Volume 214, number 1, 122-126 FEB 04568 April 1987

Insulin but not phorbol ester treatment increases phosphorylation of vinculin by protein kinase C in BC3H-1 myocytes

Denise R. Cooper, Carlos M. de Ruiz Galaretta, Luisa F. Fanjul, Ljubomir Mojsilovic, Mary L. Standaert, Robert J. Pollet and Robert V. Farese

Veterans Administration Hospital and Departments of Internal Medicine and Biochemistry, University of South Florida College of Medicine, Tampa, FL 33612, USA

Received 29 January 1987

Insulin was found to increase protein kinase C activity in BC3H-1 myocytes as determined by in vitro phosphorylation of both a lysine-rich histone fraction (histone III-S) and vinculin. TPA treatment for 20 min or 18 h provoked an apparent loss of histone-directed but not vinculin-directed phosphorylation by cytosolic C-kinase. Thus, chronic TPA-induced 'desensitization' or 'depletion' of cellular protein kinase C is more apparent than real, and is not a valid means for evaluating the role of C-kinase in hormone action.

Insulin; Phorbol ester; Protein kinase C

1. INTRODUCTION

We have reported that insulin increases diacylglycerol content in BC3H-1 myocytes [1,2], and this is associated with increases in C-kinase-mediated phosphorylation activity in cytosol and membrane fractions toward histone III-S [3]. In contrast, C-kinase-mediated phosphorylation activity toward histone H1 cleavage peptide is not altered by insulin treatment and insulin effects on ribosomal protein S6 phosphorylation are still evident after 18-h TPA treatment, which provokes a loss in C-kinase-mediated histone III-S phosphorylation and immunoprecipitable C-kinase [4]. Such 'desensitization' or 'depletion' of cellular protein kinase C has been attributed to proteolytic loss of C-kinase [5,6], but Cochet et al. [7] have recently found that acute TPA treatment of several cell types causes a loss of C-kinase-mediated phosphorylation of histone, but not vinculin and several other substrates of C-kinase. Since acute TPA treatment may alter substrate and antibody recognition by C-kinase, the loss of C-kinase activity observed following chronic TPA treatment, which has been extensively utilized to evaluate the role of protein kinase C in hormone action, may be more apparent than real. These considerations, prompted us to compare insulin and chronic TPA effects on C-kinase activity in myocytes, using both vinculin and histone III-S as substrates.

Correspondence address: D.R. Cooper, Research Service (151), James A. Haley Administration Hospital, 13000 N. Bruce B. Downs Blvd., Tampa, FL 33612, USA

Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; PS, phosphatidylserine

2. MATERIALS AND METHODS

2.1. *Materials*

We purchased PS, histone (type III-S), PMSF, ATP, TPA and BSA (RIA grade) from Sigma; [γ-^{32}P]ATP (600 mCi/mmol) from ICN Radio-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50 © 1987 Federation of European Biochemical Societies

chemicals; DEAE-Sephacel from Pharmacia; and porcine zinc insulin (25.7 IU/mg) from Elanco Products. Vinculin was purified from chicken gizzards according to Feramisco and Burridge [8] through the DEAE-cellulose chromatography step. Purity was evaluated using slab gel electrophoresis.

2.2. *Cell culture and fractionation*

BC3H-1 myocytes were cultured to confluence [1,2] in T-25 flasks containing Dulbecco's modified Eagles medium supplemented with 20% (v/v) fetal calf serum, and fed with 25 mM glucose for 18 h prior to experiments. TPA (in dimethyl sulfoxide, final vol. = 0.05%) was added in some experiments with glucose. Cells were rinsed and preincubated for 15 min at 37°C in Dulbecco's phosphate-buffered saline (DPBS) containing 1 mg/ml BSA, and then incubated for 20 min with or without insulin or TPA (as described). Reactions were stopped by decanting media and rinsing monolayers twice with ice-cold DPBS. Cells were scraped and centrifuged at 1000 × *g* for 3 min. Cell pellets were resuspended in 0.5 ml of buffer A (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 0.1 mM PMSF) and sonicated for 30 s at 50% output in a Heat Systems ultrasonicator. Sonicates were centrifuged at 105000 × *g* for 30 min in a Beckman TL-100 ultracentrifuge. Supernates (cytosol) were decanted and the pellets were resuspended in buffer B (20 mM Tris-HCl, pH 7.5, 0.25 mM sucrose, 5 mM EGTA, 2 mM EDTA, 1% Triton X-100, 0.1 mM PMSF). After 20 min at 4°C, the solubilized membrane fractions were centrifuged as described above.

