Evidence that (a) serine specific protein kinase(s) different from protein kinase C is responsible for the insulin-stimulated actin phosphorylation by placental membrane

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Partially purified phospholipid- and Ca^{2+}-dependent protein kinase C from human placenta catalyzes the Mg-ATP-dependent phosphorylation of serine residues of purified rabbit muscle actin. Two tryptic [^{32}P]-phosphopeptides were found on HPLC separation. Confirming the previous report by Machicao and Wieland [(1985) Curr. Top. Cell. Regul. 27, 95–105], actin is phosphorylated at serine residues by human placental membranes, and this is stimulated by insulin. In the absence of insulin trypsin treatment yielded eight [^{32}P]phosphopeptides, two of which coincided with the ones due to protein kinase C. Insulin led to the appearance of three new [^{32}P]phosphopeptides. These results suggest that insulin stimulates (a) serine protein kinase(s) which, like protein kinase C, is present in placental membranes.

Actin phosphorylation *Protein kinase C* *Placental membrane* *Insulin action*

1. INTRODUCTION

Since the discovery in rat brain of a protein kinase activity stimulatable by Ca^{2+} and phospholipid [1], the role of this enzyme in the regulation of several cellular processes has elicited growing interest. Protein kinase C (PK-C) turned out to be present as an inactive enzyme in the soluble fraction of many mammalian tissues [2–7]. In the presence of Ca^{2+} the enzyme binds to cellular membranes and is activated by interaction with membrane-associated lipid, mainly phosphatidylserine [2].

Protein substrates liable to phosphorylation by PK-C were found in vivo in several tissues such as brain [3], heart [4], pancreas, liver, vas deferens, adrenal [5], platelet [6] and leukemic HL-60 cells [7]. However, there exists no direct evidence that these phosphorylations play a role in cellular regulation, and the substrates themselves have not been identified. On the other hand, in vitro experiments have shown that several proteins like vinculin [8], eucaryotic initiation factor 2 [9], ribosomal protein S6 [10], smooth muscle heavy meromyosin [11], platelet myosin light chain [12], hepatic 3-hydroxy-3-methylglutaryl-CoA reductase [13], and guanylate cyclase [14] undergo phosphorylation by PK-C. In the latter two cases phosphate incorporation was accompanied by an alteration of enzyme activity.

During our studies on the insulin-dependent phosphorylation of proteins in a cell-free system we have observed that actin and ribosomal protein S6 become phosphorylated by human placenta membranes [15]. Phosphorylation taking place at serine residues suggested the implication of (a) serine protein kinase(s) which is (are) somehow activated by insulin. Although S6 protein can be phosphorylated by PK-C in vitro a direct interaction in intact membranes was ruled out previously (Carrascosa, Urumow and Wieland, unpublished). Here, we have studied the possible involvement of

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PK-C in the insulin-dependent phosphorylation of actin by placental membranes.

2. MATERIALS AND METHODS

Actin was prepared from rabbit muscle acetone powder as described in [16]. PK-C was partially purified from human placenta as reported. Human placental membranes were obtained according to [17].

Standard phosphorylation assay was carried out in 0.05 ml samples containing 25 μg actin, 10 mM $MgCl_2$, 2 mM sodium vanadate, 3.8 mM DTT and 15 μl of PK-C fraction (18.7 μg protein). Activation of PK-C was achieved by addition of 4 μg phosphatidylserine, 0.2 μg diolein and 0.5 mM $CaCl_2$. Phosphorylation was initiated by addition of [γ-^{32}P]ATP (0.5 mM, 1000 cpm/pmol). After 60 min, 6 μl of 20% SDS, 30% glycerol, 250 mM DTT solution were added, the samples boiled 5 min and the proteins analyzed on 10% SDS-PAGE and subsequent autoradiography. Phosphorylation of rabbit muscle actin by placental membranes was performed as in [18].

Actin bands were cut out from the gels and the radioactivity counted by scintillation. The protein was hydrolyzed either with 6 N HCl in order to analyze the phosphoamino acids, or with trypsin to study the tryptic phosphopeptides.

3. RESULTS AND DISCUSSION

As illustrated in fig.1 actin is phosphorylated when incubated with partially purified PK-C from human placenta and [γ-^{32}P]ATP as substrate. ^{32}P incorporation is only observed if $CaCl_2$, phosphatidylserine and diolein are added (lane b). In the presence of 1 mM EDTA and absence of the former activators no phosphorylation is seen (lane a).

