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Ca^{2+}-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE CATALYZES THE PHOSPHORYLATION OF ACTIN-BINDING PROTEINS

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Chicken gizzard vinculin and filamin were found to be phosphorylated by Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C). These two actin-binding proteins serve as substrates for protein kinase C specifically in the free form, whereas they are little phosphorylated by protein kinase C in the presence of F-actin. In contrast, α-actinin from chicken gizzard is less susceptible to phosphorylation by protein kinase C, either in the presence or in the absence of F-actin. In light of these data, the possibility that Ca^{2+} and phospholipid-dependent phosphorylation by protein kinase C may modulate the function of actin-binding proteins has to be considered.

Many lines of evidence show that changes in cytoskeletal structure in response to a variety of stimuli are regulated in a Ca^{2+}-dependent manner. It has been demonstrated that some of the proteins which comprise or interact with cytoskeletal or contractile elements are phosphorylated during cellular activation. While the biochemical pathways by which Ca^{2+} acts upon cellular processes remain obscure, one mechanism open for consideration is the phosphorylation of endogenous substrate proteins by Ca^{2+}-dependent protein kinase. In many cases, Ca^{2+} acts through calmodulin, and calmodulin-sensitive protein kinase systems have been identified, including myosin light chain kinase (1). Another system of Ca^{2+}-dependent phosphorylation which requires phospholipid as a cofactor was first described by Nishizuka and associates (2) and this deserves specific emphasis from the viewpoint of the transmembrane control (3). This Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) is widely distributed in various tissues, and some contractile elements,

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Abbreviations used are: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate; protein kinase C, Ca^{2+}-activated, phospholipid-dependent protein kinase.

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myosin light chain (4) and troponin (5) can serve as substrates for protein kinase C.

In the present study, we looked to see whether Ca^{2+} and phospholipid-dependent phosphorylation would occur in actin-binding proteins. We found that vinculin and filamin but not α-actinin were readily phosphorylated by protein kinase C, in vitro.

MATERIALS AND METHODS

Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) was partially purified from fresh human platelets (2). Vinculin, α-actinin, and filamin were isolated from chicken gizzards (6). Pooled fractions of each protein from the DEAE-cellulose column were further purified by chromatography of hydroxyapatite. As determined from the SDS-polyacrylamide gel electrophoresis, vinculin was 95% homogeneous; α-actinin, 95%; and filamin, 90%. The viscometric properties of vinculin, α-actinin and filamin in our preparation are in good agreement with those previously reported (7,8), namely vinculin decreased the steady-state viscosity of the polymerized actin solutions, α-actinin and filamin caused an increase in the rate of actin polymerization and in the steady-state viscosity of F-actin solutions, as measured in an Ostwald viscometer at 25°C. Actin was isolated from rabbit skeletal muscles(9).

Protein kinase C activities were determined by measuring incorporation of ^{32}P from [γ-^{32}P]ATP. The reaction mixture (total 0.3 ml) contained 25 mM PIPES-NaOH (pH 6.5), 10 mM $MgCl_2$, 1 mM $CaCl_2$ (10 μM $CaCl_2$ with 3 ng TPA, or 2 mM EGTA), 15 μg of phosphatidylserine (PS), 30 μM [γ-^{32}P]ATP (containing 0.7 x 10^6 - 1.2 x 10^6 cpm), 0.8 μg of protein kinase C and appropriate amounts of actin-binding proteins. The reactions were terminated by addition of 10% trichloroacetic acid (in final) and acid-precipitable materials were counted. Or alternatively, the reactions were terminated by addition of 2% SDS (in final) and electrophoresis on 0.1% SDS-10% polyacrylamide gels was carried out according to Laemmli (10). The dried gels were exposed to Kodak-X-O mat films. Protein was determined by Bio-Rad protein assay (11).

[γ-^{32}P]ATP was purchased from Amersham, phosphatidylserine (beef brain) was from Serdary Research Laboratory, Inc., TPA was from P-L Biochemicals, Inc., and all other chemicals were of reagent grade.

