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Effects of phorbol esters on insulin-induced activation of phosphatidylinositol 3-kinase, glucose transport, and glycogen synthase in rat adipocytes

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Abstract In rat adipocytes, phorbol ester-induced activation of PKC did not inhibit insulin signalling through IRS-1-dependent phosphatidylinositol (PI) 3-kinase activation. Moreover, phorbol esters alone provoked an increase in membrane PI 3-kinase activity. These findings may be relevant to the failure of phorbol esters to inhibit insulin effects on glucose transport and glycogen synthesis in rat adipocytes.

Key words: Insulin; Phorbol ester; Phosphatidylinositol 3-kinase; Glucose transport; Adipocyte; IRS-1

1. Introduction

Phorbol esters, like insulin, stimulate the phosphorylation of human insulin receptors in 3T3 fibroblasts [1] and CHO cells [2], but the consequences are uncertain. In CHO cells overexpressing protein kinase C-α (PKC-α), phorbol esters inhibit insulin-induced activation of phosphatidylinositol (PI)-3 kinase without changes in insulin receptor kinase activity, however, PI 3-kinase inhibition is not observed in untransfected CHO cells containing normal PKC-α levels [3]. In rat adipocytes, phorbol esters inhibit insulin binding and tyrosine kinase activity of purified insulin receptors [4], but have little [4,5] or no [6,7] effect on insulin-stimulated glucose transport or glycogen synthase activation [8], processes that may require PI 3-kinase activation [8,9]. Presently, we further examined the interacting effects of phorbol esters and insulin on PI 3-kinase and subsequent metabolic processes in rat adipocytes.

2. Materials and methods

Adipocytes were prepared by collagenase digestion of rat epididymal fat pads as described [9], equilibrated at 37°C in glucose-free Krebs Ringer phosphate (KRP) buffer containing 1% bovine serum albumin (BSA; Sigma), and treated for 10 min with vehicle or 500 nM tetradecanoyl phorbol-13-acetate (TPA; Sigma), followed by 300 nM insulin (Elanco) for 15 min, unless indicated otherwise. After incubation, membranes were obtained as described [9] by homogenization of cells in buffer A containing 20 mM Tris-HCl (pH, 7.4), 5 mM EGTA, 5 mM EDTA, 255 mM sucrose, 1 mM Na vanadate, 1 mM Na pyrophosphate, 1 mM NaF, 1 mM PMSF, 20 μg/ml aprotinin and 20 μg/ml leupeptin, followed by centrifugation at 300 000 × *g* for 45 min. Cell lysates were obtained by homogenization in buffer A containing 150 mM NaCl, 1% Triton X-100 and 0.5% Nonidet, and then immunoprecipitated with antiphosphotyrosine (α-PY) monoclonal antibodies [Upstate Biotechnology Incorporated (UBI) or Transduction Laboratories], or polyclonal α-IRS-1 antiserum (generously supplied by Dr. Alan Saltiel). Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with α-PI 3-kinase (UBI) or α-PY antibodies. Membranes and immunoprecipitates were also assayed for PI 3-kinase activity as described [9] by incubation for 10 min in buffer containing 8 mM Tris-HCl (pH, 7.4), 100 mM NaCl, 3.3 mM EDTA, 14 mM $MgCl_2$, 0.13 mM EGTA, 20 μg/ml phosphatidylinositol, and 58 μM [γ-^{32}P]ATP (20 μCi; NEN). After incubation, ^{32}P-labeled phosphatidylinositol 3-phosphate was extracted into $CHCl_3/CH_3OH$ (2:1) and purified by thin-layer chromatography (TLC) [9]. Autoradiograms and immunoblots [developed by ECL (Amersham)] were quantitated by autoradiography and laser scanning densitometry.

2-[^{3}H]Deoxyglucose (2-DOG) uptake was measured (see [8]) by incubation of cells for 30 min in glucose-free KRP with or without 500 nM TPA and/or 10 nM insulin, as indicated, followed by addition of 0.1 mM 2-[^{3}H]DOG (1 μCi; NEN) and measurement of uptake over 1 min. Glycogen synthesis was assessed by two methods: (a) as described [8] by measuring fractional activity of glycogen synthase [i.e. as assayed with or without 10 mM glucose 6-phosphate] in homogenates obtained from adipocytes incubated with or without 500 nM TPA and/or 10 nM insulin for 20 min in KRP buffer containing 0 or 1 mM glucose; and (b) by incubating adipocytes for 30 min in KRP buffer containing 5 mM glucose and 2 μCi D-[U-^{14}C]glucose (NEN), and measuring incorporation of label into glycogen by heating cell extracts in 4 N KOH at 100°C for 30 min and trapping glycogen on filter papers which were washed with cold ethanol.

