Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6211–6213, October 1983
Biochemistry

Phorbol esters stimulate the phosphorylation of receptors for insulin and somatomedin C

(insulin-like growth factor I/phorbol tetradecanoate acetate/phorbol dibutyrate/protein kinase C)

STEVEN JACOBS, NAJI E. SAHYOUN, ALAN R. SALTIEL, AND PEDRO CUATRECASAS

Department of Molecular Biology, The Wellcome Research Laboratories, Research Triangle Park, NC 27709

Contributed by Pedro Cuatrecasas, July 11, 1983

ABSTRACT **The effect of phorbol esters on the extent of phosphorylation of receptors for insulin and somatomedin C (insulin-like growth factor I) was studied in intact IM-9 cells that were labeled by incubation with $H_3{}^{32}PO_4$. The tumor-promoting phorbol esters phorbol tetradecanoate acetate (TPA) and phorbol dibutyrate, but not the inactive 4α-phorbol, enhanced phosphorylation of the β subunit of both receptors approximately 4-fold; 70 nM TPA maximally stimulated phosphorylation of both receptors, whereas concentrations less than or equal to 0.7 nM had no observable effect. Insulin also enhanced the phosphorylation of the β subunit of the insulin receptor, and its effects appeared to be additive to those of TPA. Peptide maps indicated that at least some of the residues phosphorylated by these two agents are distinct. These results suggest a possible role of protein kinase C in regulating insulin and somatomedin C receptors.**

Exposure of intact cells to tumor-promoting phorbol diesters results in a profound decrease in their affinity for insulin (1, 2). The rapidity of this response, which occurs within 5 min, suggests that it may be a relatively proximate response to phorbol esters. Because phorbol esters bind to and directly activate protein kinase C (3, 4), they may affect insulin binding by stimulating protein kinase C-induced phosphorylation of insulin receptors. To test this hypothesis, we investigated the effect of phorbol esters on the extent and pattern of phosphorylation of insulin receptors. Because of the extensive similarities between insulin and somatomedin C (insulin-like growth factor, type I) receptors, both with respect to their structure and their mode of regulation, the effects of phorbol esters on somatomedin C receptor phosphorylation were also studied.

METHODS

αIR-1 is a monoclonal antibody to the insulin receptor. αIR-3 is a monoclonal antibody to the somatomedin C receptor. A410 is a rabbit anti-insulin receptor antiserum. The production and properties of these antibodies have been described (5–7).

Cell Labeling. IM-9 cells (a human B-lymphocyte line) were cultured at a density of 10^5–10^6 cells per ml of RPMI 1640 medium containing 10% fetal calf serum; 10^9 cells were washed twice with phosphate-free RPMI 1640 medium, resuspended in 40 ml of phosphate-free RPMI medium containing 20 mM Hepes (pH 7.4) and 4 mCi of $H_3{}^{32}PO_4$ (1 Ci = 37 GBq), and incubated for 1 hr at 37°C. The cells were then divided into aliquots, which were incubated with no additions, phorbol derivatives, or insulin as indicated in the figure legends. The incubations were stopped by diluting the cells with 4 vol of ice-cold phosphate-buffered saline containing 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 4 mM EDTA, and 0.2 mM sodium vanadate. The cells were washed twice with this buffer.

Immunoprecipitation and $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. The washed labeled cells were solubilized with 1% Triton X-100 in 50 mM Tris·HCl (pH 7.7) containing 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.2 mM sodium vanadate, 5 mM ATP, and 1 mg of bacitracin and 20 μg of phenylmethylsulfonyl fluoride per ml. The cells were centrifuged at 200,000 × *g* for 1 hr. Supernatants were applied to 0.5-ml columns of wheat germ agglutinin-Sepharose, which were washed and then eluted with 1.5 ml of 0.5 M *N*-acetylglucosamine in 50 mM Tris containing 0.1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM NaF, 4 mM EDTA, 0.2 mM sodium vanadate, and 1 mg of bacitracin per ml.

The wheat germ agglutinin eluates were immunoprecipitated as described (6) with the antibodies indicated in the figure legends. The washed immunoprecipitates were lyophilized and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis with the Laemmli buffer system (8) and a 6.5% resolving gel. The relative amount of ^{32}P in receptor bands was determined by densitometry of autoradiograms, or by digesting excised labeled bands in H_2O_2 at 60°C, followed by liquid scintillation counting.

