Studies on the phosphorylation of protein kinase C- α

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A kinase-defective protein kinase C- α mutant is shown to be a phosphoprotein when expressed in COS-1 cells, indicating that intramolecular phosphorylation does not fully account for the phosphate content of protein kinase C- α . Furthermore, evidence is presented that the intermolecular phosphorylation of protein kinase C- α is due to an activity other than protein kinase C- α itself, and this phosphorylation appears to be necessary for protein kinase C- α activity. By contrast, the characteristic shift in apparent molecular mass consequent on phosphorylation *in vivo* can be accounted for by autophosphorylation, as demonstrated *in vitro*. The relationship between these phosphorylated protein kinase C- α species is discussed.

INTRODUCTION

Protein kinase C (PKC) constitutes a family of structurally related serine/threonine protein kinases that appear to play a central role in signal transduction events that operate through the second messenger diacylglycerol (reviewed in [1–4]). Certain PKC isoforms, i.e. PKC- α , - β and - γ , have been purified in a number of laboratories and studied in some detail (see [2] and references therein). These isoforms, when purified from tissue sources, display molecular mass values of ~ 80 kDa, although the cDNAs isolated for these isoforms predict molecular masses 2–3 kDa smaller than that of the purified protein (see [5,6]). Analysis of the primary translation product for PKC- α indicated that this was indeed of lower apparent size than the isolated 'mature' protein [6]. Similar observations have been made in an analysis of PKC- α species obtained in short pulse-labelling protocols *in vivo* [7]. Evidence that these size discrepancies are due to phosphorylation events has been provided through studies on the dephosphorylation of isolated PKC *in vitro*; it has been shown that purified PKC (probably an - α /- β /- γ mixture) shows a decrease in its apparent molecular mass following treatment with potato acid phosphatase [8].

We have previously shown that treatment with phorbol 12-myristate 13-acetate (PMA) can increase the phosphorylation state of mature PKC- α in Swiss 3T3 cells, presumably due to autophosphorylation [9], but it is not clear whether the phosphorylation events responsible for the initial shift in apparent size are due to autophosphorylation or trans-phosphorylation. We present evidence here that a trans-phosphorylation of PKC- α occurs *in vivo* and that the subsequent increase in apparent molecular mass can be accounted for by autophosphorylation. Evidence is also presented that a priming phosphorylation event is necessary for PKC- α activity.

MATERIALS AND METHODS

All reagents were obtained from BDH or from Sigma unless stated otherwise. [γ - ^{32} P]ATP, [^{35} S]methionine, peroxidase-linked donkey anti(rabbit IgG) and the ECL blotting detection system were from Amersham International. PKC- α was prepared as described previously [10]. Protein phosphatase-1 was isolated as described previously [11].

Tissue culture and labelling procedures

The glioblastoma 328 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). For labelling with [^{35} S]methionine, cells were preincubated for 2 h in methionine-free DMEM with 5% (v/v) dialysed FCS in the presence or absence of 400 nM-PMA, then [^{35} S]methionine was added at 75 μ Ci/ml for 15 min and cells were harvested after a chase of 15, 30 or 60 min. The cells were then washed in phosphate (10 mM, pH 7.0)-buffered saline (125 mM-NaCl) (PBS), lysed in SDS sample buffer and diluted 10-fold in 1% (w/v) deoxycholate/1% (v/v) Triton X-100 in PBS, and PKC was immunoprecipitated using 0442 antiserum [12].

COS-1 cells were maintained in DMEM with 10% FCS. Cells were transfected as described previously [13]. Phosphate labelling was carried out in phosphate-free DMEM with 10% FCS for 16 h.