To partially purify C-kinase, cytosolic and membrane fractions containing 200 μg protein were chromatographed on 0.5 ml DEAE-Sephacel columns which had been equilibrated in 2 column volumes of buffer C (20 mM Tris-HCl, pH 7.4, 50 mM β-mercaptoethanol, 1 mM EGTA and 1 mM EDTA). Columns were eluted stepwise with 2 column volumes (1 ml) of buffer C containing 0, 0.05, 0.15, and 0.5 M KCl. Routinely, 75% of the vinculin-directed C-kinase activity and 90% of the histone-directed C-kinase activity eluted with 0.15 M KCl. 1 ml fractions were collected and 80 μl aliquots were analyzed for C-kinase activity. Phospholipid and Ca^{2+}-dependent protein kinase activity was assayed as described [3]. The complete reaction mixtures (0.25 ml vol) contained 5 μmol Tris-HCl, pH 7.5, 1.25 μmol Mg-acetate, 2.5 nmol of [γ-^{32}P]ATP (10–20 × 104 cpm/nmol), 10 μg PS, 125 nmol of $CaCl_2$ (in excess of chelator concentrations) and 50 μg histone III-S (by weight) or 60 μg vinculin (by dye-binding protein assay). Ca^{2+}/PS-independent kinase activity was determined by incubation of samples in the absence of PS and Ca^{2+}, and in the presence of 125 nmol EGTA. Reactions were initiated by enzyme addition and terminated after 3 min by adding 2 ml of 25% (w/v) trichloroacetic acid. Precipitates were collected on nitrocellulose filters (0.45 μm, Millipore) and counted for radioactivity.

3. RESULTS

The Ca^{2+}- and phospholipid-dependence of C-kinase-mediated vinculin phosphorylation is shown in table 1. Maximal phosphorylation required the simultaneous presence of both PS and Ca^{2+}, and neither alone affected phosphorylating activity. The DEAE-cellulose column elution profile for C-kinase-mediated vinculin phosphorylation activity (not shown) was similar to that reported [3] for histone III-S phosphorylation.

The effect of insulin treatment on cytosolic and membrane-associated C-kinase activity was determined by assaying DEAE-Sephacel column fractions simultaneously with vinculin and histone III-S substrates. Histone-directed C-kinase activity in cytosolic and membrane fractions was increased 2-fold after insulin treatment. Vinculin-directed C-

Table 1

Ca^{2+} and phospholipid dependence of vinculin phosphorylation by C-kinase

Addition	pmol ^{32}P/min per fraction
PS + Ca^{2+}	21.72 ± 1.69
Ca^{2+}	7.26 ± 2.88
PS, EGTA	10.84 ± 0.56
EGTA	9.02 ± 0.58

Cytosolic extracts from insulin treated (200 nM, 20 min) were chromatographed on DEAE-Sephacel columns as described in section 2. C-kinase activity was assayed in fractions eluting with 0.15 M KCl with additions as indicated: 40 μg/ml PS, 0.5 mM Ca^{2+}, or 0.5 mM EGTA, 60 μg vinculin. Values represent means ± SE of incubations from 3 experiments

kinase activity was stimulated nearly 4-fold by insulin treatment, but this effect was only apparent in the cytosolic fraction (table 2).

Previous reports have shown that histone-directed and immunoprecipitable C-kinase is deficient or no longer detected following chronic TPA treatment of 3T3-L1 cells [6], BC3H-1 myocytes [5] and Swiss 3T3 cells [9]. Accordingly, when myocytes were treated either for 20 min or 18 h with 1 μM TPA, histone-directed C-kinase activity was usually no longer apparent in DEAE-Sephacel-purified cytosolic extracts and markedly diminished in membrane extracts (table 3), confirming the previously reported observation.