Peptide Maps. One-dimensional peptide maps were performed as described (9). Briefly, labeled bands were excised from a stained 6.5% gel, equilibrated with sample buffer, placed in the well of a 15% gel, and overlaid with 25 μg of chymotrypsin. Proteolysis was carried out during stacking.

RESULTS

IM-9 cells were incubated with $H_3{}^{32}PO_4$ for 1 hr, solubilized, and receptors for insulin and somatomedin C were purified partially on wheat germ agglutinin-Sepharose columns. When cells were incubated in the absence of phorbol esters, an antibody to the insulin receptor (αIR-1) specifically immunoprecipitated a M_r 90,000 phosphorylated band that corresponds to the β subunit of the insulin receptor (Fig. 1, lane 5), whereas an antibody to somatomedin C receptors (αIR-3) specifically immunoprecipitated a phosphorylated band (Fig. 1, lane 9) that was broader and had a slightly higher apparent molecular weight (M_r 92,000–98,000, characteristic of the β subunit of the somatomedin C receptor) than that of the β subunit of the insulin receptor. Exposure of the cells to 70 nM phorbol tetradecanoate acetate (TPA) or 250 nM phorbol dibutyrate for 7 min prior to solubilization resulted in an approximately 4-fold enhancement of the amount of ^{32}P found in both bands (Fig. 1, lanes 7, 8, 11, and 12). In contrast, 4α-phorbol, a phorbol derivative without tumor-promoting activity, which does not bind to the phorbol receptor or activate protein kinase C, failed to enhance

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: TPA, phorbol tetradecanoate acetate.

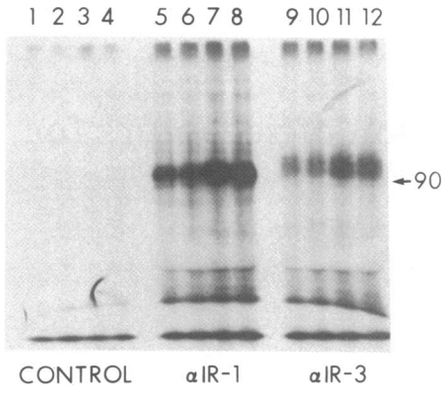

FIG. 1. Effect of phorbol esters on insulin and somatomedin C receptor phosphorylation. IM-9 cells were incubated with $H_3{}^{32}PO_4$ for 1 hr as described and then for an additional 7 min with no additions (lanes 1, 5, and 9), with 5 μM 4α-phorbol (lanes 2, 6, and 10), with 70 nM TPA (lanes 3, 7, and 11), or with 250 nM phorbol dibutyrate (lanes 4, 8, and 12). The cells were washed and solubilized, and receptors for insulin and somatomedin C were purified partially on a wheat germ agglutinin-Sepharose column. The wheat germ agglutinin-Sepharose eluate was then immunoprecipitated with a 1:300 dilution of normal mouse serum (lanes 1–4), 19 μg of αIR-1 per ml (lanes 5–8), or 11 μg of αIR-3 per ml (lanes 9–12) and was subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography. M_r is shown $\times 10^{-3}$.

phosphorylation of these bands (Fig. 1, lanes 6 and 10).

Fig. 2 shows the dose–response relationship for stimulation of the phosphorylation of insulin and somatomedin C receptors by TPA. Maximal effects on both receptors were observed with 70 nM TPA. Clear stimulation also occurred with 7 nM. However, concentrations of TPA lower than 0.7 nM produced no appreciable stimulation.

In intact IM-9 cells, insulin has been shown to stimulate phosphorylation of the β subunit of its receptor (10). To determine if the effects of insulin and phorbol esters are additive, IM-9 cells that had been labeled with $H_3{}^{32}PO_4$ were incubated