RESULTS

When the isolated vinculin, α-actinin, or filamin was incubated with protein kinase C, vinculin and filamin were readily phosphorylated by protein kinase C, whereas α-actinin was little phosphorylated. The phosphorylation reaction was dependent upon the presence of Ca^{2+} and phospholipid, as demonstrated by the finding that kinase activity was stimulated about 10 fold by addition of 1 mM $CaCl_2$ and 15 μg/0.3 ml of phosphatidylserine to the reaction mixture (Fig. 1). The autoradiography shown in Fig. 2 also indicated that ^{32}P was incorporated into vinculin and filamin, in a Ca^{2+} and phospholipid-dependent manner. Approximately 0.078 moles of phosphate per mole of vinculin

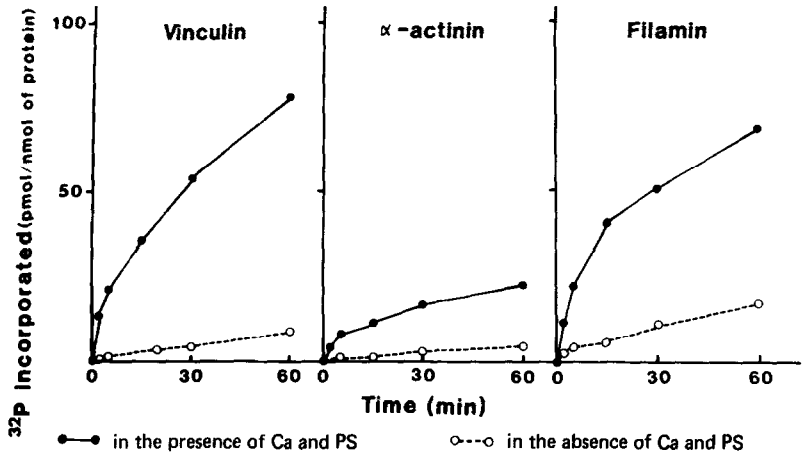

Fig. 1. Phosphorylation of vinculin, α-actinin and filamin by protein kinase C. Chicken gizzard vinculin (52 μg), α-actinin (40 μg) or filamin (96 μg) was incubated with protein kinase C in the presence or absence of 1 mM $CaCl_2$ (Ca) and 15 μg of phosphatidylserine (PS). Other assay conditions were described under "MATERIALS AND METHODS". The data presented were corrected for the blank values determined by the omission of substrate protein from the reaction.