Fig.2 shows the time course of actin phosphorylation by PK-C, maximal phosphate incorporation into actin being reached after 45 min of incubation. The phosphorylation of actin at different Mg^{2+} concentrations is shown in fig.3. Mg^{2+} concentrations of 5 mM were needed for maximal phosphorylation. Mn^{2+} was much less effective than Mg^{2+}, no plateau being reached even at 20 mM. Ca^{2+} in the absence of other divalent cations had no appreciable effect (not shown). This

Fig.1. Phosphorylation of actin by protein kinase C from human placenta. Rabbit muscle actin was phosphorylated as indicated in section 2. An autoradiogram is shown of the phosphorylated proteins analyzed on 10% SDS-PAGE. In lane a, 1 mM EDTA was added; in lane b, 0.5 mM $CaCl_2$, 4 μg phosphatidylserine and 0.2 μg diolein were used as activators of PK-C.

dependency on divalent cations is similar to that described for the phosphorylation of histone by PK-C from rat brain [19].

Fig.4 shows that phosphate incorporation into actin by partially purified PK-C takes place only at serine residues. As phosphorylation of actin by placental membranes also concerned serine residues only [18], one could speculate that PK-C

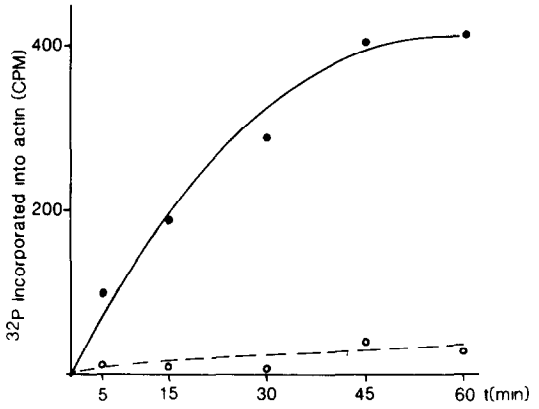


Fig.2. Time course of phosphorylation of actin by PK-C. Phosphorylation was carried out as indicated in section 2, except that the standard assays were enlarged 4-fold. At the times indicated 40-μl aliquots were taken and the reaction stopped as described in the text. Proteins were analyzed on 10% SDS-PAGE, the gels autoradiographed, and actin bands excised and counted by liquid scintillation. (●) Radioactivity incorporated into actin in the presence of activators of PK-C; (○) in the presence of 1 mM EDTA and absence of activators.

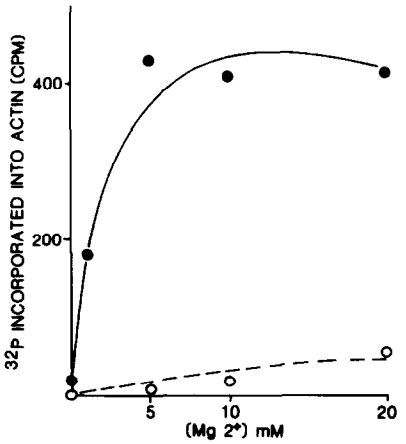


Fig.3. Effect of Mg^{2+} concentration on the phosphorylation of actin by PK-C. Phosphorylation assays were performed as indicated in section 2, except that different $MgCl_2$ concentrations were used. Phosphorylated actin was analyzed and ^{32}P incorporated quantified as in fig.2. (●) Phosphorylation in the presence of activators of PK-C; (○) in the absence of activators and in the presence of 1 mM EDTA.

