Volume 244, number 1, 174–176 FEB 06801 February 1989

Immunological evidence that insulin activates protein kinase C in BC3H-1 myocytes

Mildred Acevedo-Duncan, Denise R. Cooper, Mary L. Standaert and Robert V. Farese

James A. Haley Veterans' Hospital and Departments of Internal Medicine and Biochemistry, University of South Florida, College of Medicine, Tampa, FL 33612, USA

Received 8 November 1988; revised version received 12 December 1988

Effects of insulin on immunoreactive protein kinase C were examined in BC3H-1 myocytes. Insulin provoked rapid dose-dependent decreases in cytosolic enzyme, and transient increases and subsequent decreases in membrane-associated enzyme. Phorbol esters provoked similar changes. Our findings suggest that insulin provokes the translocative activation of protein kinase C.

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

1. INTRODUCTION

Insulin increases PKC enzyme activity in membrane fractions of BC3H-1 myocytes [1] and rat diaphragm [2], but does not decrease cytosolic enzyme activity. This activation pattern differs from that of phorbol esters [3], which decrease cytosolic and transiently increase membrane PKC activity. The latter indicates that PKC translocates from cytosol to membrane, and undergoes subsequent proteolytic degradation [4], thus leading to decreases in PKC content, particularly in the cytosol. Because insulin did not diminish cytosolic PKC enzymatic activity in the above and other [5,6] tissues, it has been questioned whether insulin truly activates PKC. However, insulin provokes large increases in DAG, which may increase cytosolic enzyme activity and mask decreases in enzyme content. Thus, simple measurement of enzyme activity may not reveal changes in content, subcellular distribution and translocation of PKC. To evaluate the latter, we measured immunoreactive PKC.

Correspondence address: R.V. Farese, Research Service (VAR 151), J.A. Haley Veterans' Hospital, 13000 Bruce Downs Blvd., Tampa, FL 33612, USA

Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide

2. EXPERIMENTAL

PKC was purified from rat (Holtzman) brains, which were homogenized in cold 0.25 M sucrose buffer containing 20 mM Tris (pH 7.5), 50 mM 2-mercaptoethanol, 0.2% Triton X-100, 10 mM EGTA, 2 mM EDTA, 0.4 mM leupeptin and 2 mM PMSF and centrifuged at 100000 × *g* for 1 h. The supernatant was applied to a DEAE-cellulose column equilibrated with buffer A [20 mM Tris-HCl (pH 7.5), 50 mM 2-mercaptoethanol, 10 mM EGTA, 2 mM EDTA, 2 mM PMSF]. The column was washed and eluted with a linear gradient of 0–0.4 M NaCl in buffer A. Fractions containing PKC enzyme activity (see [1]) were pooled, adjusted to 1.5 M NaCl and loaded on a phenyl-Sepharose CL-4B column equilibrated with buffer B [1.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 50 mM 2-mercaptoethanol, 2 mM PMSF]. The column was washed and eluted with a linear gradient of 1.5–0 M NaCl in buffer B. Fractions containing PKC were pooled and applied to a preparative SDS-PAGE slab gel. PKC was located as an 80 kDa protein by PS/Ca^{2+}-dependent autophosphorylation and used to immunize New Zealand rabbits, as described by Vaitukaitis et al. [7].

Procedures for culturing and subcellular fractionation of BC3H-1 myocytes have been described [1]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% process serum replacement-1 (Sigma), and fed 25 mM glucose

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

24 h before experimentation. Cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS) containing 0.1 mM $CaCl_2$ and 1 mg/ml glucose, and preincubated for 20 min at 37°C. Insulin (Elanco), TPA (Sigma), or vehicle (controls) was added, and after 1–20 min reactions were terminated by washing the cells twice with ice-cold DPBS. Cells were gently scraped, sonicated in buffer C [10 mM Tris-HCl (pH 7.5), 10 mM EGTA, 25 mM $MgCl_2$, 0.25 M sucrose, 2 mM PMSF, 0.4 mM leupeptin], and centrifuged at 100000 × g for 30 min to obtain cytosol and membrane fractions. The latter was resuspended in buffer C with 1% Triton X-100 and centrifuged to obtain the solubilized membrane fraction.