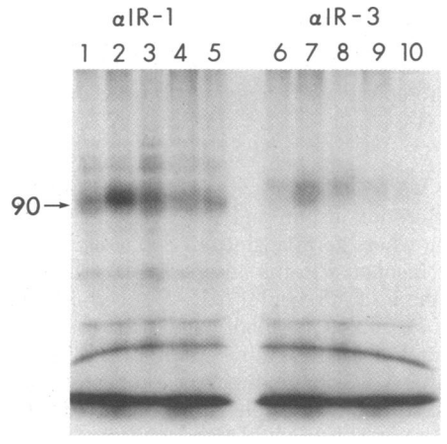

FIG. 2. Dose–response relationship of TPA-enhanced phosphorylation of insulin and somatomedin C receptors. IM-9 cells were incubated with $H_3{}^{32}PO_4$ for 1 hr as described and for an additional 7 min with no additions (lanes 1 and 6), 70 nM TPA (lanes 2 and 7), 7 nM TPA (lanes 3 and 8), 0.7 nM TPA (lanes 4 and 9), or 0.07 nM TPA (lanes 5 and 10). The cells were then washed and solubilized, and receptors for insulin and somatomedin C were purified partially on a wheat germ agglutinin-Sepharose affinity column. The wheat germ agglutinin-Sepharose eluates were then immunoprecipitated with 19 μg of αIR-1 per ml (lanes 1–5) or 11 μg of αIR-3 per ml (lanes 6–10). The immunoprecipitates were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis and analyzed by autoradiography. M_r is shown $\times 10^{-3}$.

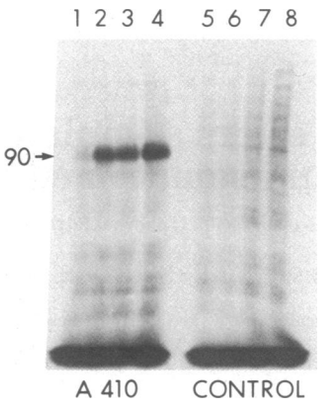

FIG. 3. Additive effects of insulin and TPA on enhancement of insulin receptor phosphorylation. IM-9 cells were incubated for 1 hr with $H_3{}^{32}PO_4$ as described and for an additional 7 min with no additions (lanes 1 and 5), with 1 μg of insulin per ml (lanes 2 and 6), with 70 ng of TPA per ml (lanes 3 and 7), or with 1 μg of insulin and 70 ng of TPA per ml simultaneously added (lanes 4 and 8). The cells were washed and solubilized, and insulin receptors were purified partially on a wheat germ agglutinin-Sepharose column. The eluate from the wheat germ agglutinin-Sepharose column was then immunoprecipitated with A410 (lanes 1–4) or normal rabbit serum (lanes 5–8) and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. Shown is an autoradiogram of the dried gel. The amounts of ^{32}P in the M_r 90,000 bands of lanes 1–4 were 54 cpm, 123 cpm, 105 cpm, and 183 cpm, respectively. M_r is shown $\times 10^{-3}$.

with no additions, with insulin, with TPA, or with TPA and insulin simultaneously added (Fig. 3). Both insulin alone (lane 2) and TPA alone (lane 3) stimulated phosphorylation of the β subunit of the insulin receptor. When added simultaneously (lane 4) their effects appeared to be additive with neither potentiation nor inhibition.

To determine the site-specificity of insulin- and TPA-directed phosphorylation, IM-9 cells were phosphorylated under the four conditions used in Fig. 3, and peptide maps of the β subunit of the insulin receptor were performed by using the Cleveland technique (9) (Fig. 4). Both insulin and TPA enhanced the phosphorylation of several peptides. Phosphorylation of some peptides was enhanced by both agents; however, phosphorylation of some was selectively enhanced by either

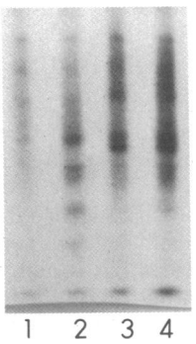

FIG. 4. Peptide maps of the β subunit of insulin receptor phosphorylated in the presence of insulin and TPA. Labeled IM-9 cells were incubated with no additions (lane 1), with 70 nM TPA (lane 2), with 1 μg of insulin per ml (lane 3), and with TPA and insulin simultaneously added (lane 4) as described in the legend to Fig. 3. The cells were then washed and solubilized, and the insulin receptors were purified on a wheat germ agglutinin-Sepharose column followed by immunoprecipitation by A410. The immunoprecipitates were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. Bands corresponding to the labeled M_r 90,000 subunit were excised and subjected to one-dimensional peptide mapping with 25 μg of α-chymotrypsin as described.

insulin or TPA. Thus, at least some of the residues on the β subunit of the insulin receptor are differentially phosphorylated by insulin and phorbol esters.