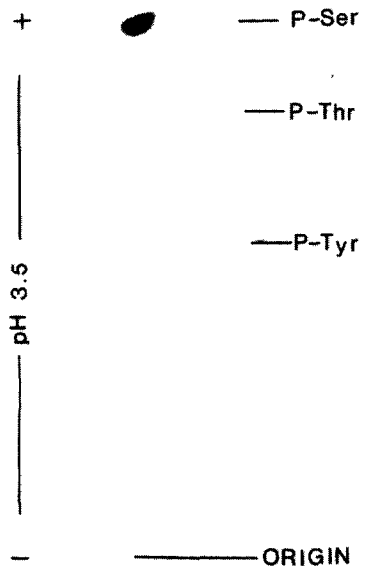
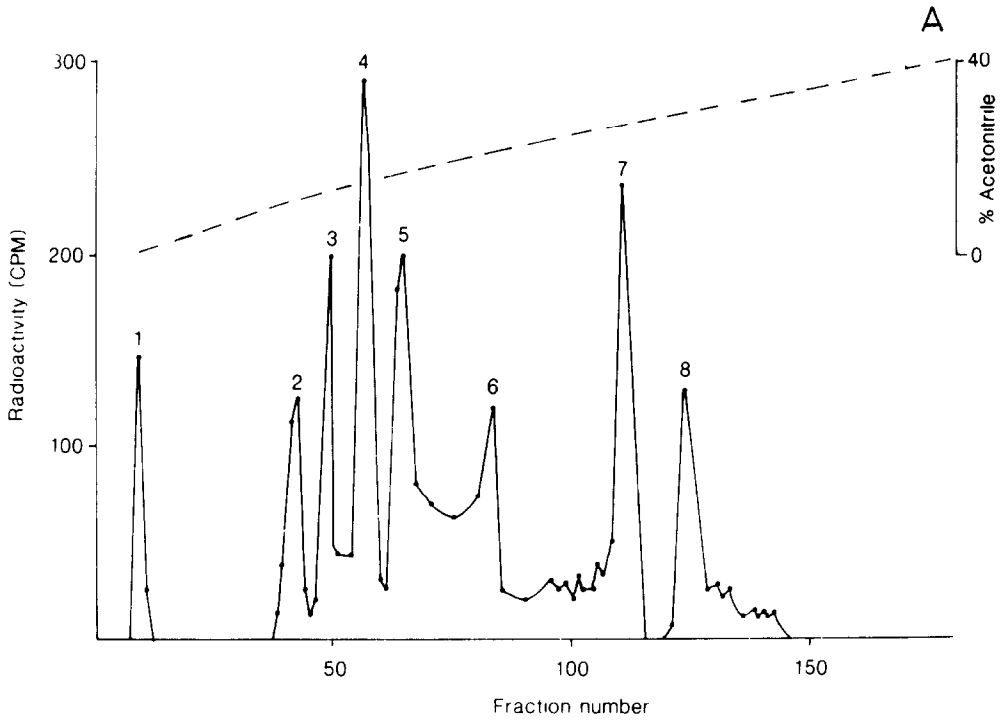


Fig.4. Identification of phosphoamino acids. The excised bands corresponding to actin from the experiments in the former figures were washed with dioxane, methanol and 10% methanol, respectively. The gel fragments were hydrolyzed with 6 N HCl (2 h) and analyzed by electrophoresis as indicated in [16]. Phosphoamino acid standards were localized with ninhydrin and ^{32}P-labelled amino acids by autoradiography.

