

Studies of in vivo phosphorylated proteins in BC3H-1 myocytes suggest that protein kinase C is involved in insulin action

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Insulin (10 and 100 nM) and phorbol esters increased the phosphorylation of several proteins, including 40, 47 and 80 kDa proteins, which are markers for protein kinase C activation. Insulin effects were evident at 2 min and increased over 20 min. These findings suggest that insulin activates protein kinase C in BC3H-1 myocytes.

Insulin; Phorbol ester; Protein phosphorylation; Protein kinase C; Myocyte; Diacylglycerol; Phospholipid

1. INTRODUCTION

Insulin increases protein kinase C (PKC) activity in BC3H-1 myocytes [1] and rat diaphragm [2], as measured in in vitro assays. To test further the hypothesis that insulin activates PKC in vivo, we examined the effects of insulin and TPA, a known activator of PKC, on protein phosphorylation in BC3H-1 myocytes.

2. MATERIALS AND METHODS

BC3H-1 myocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with controlled process serum replacement-I (Sigma), as described [3]. Cells were incubated for 2 (or 16) h with serum-free DMEM containing 0.1% bovine serum albumin (BSA), and 100 μ Ci/ml of $H_3^{32}PO_4$ (ICN). Insulin (Elanco) in DMEM, TPA (Sigma) in 0.02% (final) DMSO or vehicle alone (which was without effect) was then added, and after 1–20 min, the cells were washed 3 times with ice-cold Dulbecco's phosphate-buffered saline (DPBS), gently scraped, collected by centrifugation, sonicated

in 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 1.2 mM EGTA, 0.4 μ M leupeptin and 2 mM PMSF, and centrifuged at $105\,000 \times g$ for 30 min at 4°C to obtain cytosol and membrane fractions. Proteins were determined according to Bradford [4] and equal amounts were subjected to SDS-PAGE in 10% slab gels according to Laemmli [5], and evaluated by autoradiography and densitometric analysis of X-ray films using an LKB laser scanner. Two-dimensional PAGE was conducted by a slight modification of the method of O'Farrell [6]: gels contained 2% Triton X-100 instead of 2% Nonidet P-40, and 1.6% pH 3–5 ampholytes and 0.4% pH 3.5–10 ampholytes were used for isoelectrofocusing. The isoelectrofocusing gels were layered on 10% polyacrylamide separating gels. PKC-dependent protein phosphorylation in vitro was examined as in [1]. The reaction was conducted for 3 min at 30°C in 250 μ l buffer containing: 5 μ mol Tris-HCl (pH 7.5), 1.25 μ mol magnesium acetate, 2.5 nmol [γ - ^{32}P]ATP (5×10^6 cpm), 10 μ g PS and 0.5 mM $CaCl_2$ or 0.5 mM EGTA (as indicated) and cytosol fraction from BC3H-1 myocytes. The reaction was stopped by addition of 63 μ l buffer containing 0.1 M Tris-HCl (pH 6.8), 2% SDS, 0.003% bromophenol blue, 3% 2-mercaptoethanol and 30% glycerol, and then heated at 100°C for 3 min. Samples were separated on SDS-PAGE.

3. RESULTS

We analyzed equal amounts of proteins from control and treated cells by SDS-PAGE, since insulin is reported to provoke an overall increase in protein phosphorylation [7]. Accordingly, there were small, but insignificant, increases in total and

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Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; PKC, protein kinase C; DMSO, dimethyl sulfoxide