DISCUSSION

The main findings of these studies are that tumor-promoting phorbol esters enhance the phosphorylation of both insulin and somatomedin C receptors. The concentrations of TPA at which these effects were observed were somewhat higher than those required to bind to the phorbol ester receptor (11, 12) and activate protein kinase C (3, 4). There are two likely explanations for this. TPA is known to nonspecifically partition into a hydrophobic environment (10). Because a high density of cells was used, the free concentration of TPA available to interact with its receptor may have been considerably less than the total amount added. Second, phosphorylation was measured after a brief exposure to TPA, possibly before full equilibration with its receptor was possible. The fact that TPA and phorbol dibutyrate activate phosphorylation of the receptors while 4α-phorbol does not suggests that these agents are working through the phorbol receptor. However, on the basis of these studies, we cannot determine if protein kinase C directly phosphorylates insulin and somatomedin C receptors or if enhanced phosphorylation results from the activation of an intermediate kinase or from the inhibition of an intermediate phosphatase. *In vitro* phosphorylation studies using purified protein kinase C and purified receptors may help to distinguish between these possibilities.

Peptide maps (Fig. 4) indicate that TPA enhances the phosphorylation of at least some residues whose phosphorylation is not enhanced by insulin and vice versa. Whereas some large peptide fragments are phosphorylated by both TPA and insulin, these peptides could contain more than one phosphorylation site. Thus, the possibility remains that those residues whose phosphorylation is enhanced by TPA are totally distinct from those whose phosphorylation is enhanced by insulin.

When TPA and insulin were added simultaneously, enhancement of phosphorylation appeared to be additive (Fig. 3). Neither agent appeared to either potentiate or inhibit the effects of the other. However, because we were unable to determine what fraction of the receptors were phosphorylated, these results should be interpreted with caution. If only a small fraction of the receptors were modified by TPA, a large difference in the enhancement of phosphorylation by insulin would not be expected. Furthermore, because a high concentration of insulin (1 μg per ml) was used in this study, an effect of TPA on the affinity for insulin could easily be missed.

Protein kinase C is present in a wide variety of tissues, with some of the highest levels occurring in lymphocytes (13). Therefore, it is interesting that, in several cell lines tested, the magnitude of the effects of phorbol esters on insulin binding affinity was greatest in IM-9 cells (1). Little or no effect was observed in fibroblasts or HeLa cells (14, 15).

The activity of protein kinase C is regulated by calcium, phosphatidylserine, and diacylglycerol (13). A variety of hormones have been postulated to exert their effects, at least in part, through the activation of protein kinase C (13). In view of the present findings, it is interesting to speculate that protein kinase C may mediate the alterations of affinity of insulin and somatomedin C, which occur under a variety of physiological and pathological circumstances.

We thank Stella Cook for excellent technical assistance.

1. Grunberger, G. & Gordon, P. (1982) *Am. J. Physiol.* **243,** E319–E324.
2. Thomopoulos, P., Testa, U., Gourdin, M.-F., Hervy, C., Titeux, M. & Vainchenker, W. (1982) *Eur. J. Biochem.* **29,** 389–393.
3. Niedel, J. E., Kuhn, L. J. & Vandenbark, G. R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 36–40.
4. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 7847–7851.
5. Kull, F. C., Jr., Jacobs, S., Su, Y.-F. & Cuatrecasas, P. (1982) *Biochem. Biophys. Res. Commun.* **106,** 1019–1026.
6. Kull, F. C., Jr., Jacobs, S., Su, Y.-F., Svoboda, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1983) *J. Biol. Chem.* **258,** 6561–6566.
7. Jacobs, S., Chang, K.-J. & Cuatrecasas, P. (1978) *Science* **200,** 1283–1284.
8. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
9. Cleveland, D. W., Fisher, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
10. Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982) *Science* **215,** 185–187.
11. Driedger, P. E. & Blumberg, P. M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 567–571.
12. Shoyab, M. & Todaro, G. J. (1980) *Nature (London)* **288,** 451–455.
13. Nishizuka, Y. & Takai, Y. (1981) in *Protein Phoshorylation,* Cold Spring Harbor Conferences on Cell Proliferation, eds. Rosen, O. & Krebs, E. G. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 8, pp. 237–249.
14. Shoyab, M., DeLarco, J. E. & Todaro, G. J. (1979) *Nature (London)* **279,** 387–391.
15. Lee, L.-S. & Weinstein, I. B. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5168–5172.