Volume 200, number 1 FEBS 3646 May 1986

# Protein phosphorylation in isolated mitochondria and the effects of protein kinase C

J.M. Backer, J.P. Arcoleo and I.B. Weinstein

*Comprehensive Cancer Center, Columbia University, New York, NY 10032, USA*

Received 14 February 1986; revised version received 17 March 1986

When isolated intact rat liver mitochondria are incubated with [$\gamma$-$^{32}$P]ATP the major phosphorylated proteins are those of 47 and 36 kDa. Phosphorylation of the 47 kDa protein, but not of the 36 kDa protein, is inhibited by carboxyatractyloside, an inhibitor of mitochondrial ATP uptake, while phosphorylation of the 36 kDa protein is inhibited by various uncouplers and an inhibitor of mitochondrial respiration. Addition of purified protein kinase C to the isolated mitochondria leads to the phosphorylation of 69, 37 and 17 kDa proteins. As with other substrates for protein kinase C, phosphorylation of these proteins is dependent on $Ca^{2+}$ and markedly stimulated by various tumor promoters.

*Mitochondrial protein* *Protein phosphorylation* *Protein kinase C*

## 1. INTRODUCTION

Recent studies have implicated $Ca^{2+}$ and phospholipid-dependent protein kinase C (PKC) in the action of certain tumor promoters, hormones and growth factors [1]. In intact cells soluble PKC is presumably activated by association with cellular membranes [1]. Outer mitochondrial membranes constitute a substantial fraction of total cellular membranes that might be accessible to PKC. It is conceivable, therefore, that mitochondrial membranes might activate PKC and also contain proteins that are targets for phosphorylation by PKC. We have, therefore, investigated protein phosphorylation in isolated rat liver mitochondria in the absence and presence of partially purified bovine brain PKC.

## 2. MATERIALS AND METHODS

Mitochondria from female Sprague-Dawley rats (90–150 days old) were isolated as described [2]. Assays for the phosphorylation of mitochondrial proteins were performed in a 0.1 ml reaction mixture that contained 0.21 M D-mannitol, 0.07 M sucrose, 1 mM Hepes at pH 6.8, 2.5 mM $MgCl_2$, 10 mM potassium succinate, 2 $\mu$g/ml rotenone (Sigma), 2 $\mu$g/ml oligomycin (Sigma) and 100–200 $\mu$g/ml mitochondrial protein. Reactions were started by the addition of 5 $\mu$Ci [$\gamma$-$^{32}$P]ATP (>5000 Ci/mmol, Amersham) and terminated after 1 min incubation at 20°C by the addition of 0.02 ml of 20% (w/v) SDS. Samples were analyzed by SDS-polyacrylamide gel electrophoresis with 10% running and 3% stacking gels and with an acrylamide/bisacrylamide ratio of 20:1. Molecular mass markers were run on the same gels. Gels were fixed with 20% 2-propanol prior to autoradiography. The relative intensities of the bands on the autoradiographs were measured with a Chromoscan 3 Joyce Loeble densitometer. In some experiments, autoradiographed gels were treated overnight with a 15% acetic acid/15% methanol mixture and autoradiographed again. Bovine brain PKC was isolated and partially purified as in [3]. Experiments with PKC were performed as described above, but all reaction mixtures contained 5 $\mu$g/ml carboxyatractyloside (Sigma). 12-*O*-Tetradecanoyl phorbol-13 acetate (TPA), phorbol dibutyrate (PDBu) and 4$\alpha$-phorbol didecanoate

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/86/$3.50 

(4$\alpha$-PDD) were from LC Services (Boston, USA). Teleocidin B and aplysiatoxin were generously provided by Dr H. Fujiki.

## 3. RESULTS AND DISCUSSION

We found that when intact respiring rat liver mitochondria were incubated with [$\gamma$-$^{32}$P]ATP, and the phosphorylated proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis the major phosphorylated proteins had apparent molecular masses of 47 and 36 kDa (fig.1, lane 1). In addition to these proteins, we observed much weaker and less reproducible bands of phosphorylated proteins at positions corresponding to 107, 70, 53 and 17 kDa. Several compounds known to affect mitochondrial functions altered the phosphorylation of the 47 and 36 kDa proteins. Phosphorylation of the 36 kDa protein, but not of the 47 kDa protein, was inhibited by an uncoupler of mitochondrial respiration carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (fig. 1, lane 2). A similar effect was observed when mitochondrial respiration was uncoupled by a combination of the potassium ionophore valinomycin and 50 mM $K^+$ (fig.1, lane 3). $Ca^{2+}$ (0.01–0.1 mM) did not affect phosphorylation of mitochondrial proteins, while a combination of $Ca^{2+}$ (0.1 mM) with the calcium ionophore A23187 (which uncouples mitochondria) inhibited phosphorylation of the 36 kDa protein (not shown). Phosphorylation of the 36 kDa protein was also inhibited when mitochondrial respiration was blocked by 2 $\mu$g/ml of antimycin (fig.1, lane 4). On the other hand, phosphorylation of the 47 kDa protein, but not of the 36 kDa protein, was inhibited when influx of ATP into mitochondria was blocked by carboxyatractyloside, suggesting that the 47 kDa protein is phosphorylated inside mitochondria (fig.1, lane 5).