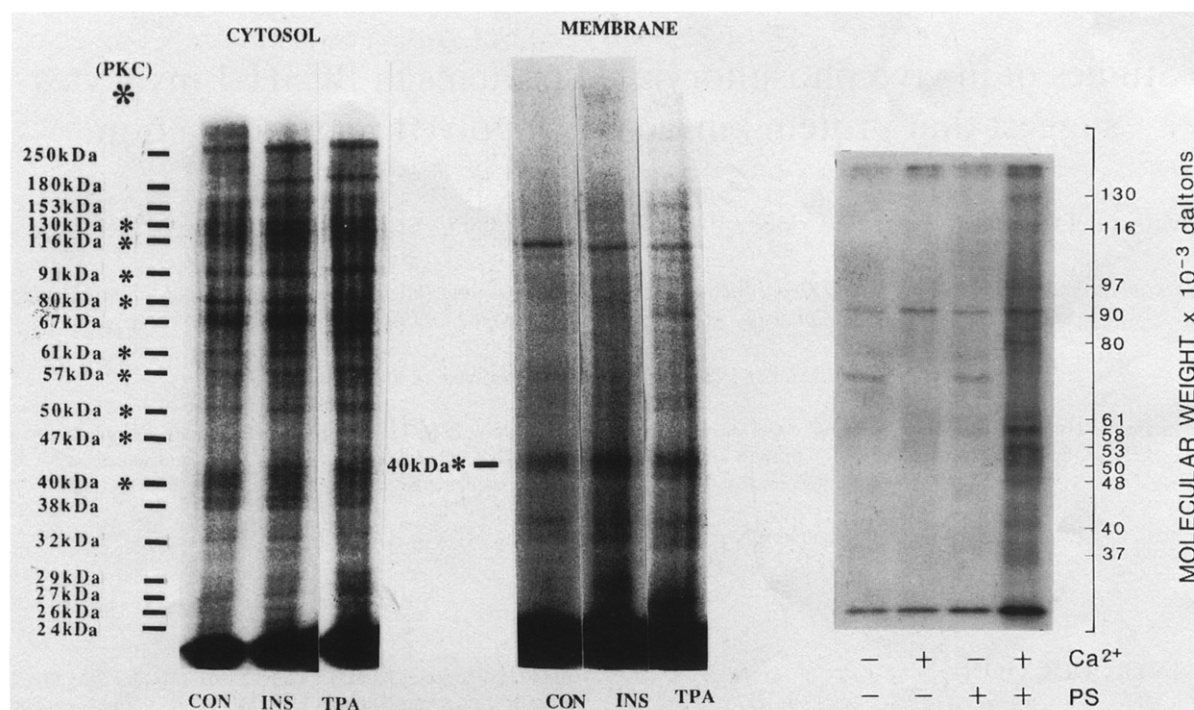


Fig.1. Effects of insulin (100 nM) or TPA (500 nM) on phosphorylation of proteins recovered in cytosol (left) and membrane fractions (center) of BC3H-1 myocytes. Cells were labeled with ^{32}P for 2 h and then treated for 20 min. The panel at the right shows endogenous cytosolic substrates of PKC phosphorylated in vitro, and these are also designated by asterisks in the left and center autoradiographs.

trichloroacetic acid-precipitable radioactivity in the insulin- and TPA-treated samples. In the cytosol, total ^{32}P radioactivity in insulin-treated and TPA-treated samples was: $114 \pm 7\%$ ($n = 23$) and $116 \pm 12\%$ ($n = 9$), respectively, of their corresponding controls, and the trichloroacetic acid-precipitable counts were $115 \pm 7\%$ ($n = 15$) and $116 \pm 14\%$ ($n = 9$). In the membrane fraction, total ^{32}P radioactivity in insulin-treated and TPA-treated samples was $109 \pm 7\%$ ($n = 22$) and $110 \pm 15\%$ ($n = 7$), respectively, of their corresponding controls, and trichloroacetic acid-precipitable counts were $114 \pm 5\%$ ($n = 15$) and $145 \pm 28\%$ ($n = 8$). Labeling of the acid-soluble fraction and ATP was not affected by insulin or TPA treatment (not shown).