Immunoprecipitation

Transfected COS-1 cells (9 cm dish) labelled with [^{32} P]P_i were rapidly rinsed twice with 10 mM-Tris/HCl (pH 7.5)/125 mM-NaCl at 4 °C to remove excess [^{32} P]P_i and then lysed in 0.5 ml of Laemmli [14] sample buffer. DNA was sheared by passage through a 21-gauge needle and the extract was diluted with an equal volume of 2% (v/v) Triton X-100, 4 mM-EDTA, 20 mM-benzamidine, 40 mM-Tris/HCl, pH 8.0, 10 mM-NaF, 0.4 mM-sodium orthovanadate, 100 μ g of phenylmethanesulphonyl fluoride/ml, 50 μ g of leupeptin/ml, 2 mM-dithiothreitol and 20 mM-*p*-nitrophenyl phosphate. The extracts were cleared in an Eppendorf centrifuge (10 min at 12000 g) and the supernatants were subjected to immunoprecipitation with the anti-PKC antiserum 0442 [12].

Dephosphorylation and rephosphorylation of purified PKC

PKC- α (6–15 units; 1000 units/mg) was dephosphorylated using potato acid phosphatase (0.15 unit) at 30 °C in the presence of 67 mM-Mes, pH 5.5, 2 mM-dithiothreitol, 0.02% (v/v) Triton X-100 and 0.1 mg of BSA/ml in a total volume of 20 μ l. In control incubations, 10 mM-sodium phosphate (pH 5.5) was included. Reactions were terminated by (i) boiling a sample in an equal volume of 4 $\times$ concentrated Laemmli sample buffer for

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C (EC 2.7.1.37); DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; PDBu, phorbol dibutyrate; PPase, protein phosphatase.

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PAGE, and (ii) diluting a sample 200-fold in 20 mM-Tris, pH 7.5, 2 mM-EDTA, 0.02% (v/v) Triton X-100, 10 mM-sodium phosphate, pH 7.5, and 2 mM-dithiothreitol for determination of activity.

Where dephosphorylated PKC- α was subjected to re-phosphorylation, samples of dephosphorylated PKC were diluted 2-fold in the buffer described above in (ii), and then incubated under standard assay conditions but with substrate (histone III-S) replaced by buffer (200 mM-Hepes, pH 7.5, and 2 mM-EGTA). Samples were taken from this phosphorylation mixture for analysis by SDS/PAGE. Because of the dilution of the PKC- α through this two-step procedure, for these rephosphorylation experiments PKC- α was visualized by immunoblot rather than protein staining (see the Results and discussion section).

Dephosphorylation of PKC- α (0.5 unit/ml) with protein phosphatase-1 (PPase-1; 20 units/ml) was performed in a buffer containing 20 mM-Tris/HCl, pH 7.5, 0.1% (v/v) β -mercapto-ethanol, 0.02% (v/v) Triton X-100 and 200 μ g of histone III-S/ml. After incubation for the indicated times at 30 °C, the dephosphorylation reaction was stopped by the addition of the phosphatase inhibitor microcystin at 20 μ M. In control incubations, microcystin (20 μ M) was included. The kinase activity of PKC- α treated with PPase-1, in the presence and absence of microcystin, was then determined by measuring protamine phosphorylation as previously described [15].

Other methods

PKC assays, gel electrophoresis, immunoblotting and protein determination were as described previously [13].

RESULTS AND DISCUSSION

Bacterially expressed PKC, while displaying a normal affinity for [^{3} H]phorbol dibutyrate ([^{3} H]PDBu), displays no detectable kinase activity (e.g. [16]). Similarly, PKC translated *in vitro* using the reticulocyte lysate system shows no detectable kinase activity despite being expressed at a level equivalent to the endogenous reticulocyte PKC, which is itself readily detected as a phospholipid-dependent histone kinase activity (S. Stabel & P. J. Parker, unpublished work). This is consistent with neither of these systems being able to carry out a modification, such as a trans-phosphorylation, required for kinase activity. In both of the above situations, the PKC remains the size of the primary translation product; this lack of apparent size shift would be consistent with the lack of accumulation of phosphate groups on the protein [8]. If phosphorylation is indeed involved, these observations would imply that PKC itself is not responsible for carrying this out.