Table 2

Effect of insulin treatment on C-kinase-mediated phosphorylation of vinculin and histone III-S

Substrate	Subcellular fraction	pmol ^{32}P/min per fraction	
		Control	Insulin treatment
Vinculin	cytosol (n = 3)	2.18 ± 0.85	8.31 ± 1.66[a]
	membrane (n = 3)	3.54 ± 1.62	3.84 ± 0.33
Histone III-S	cytosol (n = 5)	21.10 ± 0.57	44.69 ± 5.25[b]
	membrane (n = 5)	5.83 ± 0.63	12.17 ± 0.87[c]

[a] $p<0.025$
[b] $p<0.01$
[c] $p<0.001$

BC3H-1 myocytes were treated with insulin (200 nM) for 20 min. Cytosolic and membrane extracts (200 μg protein) were chromatographed on DEAE-Sephacel and the (C-kinase-rich) fraction was assayed as described in section 2. Activity is expressed as pmol of PS and Ca^{2+}-dependent phosphorylation of vinculin (60 μg) or histone III-S (50 μg)/min per fraction of DEAE-Sephacel eluate. Data shown are means ± SD of 3 or 5 separate experiments as indicated

Table 3

Effect of TPA treatment on C-kinase-mediated phosphorylation of histone III-S and vinculin in BC3H-1 myocytes

Addition	pmol ^{32}P transferred/min per fraction			
	Histone III-S		Vinculin	
	Cytosol	Membrane	Cytosol	Membrane
Control	18.4	65.0	1.91	3.76
TPA (20 min)	0	4.25	1.93	0.73
TPA (18 h)	0	5.95	2.12	0

Myocytes were treated for 20 min or 18 h with 1 μM TPA. DEAE-eluates of the 0.15 M KCl fraction from cytosolic and membrane extracts were assayed for C-kinase activity as described in section 2. Activity is expressed as pmol of PS and Ca^{2+}-dependent phosphorylation of histone III-S (50 μg) or vinculin (60 μg)/min per fraction. Data are representative of an experiment which was repeated 3 times

However, residual histone-phosphorylating activity always remained detectable in membrane extracts and occasionally in cytosolic extracts (not shown), even following 16 μM TPA treatment for 18 h. Furthermore, in contrast to the loss of histone III-S phosphorylation in cytosolic extracts after 20 min or 18 h of 1 μM TPA treatment, cytosolic vinculin-directed C-kinase activity was not depleted or desensitized and remained fully active (table 3). On the other hand, membrane-associated vinculin-phosphorylating activity was diminished following TPA treatment.

4. DISCUSSION

Our findings demonstrate that both vinculin and histone are substrates for C-kinase in the BC3H-1 myocytes. The requirement for both PS and Ca^{2+} suggests that C-kinase rather than protease-activated kinase II [10] is responsible for vinculin phosphorylation. Further, when myocytes were treated with insulin, increased phosphorylation of both substrates was apparent in C-kinase assays using cytosolic extracts. The reason for the difference in effects of insulin on vinculin and histone III-S phosphorylation by membrane-associated C-kinase is not apparent.

Of further interest, after 20-min or 18-h TPA-treatment, histone III-S was no longer or poorly phosphorylated by cytosolic C-kinase, but vinculin phosphorylation was virtually unchanged in the cytosol. (It is unclear why vinculin phosphorylation diminished in the membrane fraction with TPA treatment.) Thus, despite previous suggestions to the contrary [4], C-kinase activity is still present in TPA-treated myocytes, albeit in an altered form with respect to substrate and antibody recognition.

After TPA treatment, the failure of C-kinase to recognize histone III-S as a substrate or to be recognized as a ligand for antibody binding [4], may be indicative of an altered catalytic state of the enzyme as has been suggested [7], or may reflect different forms of the enzyme which may selectively phosphorylate different substrates. Several forms of C-kinase activity have now been described as products of different genes or differently processed mRNAs [11–14]. Whatever the explanation, our results and those of Cochet et al. [7] emphasize the fact that C-kinase cannot be considered to be absent or depleted in 'TPA-desensitized' cells, regardless of apparent decreases in histone phosphorylation and immunoprecipitable C-kinase activity. As a corollary, continued hormonal actions in TPA-desensitized cells cannot be taken as definitive evidence that the C-kinase system is not involved in these actions (e.g [5,9]).