Insulin- and TPA-induced changes in protein phosphorylation were similar over a 20 min period (fig.1). Both agonists increased the phosphorylation of many proteins, including those migrating in bands at 40, 47 and 80 kDa, which are markers for PKC activation [8–11]. The 80 and 47 kDa pro-

teins were mainly cytosolic, and the 40 kDa protein(s) was mainly membrane-associated, but these were found in both subcellular fractions, as previously reported [9,11,12]. Upon analysis of the densitometric scans of cytosolic and membrane

Table 1

Effects of insulin on phosphorylation of 40, 47 and 80 kDa proteins in BC3H-1 myocytes

Phosphorylated proteins (kDa)	Percent increase in phosphorylation (insulin vs control)	
	10 nM insulin	100 nM insulin
40	55 ± 12 (4) ^a	79 ± 12 (8) ^a
47	75 ± 16 (4) ^a	116 ± 31 (4) ^a
80	112 ± 31 (6) ^a	110 ± 19 (6) ^a

^a $P < 0.05$ (paired *t*-test)

BC3H-1 myocytes were labeled with ^{32}P for 2 or 16 h (results were similar and pooled) and then treated without or with 10 or 100 nM insulin for 20 min. Mean \pm SE of (*n*) determinations

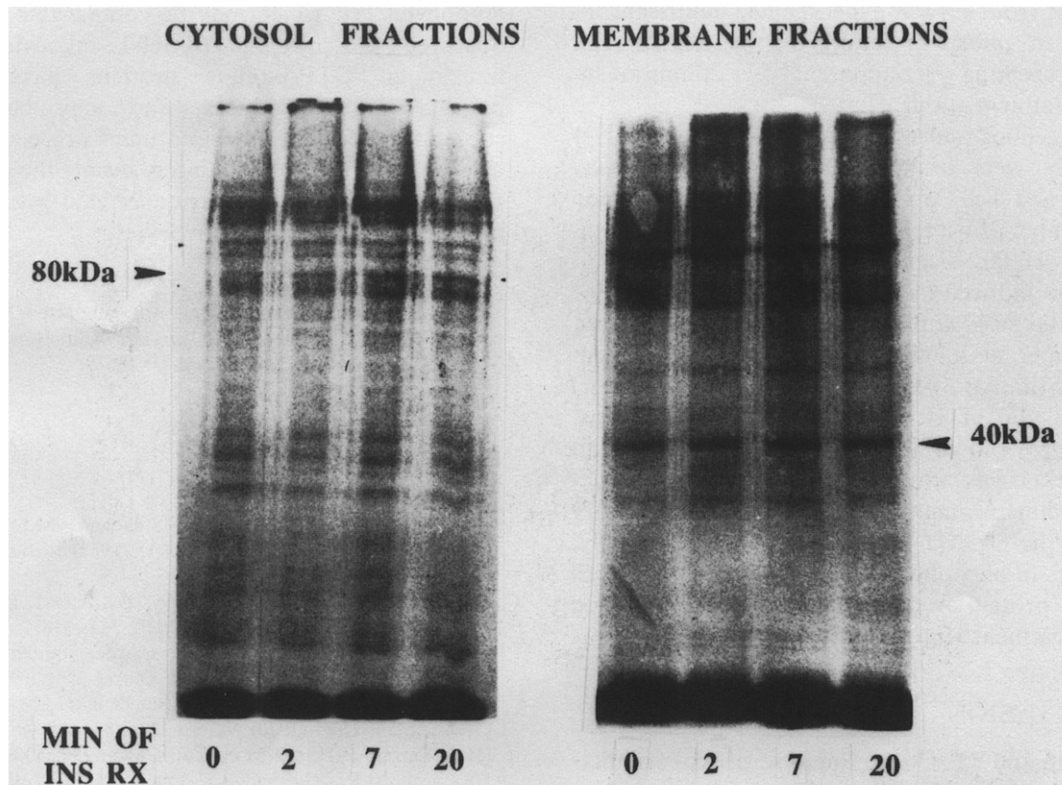


Fig.2. Time course of insulin (100 nM) effects on phosphorylation of 40 and 80 kDa proteins in BC3H-1 myocytes.