In order to determine whether intramolecular autophosphorylation of PKC accounts for the entire phosphate content of the protein *in vivo*, we have employed a mutant PKC defective in intrinsic kinase activity which was constructed by conversion of the conserved lysine at the ATP-binding site to a methionine residue [17]. The wild-type PKC- α and this kinase-defective species, PKC- α kin $^-$, were transiently expressed in COS-1 cells; phosphate labelling of these cells ([^{32} P]P $_i$) and immunoprecipitation revealed that both the wild-type and mutant forms of PKC were phosphorylated (Fig. 1). It has previously been confirmed that this mutant PKC- α is indeed devoid of kinase activity, while expressing a normal PDBu-binding activity [17]. The presence of phosphate on the transiently expressed PKC- α kin $^-$ mutant implies that, in COS-1 cells, PKC is in part phosphorylated by an intermolecular reaction (as opposed to an intramolecular one). However, since COS-1 cells express endogenous PKC activity, it is possible that this PKC activity is responsible for phosphorylation of PKC- α kin $^-$.

To test the role of PKC in the trans-phosphorylation of newly synthesized PKC, use has been made of the 328 glioblastoma cell line, which expresses high levels of PKC- α [12]. In these cells the accumulation of phosphate on newly synthesized [^{35} S]-methionine-labelled PKC can be observed through the mobility change on SDS/PAGE [7]. To test the role of mature PKC in the phosphorylation of newly synthesized PKC, cells were pulse-labelled and chased in the presence or absence of PMA (Fig. 2). It can be seen that PMA in fact decreases the rate of phosphorylation, as judged by the 76 kDa to 80 kDa conversion. Since PMA can activate the endogenous PKC, then if PKC was itself responsible an increased rate of conversion would be expected. The result obtained implies that an undefined kinase is

Fig. 1. Phosphate content of a kinase-deficient PKC- α

Wild-type PKC- α (wt) or a kinase defective form (k $^-$) were transiently expressed in COS-1 cells and labelled by incubation for 16 h with [^{32} P]P $_i$. The PKC was immunoprecipitated using the 0442 antiserum in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of competing antigenic peptide, the precipitated proteins were resolved by SDS/PAGE and the presence of phosphate was confirmed by autoradiography. The expression of comparable amounts of the two proteins was confirmed by Western blot analysis (not shown). This is one of three similar experiments.

Fig. 2. Inhibition of PKC- α maturation by PMA

328 cells were pulse-labelled with [^{35} S]methionine and chased for the times shown in the absence (-) or presence (+) of 400 nM-PMA. PKC was immunoprecipitated using the 0442 antiserum, resolved by SDS/PAGE and visualized by autoradiography. The specificity was confirmed for the 60 min time point by the inclusion of competing antigenic peptide in parallel immunoprecipitations (60+). This is one of two similar experiments.

Fig. 3. Dephosphorylation and autophosphorylation of PKC- α

(a) PKC- α purified from bovine brain was incubated at pH 5.5 with potato acid phosphatase for the times indicated in the presence (+) or absence (–) of 10 mM-phosphate as a competitive inhibitor of the phosphatase. The resulting changes in molecular mass were visualized by silver staining after SDS/PAGE. The migration of molecular mass markers is indicated. A contaminant in the PKC- α preparation at 90 kDa does not alter migration on phosphatase treatment and serves to illustrate the altered mobility of PKC- α . This is one of eight similar experiments with three different PKC preparations. (b) PKC- α purified from bovine brain was dephosphorylated by incubation with potato acid phosphatase for 10 min as described for (a). After inhibition of the phosphatase by dilution in a buffer containing 10 mM-phosphate at pH 7.5, the kinase was then allowed to autophosphorylate for 5, 15, 30 and 60 min (see the Materials and methods section). The resulting increase in apparent molecular mass was visualized by Western analysis using an antiserum specific for PKC- α [15]. The extreme upper and lower PKC- α species are indicated by arrowheads. This is one of three similar experiments.