might represent the responsible enzyme. This was further studied by analysis of the phosphopeptides of actin that had been phosphorylated either by placental membranes or partially purified PK-C from placenta. As shown in fig.5A eight phosphopeptides are obtained if actin is phosphorylated by placental membranes in the absence of insulin. Interestingly, this pattern changes markedly if the membranes are previously incubated with insulin. Thus peak 1 disappears, and three new peaks (1a, 6a, 6b) are observed (fig.5B). As illustrated in fig.5C only two phosphopeptides appear after phosphorylation of actin by PK-C, which seem to coincide with peak nos 7 and 8 in fig.5A. Thus, the insulin-dependent phosphopeptides of actin must be formed by (a) yet unknown protein kinase(s) different from PK-C. Similar conclusions were drawn by others [20], who studied the insulin and phorbol ester-dependent phosphorylation of ribosomal protein S6, in vivo.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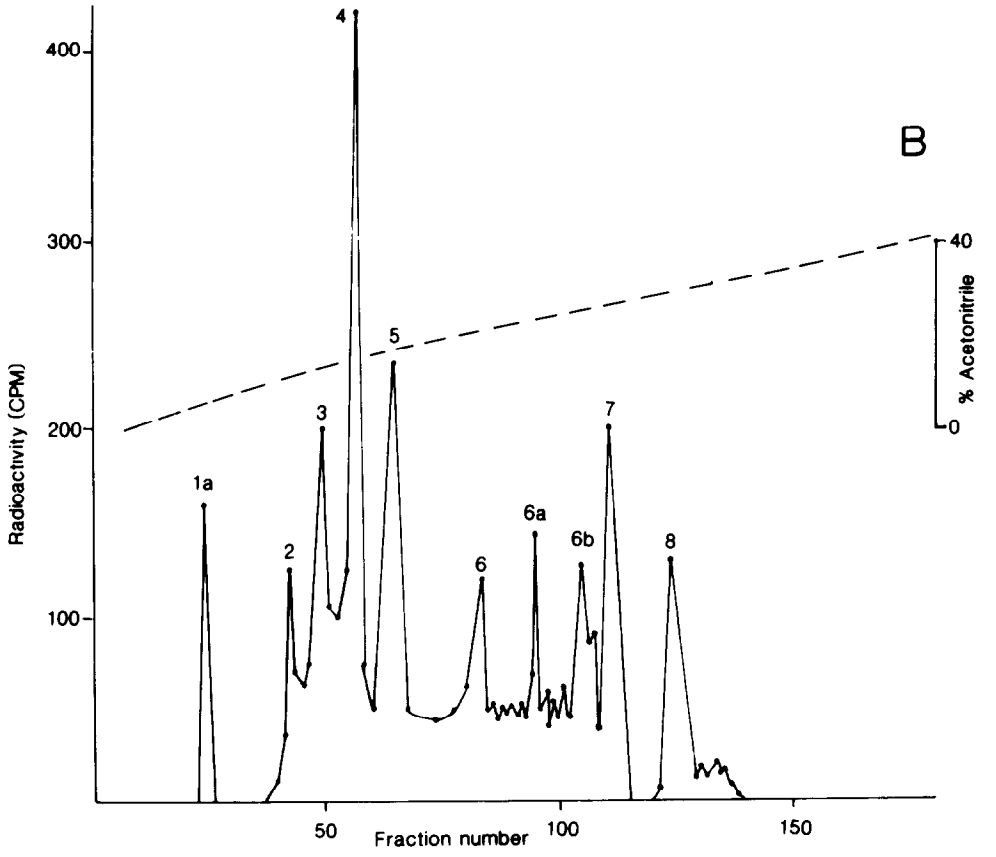
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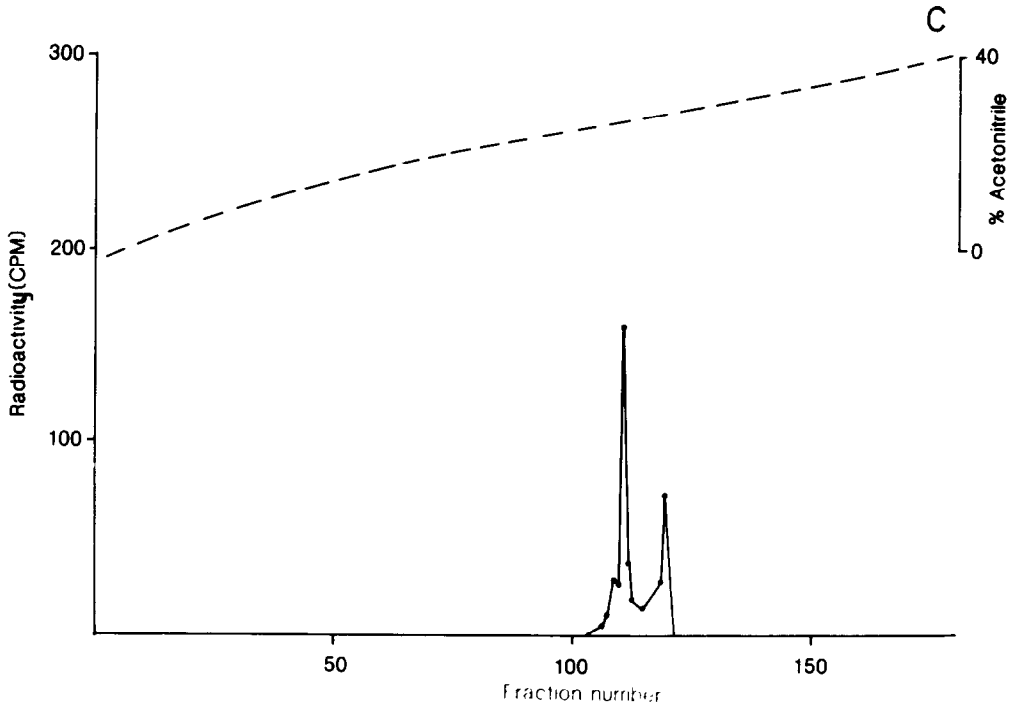

Fig.5. Tryptic phosphopeptide analysis of ^{32}P-actin phosphorylated by human placenta membranes in the absence (A) and presence (B) of insulin, or by protein kinase C (C). Bands corresponding to [^{32}P]phosphoactin were cut out of the gels, and after washing with water for 45 min and 0.2 M $(NH_4)HCO_3$, pH 8.6, for another 45 min digested 20 h at 37°C in 0.5 ml/slice of 0.2 M $(NH_4)HCO_3$, pH 8.6, containing 100 μg trypsin (Sigma) followed by additional 4 h incubation with 50 μg trypsin/slice. After removal of the gel slice and addition of a little glacial acetic acid the solution was lyophilized, the residue taken up in 50 μl of 0.1% trifluoroacetic acid, and applied to an Ultropac TSK ODS-120T reversed-phase HPLC column (LKB). Phosphopeptides were eluted with a 0–40% linear gradient of acetonitrile in 0.1% trifluoroacetic acid.

Previous work from this laboratory provided evidence that phosphorylation of actin leads to specific structural changes of the protein [18]. In this context it seems worth noting that insulin has been reported to provoke alterations in the shape of cellular plasma membranes (membrane ruffling) that are caused by structural changes of the cytoskeleton [21].

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