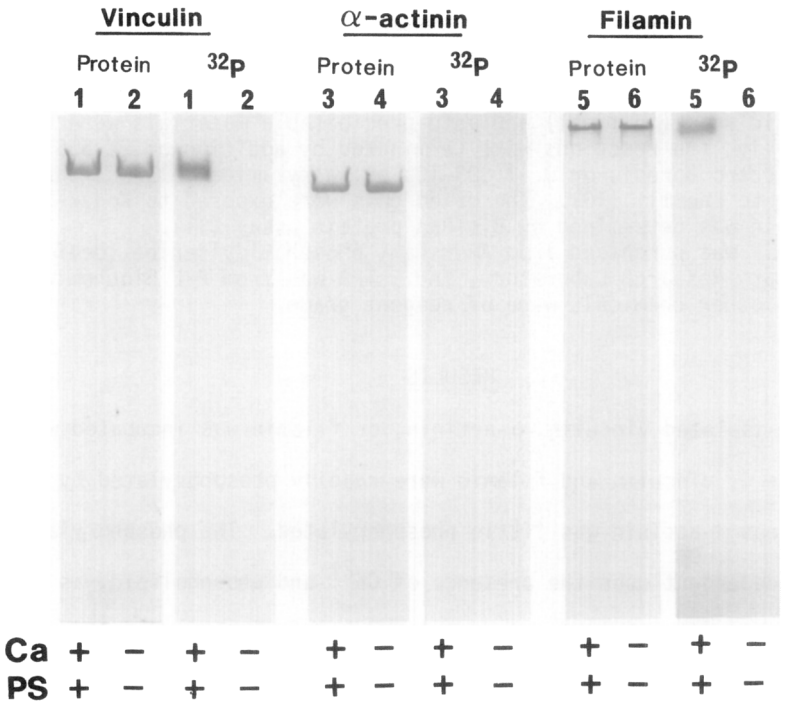

Fig. 2. Autoradiograms of phosphorylated vinculin, α-actinin and filamin by protein kinase C. The reactions were carried out for 15 min under the same conditions as described in Fig. 1. The reaction products were analyzed by SDS-10% polyacrylamide gel electrophoresis.

and 0.068 moles of phosphate per mole of filamin were incorporated over a 60 min period. The relatively high concentrations (10^{-4} - 10^{-3} M) of Ca^{2+} were needed for full activation of protein kinase C in the presence of phospholipid alone. However, either TPA or diolein greatly increased the apparent affinity of the enzyme for Ca^{2+} as well as for phospholipid (12). Vinculin and filamin were phosphorylated by protein kinase C at a lower concentration (10 μM) of Ca^{2+} in the presence of both TPA (3 ng/0.3 ml) and phosphatidylserine (15 μg/0.3 ml)(data not shown).

Each actin-binding protein interacts specifically with actin (7,8,13), thus it had to be determined whether phosphorylation of these actin-binding proteins would be catalyzed by protein kinase C in the presence of F-actin. Since F-actin from skeletal muscles is to a small extent phosphorylated by protein kinase C (about 0.017 moles of phosphate per mole of actin were incorporated during 60 min), the amount of ^{32}P incorporated into each protein was analyzed using autoradiography after separation on SDS-polyacrylamide gels. As shown in Fig. 3, incubation of vinculin with F-actin prior to addition of the enzyme caused a significant reduction in phosphorylation of vinculin, with only a slight effect on phosphorylation of actin. When filamin crosslinked with F-actin to form a highly viscous gel, protein kinase C failed to catalyze phosphorylation of either filamin or actin. The interaction of actin filaments with α-actinin and which forms a crosslinked network of actin, had little effect on the phosphorylation of both these proteins. Thus, vinculin and filamin can serve as substrates for protein kinase C, specifically in the free form, yet they are little phosphorylated by protein kinase C, in the actin-binding form. In contrast, α-actinin is less susceptible to phosphorylation by protein kinase C, either in the free or actin-binding form.

DISCUSSION

We obtained data which show that protein kinase C catalyzes the phosphorylation of vinculin and filamin. The somewhat low reaction velocity of phosphorylation of these actin-binding proteins may represent residual phsophate in the purified proteins or alternatively, the presence of modified or