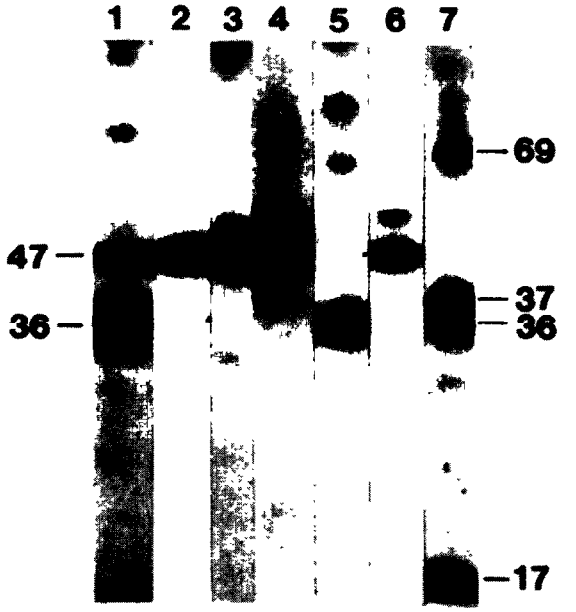


Fig.1. In vitro phosphorylation of mitochondrial proteins by mitochondrial protein kinases and by PKC. Experiments were performed as described in section 2. Lanes: 1, mitochondria incubated alone; 2, mitochondria incubated with 10 $\mu$M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); 3, mitochondria incubated with 50 mM $K^+$ and 0.05 $\mu$g/ml valinomycin; 4, mitochondria incubated with 2 $\mu$g/ml antimycin; 5, mitochondria incubated with 5 $\mu$g/ml carboxyatractyloside; 6, same as 1, but the gel was treated with 15% acetic acid:15% methanol solution overnight, before autoradiography; 7, mitochondria incubated with 5 $\mu$g/ml carboxyatractyloside, 0.1 mM $Ca^{2+}$ and 20 $\mu$g/ml PKC.

In the above studies the gels were fixed with 2-propanol prior to autoradiography. We found that when gels were fixed in 15% methanol-acetic acid prior to autoradiography, the 36 kDa band (and the less reproducible 107 and 70 kDa bands), but not the 47 kDa band, was lost (fig.1, lane 6), suggesting that phosphorylation of the 36 kDa protein occurred on basic amino acid residues with formation of acid-labile phosphoamide bonds [4].

The 47 kDa protein probably corresponds to the $\alpha$-subunit of the intramitochondrial pyruvate dehydrogenase, since the latter protein has a similar molecular mass and its phosphorylation has been previously observed in mitochondria from various tissues [5–7]. To our knowledge, the phosphorylated 36 kDa protein has not been previously described, perhaps because the phosphorylation of the 36 kDa protein requires conditions which maintain coupled respiration of mitochondria. It appears that the 36 kDa protein resides in the outer mitochondrial membrane or the outer face of the inner mitochondrial membrane since its phosphorylation is not blocked by carboxyatractyloside (fig.1, lane 6). For reasons

that are not apparent at present, phosphorylation of the 36 kDa protein is inhibited by various uncouplers and an inhibitor of mitochondrial respiration (fig.1).

The addition of PKC to mitochondria incubated with carboxyatractyloside and 0.1 mM $Ca^{2+}$ resulted in the appearance of additional phosphorylated proteins. The major new bands corresponded to proteins of 69, 37 and 17 kDa (fig.1, lane 7). These proteins are apparently tightly associated with mitochondria, since they persisted when mitochondria were resedimented after phosphorylation in the presence of PKC. Cooper et al. [8] have described a 16 kDa protein associated with rat liver cytosol and plasma membrane that can apparently be phosphorylated by an endogenous PKC. Further studies are required to determine whether this protein bears any resemblance to the above-described 17 kDa protein. The radioactive 69, 37 and 17 kDa bands seen in the presence of PKC were acid-stable, as expected for phosphorylated serine and threonine residues which are the products of the action of PKC [1]. In these studies the mitochondria apparently provided both the lipid cofactor necessary for the action of PKC and specific substrate proteins. In separate studies (not shown) we found that mitochondria also completely satisfied the phospholipid requirement of PKC in the phosphorylation of exogenously added histone H1.