Cytosol and solubilized membrane fractions (50 μg protein) were subjected to SDS-PAGE (8% gel) and proteins were electrophoretically transferred to nitrocellulose [8]. Nonspecific binding sites were blocked with 3% gelatin in 20 mM Tris-HCl (pH 7.5) containing 500 mM NaCl (TBS). The nitrocellulose sheets were washed with TBS containing 0.05% Tween 20 (TTBS), incubated for 2 h with PKC antiserum in 1% gelatin in TTBS, washed and incubated for 1 h with goat anti-rabbit antibody coupled to horseradish peroxidase in 1% gelatin-TTBS. Immunoreactive proteins were visualized with 4-chloro-1-naphthol and H_2O_2, and quantified with a laser densitometric scanner (LKB). Hydroxyapatite-purified, 80 kDa rat brain protein kinase C was used as a molecular mass standard.

3. RESULTS

Our polyclonal antiserum detected a major 80 kDa immunoreactive protein in crude cytosol and membrane fractions of BC3H-1 myocytes (figs 1,2). Identical results were obtained after purification of PKC by Mono Q column chromatography, followed by resolution of type I–III isozymes by hydroxyapatite column chromatography [9], or when we used an antiserum (provided by Drs Bryan Roth and John Mehegan), which was raised to a synthetic type II peptide sequence and specifically recognizes type II PKC (not shown).

Temporal effects of 100 nM insulin on immunoreactive PKC are depicted in fig.1 by representative immunoblots and densitometric scans. Results of scans of control and insulin-treated samples from several experiments were pooled, and cytosolic immunoreactive PKC decreased 8 ± 4% (mean ± SE, n = 3) at 1 min, 53 ± 10% (n = 5) at 10 min, and 77 ± % (n = 4) at 20 min of insulin treatment. Immunoreactive PKC in membrane fractions increased 35 ± 20% (n = 3) at 1 min, and then decreased 45 ± 6% (n = 3) at 10 min, and 65 ± 9% (n = 6) at 20 min of insulin treatment.

As shown in fig.2, increasing concentrations of insulin provoked graded decreases in cytosolic

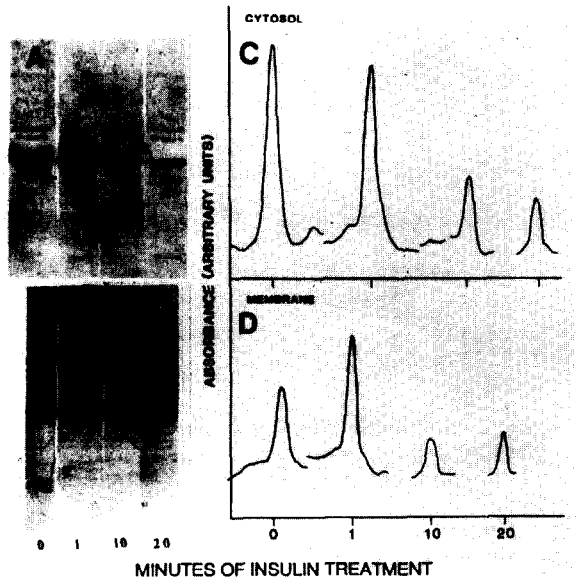

Fig.1. Western blots (left) and densitometric scans (right) of protein kinase C in cytosol (A,C) and membrane (B,D) fractions of BC3H-1 myocytes. Cells were treated with 100 nM insulin for 1, 10, or 20 min, or with buffer alone (controls) for 20 min.

PKC immunoreactivity over a 20 min treatment period. Increases in membrane PKC immunoreactivity were observed with 1 nM insulin, but higher concentrations provoked decreases.

TPA (2 μM) provoked decreases in cytosolic and membrane-bound immunoreactive PKC, which were similar to, although slightly less pronounced than, those provoked by insulin (fig.3).

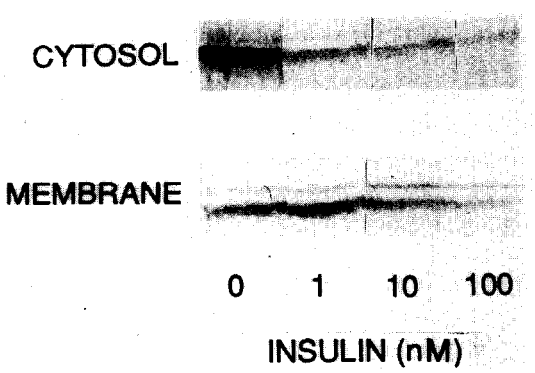

Fig.2. Dose-related effects of insulin (over 20 min) on immunoreactive protein kinase C in cytosol and membrane fractions of BC3H-1 myocytes.