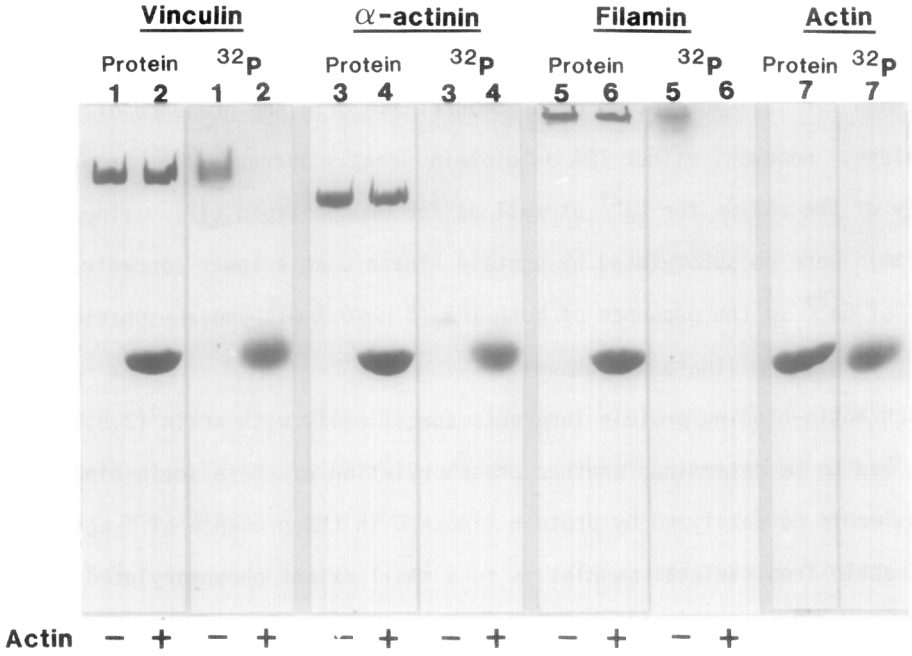

Fig. 3. Effects of F-actin on phosphorylation of vinculin, α-actinin and filamin by protein kinase C. Vinculin (52 μg), α-actinin (40 μg) or filamin (96 μg) was preincubated at 30°C for 2 min, with or without F-actin of skeletal muscle (121 μg) in the standard reaction mixture in which the enzyme and ATP were omitted. The phosphorylation reaction was then initiated by the addition of protein kinase C and [γ-32P]ATP and the incubation was carried out for 15 min. The reaction products were analyzed by SDS-10% polyacrylamide gel electrophoresis followed by autoradiography. Proteins were stained with Coomassie brilliant blue.

denatured proteins not amenable to phosphorylation. Moreover, the activity of protein kinase C varied greatly depending on the protein substrate used. Similar variation in the activity of other protein kinase, e.g. the src kinase or casein kinase, was observed, and the velocity of the phosphorylation of natural substrates is sometimes lower than that of artificial substrates (14,15). This variation appears to be a general phenomenon when both enzyme and substrate are macromolecules.

Vinculin is localized at the area where microfilament bundles terminate at the cell membrane, as determined by immunological techniques (16,17). The enzyme and substrate interaction requires membrane phospholipids for the activation of protein kinase C. Thus, vinculin is well located within the cell for susceptibility to the action of protein kinase C. One recent finding

(18) of particular interest is that phosphorylation of vinculin by the src kinase is dramatically stimulated by phospholipids in vitro. Vinculin was the only substrate for the src kinase, the phosphorylation of which was enhanced by phospholipids, whereas phosphorylation of other substrate proteins, casein and actin, by the src kinase was reduced by phospholipids. Therefore, phospholipids may possibly play a role in the underlying mechanism of vinculin phosphorylation. Filamin seems to have no specific association with membrane phospholipids. However, the activities of protein kinase C are distributed in both the membrane and cytosolic fractions. In addition, the endogenous substrate proteins for protein kinase C are reportedly located in the cytosolic fraction of various tissues (3).

The physiological significance of Ca^{2+} and phospholipid-dependent phosphorylation of actin-binding proteins remains to be determined, however, the finding that protein kinase C failed to catalyze the phosphorylation of vinculin and filamin in the presence of actin does suggest that the actin-binding protein molecule may be closely associated with actin in the specific peptide region where phosphate could be transferred from ATP. Protein kinase C is directly activated by TPA, a potent tumor promoter (12), and this protein kinase may actually be a "receptor" for the biologically active phorbol esters (19,20). TPA induces changes in actin filament organization which resemble those seen following transformation of cells in culture, by the Rous sarcoma virus (21). There is circumstantial evidence suggesting that vinculin phosphorylation on the tyrosyl residue catalyzed by the src kinase leads to cytoskeletal disorganization (22). If phorbol esters do enhance vinculin and/or filamin phosphorylation in living cells, the protein kinase C-catalyzed phosphorylation of these actin-binding proteins may be implicated in the morphological changes induced by phorbol esters.

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