We found that phosphorylation of the 69, 37 and 17 kDa proteins in the presence of PKC was $Ca^{2+}$-dependent and was enhanced by tumor promoters, when assays were done at suboptimal $Ca^{2+}$ concentrations (<0.1 mM). The stimulatory effects of $Ca^{2+}$ and different tumor promoters on phosphorylation of the 37 kDa protein were quantitated by densitometry and are shown in fig.2. These assays were performed in the presence of 0.04 mM EGTA to complex any $Ca^{2+}$ endogenous to the system. It is apparent that the biologically active phorbol esters TPA and PDBu (but not the inactive analog 4$\alpha$-phorbol acetate), as well as the indole alkaloid tumor promoter teleocidin B and the polyacetate compound aplysiatoxin, lower the $Ca^{2+}$ requirement for this reaction, a finding consistent with previous studies on PKC using purified phospholipids as cofactors and histones as substrates for phosphorylation [3,9,10]. Teleocidin

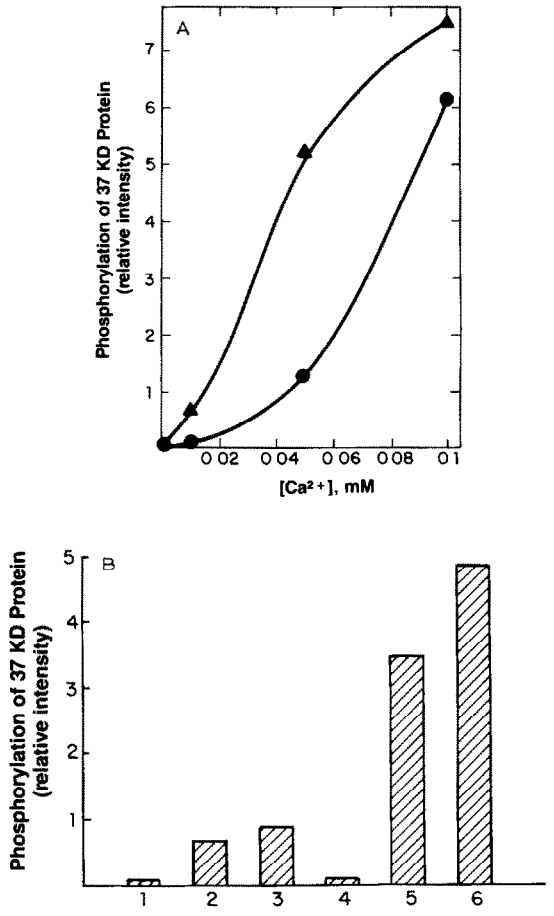


Fig.2. $Ca^{2+}$ and tumor promoters stimulate PKC-induced phosphorylation of the 37 kDa mitochondrial protein. Experiments were performed as described in section 2. All incubation mixtures contained 5 $\mu$g/ml carboxyatractyloside, 20 $\mu$g/ml PKC and, where indicated, 200 ng/ml of the tumor promoters. Reaction mixtures contained (A) the indicated amounts of $Ca^{2+}$ without (●), or with (▲) TPA; (B) 0.01 mM $Ca^{2+}$ and (1) no additions, (2) TPA, (3) PDBu, (4) 4$\alpha$-PDD, (5) teleocidin B, (6) aplysiatoxin.

B and aplysiatoxin provided the greatest stimulation (fig.2B).

These results raise the possibility that specific mitochondrial proteins may be targets for phosphorylation by PKC in vivo. Consistent with this possibility are studies employing fluorescent-labelled TPA indicating that the receptors for TPA are not confined to the plasma membrane, since they are also associated with cytoplasmic struc-

tures, including mitochondria [11,12]. Fractionation studies also indicate that in adrenal tissue a portion of cellular PKC is associated with mitochondria [13]. A recent study indicates that the phosphorylation of intact mitochondria by a mixture of cytosolic protein kinases decreased the apparent $K_m$ of mitochondrial monoaminooxidase [14]. Thus, it is possible that phosphorylation of specific mitochondrial proteins by their endogenous protein kinases and by cytosolic PKC may play an important role in modulating mitochondrial functions. Altered phosphorylation of mitochondrial proteins may also explain our previously described effects of TPA on mitochondrial respiration in intact fibroblasts [15].

## ACKNOWLEDGEMENTS

This work was supported by National Cancer Institute grant CA 02332 and funds from the National Foundation for Cancer Research.

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