The present results also serve to emphasize other points in interpreting C-kinase data. First, hormonal effects on the C-kinase system may be similar in some respects to those of TPA and other tumor promoters, but patterns of C-kinase activation, subcellular distribution, and ultimately phosphorylation effects on various substrates may be considerably different when C-kinase is activated by endogenously produced diacylglycerols versus unphysiological phorbol esters [15–17]. Thus, changes observed with TPA should not serve as the absolute criterion to determine wheter the C-kinase system has been activated by physiological agonists. Second, the C-kinase system, through variations in substrate recognition or variable activation of several forms of C-kinase [18], in addition to variations in subcellular distribution, may provide greater flexibility for biological expression than has been appreciated.

In summary, the present findings provide further support for the hypothesis that insulin activates the C-kinase system. This activation by insulin is decidedly different from that provoked by TPA. The divergent changes in substrate-specific C-kinase activity observed presently are best explained by postulating that agonists may alter substrate recognition, or activate different forms of C-kinase. Our results also indicate that TPA desensitization does not cause a full loss of all forms or all activities of C-kinase. These caveats emphasize the need for caution in evaluating changes in the C-kinase system.

ACKNOWLEDGEMENTS

This work was supported by funds from the Research Service of the Veterans Administration (R.V.F.), and Spanish American Joint Committee for Scientific and Technological Cooperation (L.F.F. and C.M.d.R.G.).

REFERENCES

[1] Farese, R.V., Barnes, D.E., Davis, J.S., Standaert, M.L. and Pollet, R.J. (1984) J. Biol. Chem. 259, 7094–7100.

[2] Farese, R.V., Davis, J.S., Barnes, D.E., Standaert, M.L., Babischkin, J.S., Hock, R., Rosic, N.K. and Pollet, R.J. (1985) Biochem. J. 231, 269–278.

[3] Cooper, D.R., Konda, T.S., Standaert, M.L., Davis, J.S., Pollet, R.J. and Farese, R.V. J. Biol. Chem., in press.

[4] Spach, D.H., Nemenoff, R.A. and Blackshear, P.J. (1986) J. Biol. Chem. 261, 12750–12753.

[5] Blackshear, P.J., Witters, L.A., Girard, P.R., Kuo, J.F. and Quamo, S.N. (1985) J. Biol. Chem. 260, 13304–13315.

[6] Ballester, R. and Rosen, O.M. (1985) J. Biol. Chem. 260, 15194–15199.

[7] Cochet, C., Souvignet, C., Keramidas, M. and Chambaz, E.M. (1986) Biochem. Biophys. Res. Commun. 134, 1031–1037.

[8] Feramisco, J.R. and Burridge, K. (1980) J. Biol. Chem. 255, 1194–1199.

[9] Kitigawa, K., Nishino, H. and Iwashima, A. (1986) Biochim. Biophys. Acta 887, 100–104.

[10] Gonzatti-Haces, M.I. and Traugh, J.A. (1986) J. Biol. Chem. 261, 15266–15272.

[11] Coussens, L., Parker, P.J., Rhee, L., Yang-Feng, T.L., Chen, E., Stable, S., Waterfield, M.D. and Ullrich, A. (1986) Science 233, 853–859.

[12] Makowske, M., Birnbaum, M.J., Ballester, R. and Rosen, O.M. (1986) J. Biol. Chem. 261, 13389–13392.

[13] Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K. and Nishizuka, Y. (1986) FEBS Lett. 206, 347–352.

[14] Huang, K.P., Nakabayashi, H. and Huang, F.L. (1986) Proc. Natl. Acad. Sci. USA 83, 8535–8539.

[15] Gopalakrishna, R., Barsky, S.H., Thomas, T.P. and Anderson, W.B. (1986) J. Biol. Chem. 261, 16438–16445.

[16] Shinohara, O., Knecht, M. and Catt, K.J. (1985) Biochem. Biophys. Res. Commun. 133, 468–474.

[17] Pontremoli, S., Melloni, E., Michetti, M., Salamino, F., Sparatore, B., Sacco, O. and Horecker, B.L. (1986) Biochem. Biophys. Res. Commun. 136, 228–234.

[18] Ramsdell, J.S., Pettit, G.R. and Tashjian, A.H. (1986) J. Biol. Chem. 261, 17073–17080.