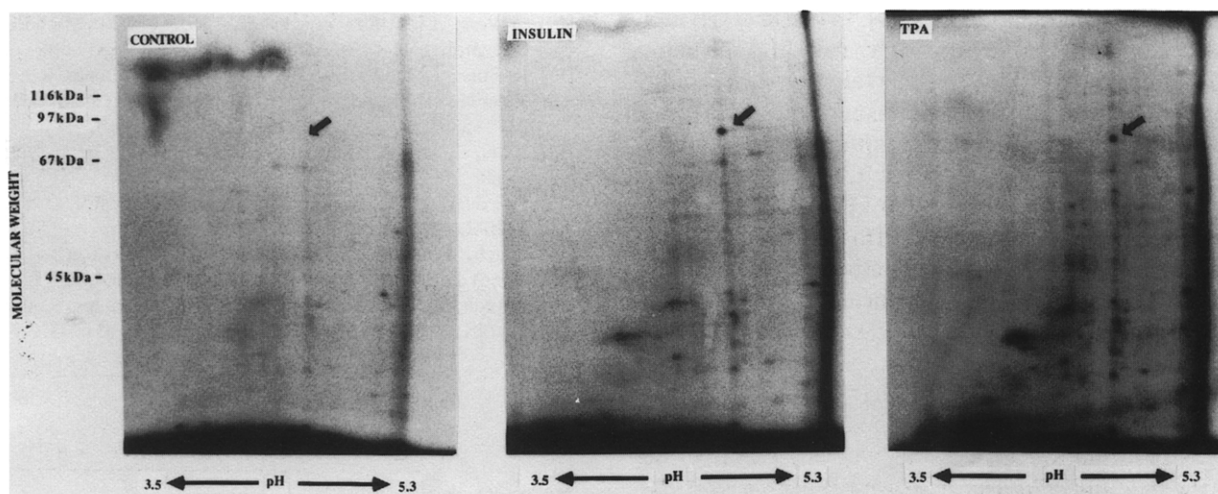


Fig.3. Effects of 100 nM insulin and 500 nM TPA on phosphorylation of the 80 kDa protein (arrows) as purified by 2-dimensional PAGE. Myocytes were labeled for 16 h with ^{32}P , and then treated for 20 min.

fractions from several experiments, significant increases in phosphorylation of the 40, 47 and 80 kDa proteins were apparent after 20 min of insulin treatment (table 1).

Endogenous substrates of PKC in BC3H-1 myocytes were examined in an *in vitro* assay (fig.1), and PS/Ca²⁺-dependent phosphorylation of a number of proteins could be demonstrated, including the 80, 47 and 40 kDa proteins.

Insulin-induced increases in phosphorylation of the 40 and 80 kDa proteins were evident at 2 min, but greater at longer times of insulin treatment (fig.2). Stimulatory effects were observed (table 1) using insulin at 10 and 100 nM concentrations, which are known to provoke submaximal and maximal effects on glucose transport [13].

Two-dimensional PAGE was used to identify further the 80 kDa protein [10,14]. Accordingly, increases in phosphorylation of an acidic (pI 4–5) 80 kDa protein were identical after insulin and TPA treatment (fig.3).

4. DISCUSSION

Insulin and TPA were found to provoke similar increases in the phosphorylation of a number of proteins, several of which were phosphorylated *in vitro* in response to PS and Ca²⁺ and may therefore be substrates of PKC, and several of which have been used as markers for activation of protein kinase C, viz. proteins of apparent molecular mass 40 kDa [8,9], 47 kDa [11] and 80 kDa [10]. In the case of the 80 kDa protein, 2-dimensional SDS-PAGE verified the acidic nature of this protein and insulin and TPA provoked comparable increases in its phosphorylation (it is unclear why insulin did not stimulate phosphorylation of the 80 kDa protein in a previous study [14]). A 40 kDa protein has commonly been used as a PKC activation marker [8], and insulin and TPA reportedly increase the phosphorylation of a 40 kDa protein in rat

adipocytes [9], which may be comparable to that observed presently in BC3H-1 myocytes. Interestingly, GTP-binding proteins have comparable molecular mass, and may be PKC substrates [15], but further studies are needed to evaluate this possibility. In any event, the present findings provide further evidence that insulin activates PKC in BC3H-1 myocytes.

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