Table 1. Effect of potato acid phosphatase treatment on PKC- α activity

PKC- α was dephosphorylated with potato acid phosphatase (PAP), diluted and assayed as described in the Materials and methods section. The activities are expressed in units/ml, with the percentage of control (PAP+phosphate) given in parentheses. The activity prior to incubation was 2.8 units/ml. This is one of five similar experiments.

Treatment	Time (min)...	PKC- α activity (units/ml)		
		2	5	10
PAP + phosphate		2.4	2.2	1.7
PAP		2.3 (99)	2.0 (91)	1.9 (111)

responsible at least for initiating the phosphorylation (and altered migration) of PKC- α .

In order to determine the possible contribution of subsequent autophosphorylation to the observed altered migration, fully active purified PKC- α was dephosphorylated and allowed to autophosphorylate *in vitro*. Dephosphorylation by potato acid phosphatase induced an increased mobility in SDS/PAGE (Fig.

Fig. 4. Inactivation of PKC- α treated with PPase-1

PKC- α was incubated with PPase-1 as described in the Materials and methods section either in presence (□) or in the absence (■) of microcystin for the indicated times. The kinase activity was then measured by assessing [^{32} P]P $_i$ incorporation into protamine sulphate. Data are expressed as percentages of activity with respect to untreated PKC- α .

Fig. 5. Scheme for the phosphorylation of PKC- α

The scheme indicates the trans-phosphorylation (Trans) of the PKC- α primary translation product by a (non-PKC) kinase, followed by autophosphorylation of the now activatable PKC (Auto). All species of PKC- α are expected to bind to membranes in the presence of PMA ($\gg$), and this sequestration may account for the inhibition of the initial phosphorylation event.

3a), as has been described previously [8]. Under the conditions employed here (see the Materials and methods section), activity is preserved (Table 1) (the moderate phosphatase-independent loss of activity observed on incubation is a consequence of the low pH; PKC- α remains stable on incubation at neutral pH). Following dephosphorylation of PKC- α by potato acid phosphatase, incubation with effectors and Mg-ATP permits autophosphorylation to proceed, and this is associated with conversion back to a more slowly migrating (80 kDa) form (Fig. 3b).

Thus, in principle, autophosphorylation can account for the shift in PKC- α mobility observed *in vivo*.

Formal proof that at least one site of phosphorylation is necessary for PKC- α activity has come from studies with PPase-1 (similar observations have been made with PPase-2A). Treatment of purified (80 kDa) PKC- α with PPase-1, as described in the Materials and methods section, induces a time dependent inactivation of the kinase (Fig. 4). This inactivation is completely inhibited by inclusion of the PPase inhibitor microcystin. This observed inactivation of PKC- α by PPase-1 does not correlate with an altered mobility on SDS/PAGE (conversion to an intermediate form is observed; results not shown), consistent with the effect of potato acid phosphatase in altering migration without affecting activity (Table 1).

These observations, in conjunction with the previously published data showing that PMA treatment can lead to the accumulation of an inactive form of PKC that co-migrates with the primary translation product [18], suggest the scheme illustrated in Fig. 5. It is proposed that the PKC primary translation product is phosphorylated by an as yet unidentified protein kinase; *in vitro* this phosphate(s) can be removed by PPase-1. This initial step does not correlate with any altered mobility of PKC- α on SDS/PAGE. Subsequently the PKC- α can autophosphorylate, inducing an altered mobility. In intact cells PMA treatment decreases the rate of trans-phosphorylation (and consequently the subsequent autophosphorylation), perhaps by sequestering the PKC- α in a membrane fraction. Evidence that the primary translation product of PKC binds phorbol esters and so could undergo translocation to the membrane is provided by a number of reports concerning the bacterially expressed polypeptide (e.g. [16,19]).

Direct evidence for the relationship between the modifications *in vivo* and functional states of the different species is technically very difficult to obtain due to the low concentration of the intermediate species. However, it is anticipated that a direct

approach through the isolation of a putative 'PKC kinase' will shed further light on the biosynthesis and control of PKC.

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Received 16 August 1991/4 November 1991; accepted 11 November 1991