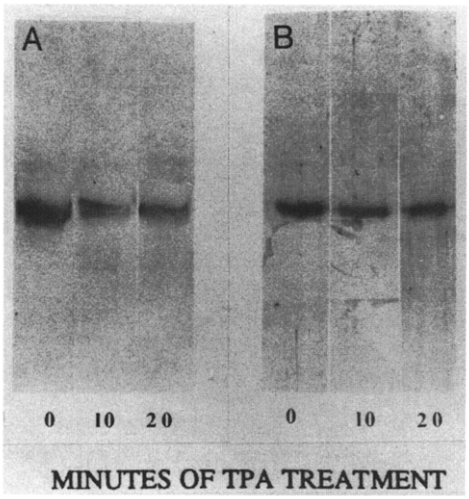

Fig.3. Western blot analysis of TPA effects on protein kinase C in cytosol (A) and membrane (B) fractions of BC3H-1 myocytes. Cells were treated for 10 or 20 min with 2 μM TPA in DMSO, or DMSO alone.

4. DISCUSSION

Although not apparent in studies of insulin effects on enzymatic activity of PKC [1,2,5,6], insulin provoked rapid decreases in cytosolic, and transient increases and subsequent decreases in membrane-associated immunoreactive PKC. These findings suggest that translocation and subsequent degradation of PKC occur during insulin treatment, and indicate that the enzyme has been activated in vivo. In keeping with this hypothesis, similar changes in cytosolic and membrane-associated immunoreactive PKC were observed with TPA, a known activator of the enzyme [1].

The observed changes in immunoreactive PKC during insulin and TPA treatment presumably reflect changes in enzyme content. It may therefore seem paradoxical that insulin-induced decreases in apparent content of cytosolic PKC were not paralleled by observed decreases in enzymatic activity [1]. However, enzyme activity was previously assayed with crude cytosol, and insulin-induced increases in DAG may increase the V_{max} of PKC, even in the cytosol fraction. Thus, a decrease in enzyme content may be offset by an increase in enzymatic activity. Indeed, if the cytosolic PKC is purified by Mono Q column chromatography to remove sufficient endogenous DAG, and then assayed with optimal concentrations of diolein, control cytosolic PKC activity is 2-fold greater than that measured in insulin-treated cytosol (unpublished): these results, like those of the present study, indicate that cytosolic PKC content is decreased by insulin treatment. Along these lines, whether the endogenous DAG which activates cytosolic PKC in in vitro assays of crude cytosol is present in this fraction in vivo, or is an artifact of homogenization, is presently uncertain. Nevertheless, insulin probably activates PKC both by DAG-induced activation, as well as by translocation of the enzyme to the membrane fraction.

Acknowledgements: This research was supported by funds from the Research Service of the Veterans' Administration and National Institutes of Health grant DK38079.

REFERENCES

[1] Cooper, D.R., Konda, T.S., Standaert, M.L., Davis, J.S., Pollet, R.J. and Farese, R.V. (1987) J. Biol. Chem. 262, 3633–3639.
[2] Walaas, S.I., Horn, R.S., Alder, A., Albert, K.A. and Walaas, O. (1987) FEBS Lett. 220, 311–318.
[3] Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621–623.
[4] Kishimoto, A., Kajikawa, N., Shirota, M. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156–1164.
[5] Glynn, B.P., Colliton, J.W., McDermott, J.M. and Witters, L.A. (1986) Biochem. Biophys. Res. Commun. 135, 1119–1125.
[6] Vaartjes, W.J., De Haas, C.G.M. and Van Den Bergh, S.G. (1986) Biochem. Biophys. Res. Commun. 138, 1328–1333.
[7] Vaitukaitas, J., Robbin, J.B., Nieschlag, E. and Ross, G.T. (1971) J. Clin. Endocrinol. Metab. 33, 988–991.
[8] Towbin, H., Staehelin, T. and Greengard, P. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[9] Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K. and Nishizuka, Y. (1987) Science 236, 1116–1120.