorylation of this domain might be an important regulatory mechanism for both proteins. Although A-I and A-II_t are both phosphorylated by protein kinase C in the regulatory N-terminal domain, it was unclear if phosphorylation similarly affected the *in vitro* activities of these proteins. An early attempt to investigate the regulatory role of the N-terminal domain on the *in vitro* activities of these proteins involved the removal of the domain by selective proteolysis. Ando et al. (1989) reported that the proteolysis of the N-terminal domain of A-I resulted in a dramatic increase in the Ca²⁺ sensitivity of phospholipid binding. In contrast, the proteolysis of the N-terminal domain of A-II_t was reported to decrease the Ca²⁺ sensitivity of the protein (Drust and Creutz, 1988). This suggested that the N-terminal regulatory domains of A-I and A-II_t exerted opposite effects on these proteins. However, we have recently reported that the phosphorylation of A-II_t by protein kinase C results in the inhibition of the phospholipid-aggregation activity of the protein (Johnstone et al., 1992). This result, taken with the results presented in the current paper, suggests that the phosphorylation of A-I and A-II_t by protein kinase C represents an inhibitory signal for these proteins. Recently, Wang and Creutz (1992) reported that the protein-kinase-C dependent phosphorylation of A-I inhibited the ability of the protein to aggregate chromaffin granules by increasing the Ca²⁺ requirement 4-fold. The inhibition was not due to the inhibition of binding since the authors reported that the phosphorylated protein bound to the chromaffin granules at a slightly lower Ca²⁺ concentration than does the native protein. These results were obtained at a stoichiometry of phosphorylation of 1 mol of phosphate per mol of A-I. Taken collectively, our results and those of Wang and Creutz (1992) suggest that the protein-kinase-C dependent phosphorylation of A-I is a general inhibitory signal.

The mechanism by which the aggregation activity of A-I is inhibited by protein kinase C is unclear. Zaks and Creutz (1991) and Meers et al. (1992) have suggested that annexin self-association may be required to allow the annexins to aggregate vesicles. If this model is correct then it is reasonable to suggest that the phosphorylation of A-I by protein kinase C may disrupt the self-association of the molecule, thereby blocking the aggregation activity of the protein without affecting the phospholipid-binding properties of the molecule.

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