3. Results

As shown in Figs. 1 and 2, the addition of 500 nM TPA 20 min before insulin failed to alter basal or insulin-induced increases in the co-immunoprecipitation of immunoreactive PI 3-kinase with either IRS-1 or total PY-containing proteins in total cell lysates of rat adipocytes. Similarly, insulin-induced increases in PI 3-kinase enzyme activity in both α-IRS-1 and α-PY immunoprecipitates were not diminished by TPA pretreatment (Fig. 2).

Since TPA failed to diminish IRS-1-dependent immunoprecipitation and activation of PI 3-kinase, it was of interest to determine whether insulin effects on overall tyrosine phosphorylation of IRS-1 would be altered by TPA. Accordingly, as shown in Fig. 2, TPA failed to diminish insulin effects on tyrosine-phosphorylation of IRS-1.

In addition to finding that insulin effects on overall IRS-1 tyrosine phosphorylation, and IRS-1-dependent PI 3-kinase activation were not inhibited by TPA, we also surprisingly found that TPA alone increased PI 3-kinase activity in adipocyte membranes nearly as well as insulin (Fig. 3).

Since TPA failed to inhibit insulin effects on IRS-1-dependent activation of PI 3-kinase, it was of interest to examine the effects of TPA on metabolic processes that are wortmannin-sensitive (and thus possibly PI 3-kinase-dependent) in rat adipocytes, viz. glucose transport and glycogen synthesis. As shown in Fig. 4, TPA increased 2-DOG uptake modestly relative to insulin, and, moreover, had no effect on: (a) insulin-stimulated 2-DOG uptake; (b) basal- or insulin-stimulated

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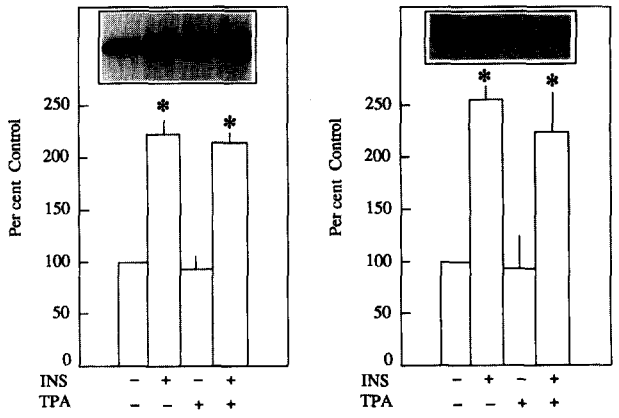

Fig. 1. Effects of TPA and insulin on levels of immunoreactive PI 3-kinase in α-IRS-1 (left) and α-PY (right) immunoprecipitates. Representative immunoblots of the p85 subunit of PI 3-kinase are shown in insets. Bars depict mean ± S.E.M. results of densitometric scans of 3–5 experiments with the control set at 100%. Asterisk indicates $p < 0.05$ (paired t-test).

[U-^{14}C]glucose incorporation into glycogen, or (c) basal or insulin-stimulated glycogen synthase activity (later data not shown, but see [9]).

4. Discussion

Although insulin receptor tyrosine kinase is inhibited by TPA in certain circumstances, we presently found in rat adipocytes that TPA had relatively little or no effect on both insulin signalling through IRS-1-dependent PI 3-kinase activation, and subsequent activation of putative downstream metabolic processes, viz. glucose transport and glycogen synthesis. Effects of TPA and PKC on this signalling system in other cell types are not entirely clear, but in rodent skeletal muscles, both TPA-induced [10,11] and denervation-induced [12,13] PKC activation do not inhibit insulin-induced activation of insulin receptor tyrosine kinase or PI 3-kinase. Thus, the inhibitory effect of TPA on PI 3-kinase activation observed in CHO cells overexpressing PKC-α [3] may not be relevant to adipose and muscle tissues of intact rodents.

The failure of TPA to inhibit glycogen synthesis in rat adipocytes contrasts with inhibition observed in rat skeletal muscle ([14]; also confirmed in our laboratory) and liver [15]. The reason for this difference is uncertain. However, in view of the present and previous [10–13] findings, the inhibitory effects of TPA on glycogen synthesis in muscle and liver may be provoked through mechanisms independent of IRS-1-dependent PI 3-kinase activation, perhaps by phosphorylation of distal regulatory factors and/or glycogen synthase itself.

The stimulatory effect of TPA on adipocyte membrane PI 3-kinase activity is of interest, as it has also been noted in platelets [16] and 3T3/L1 adipocytes [17], and such cross-talk between PKC and PI 3-kinase has many implications. In 3T3/L1 adipocytes, TPA-induced activation of PI 3-kinase may explain how TPA stimulates glucose transport, as this effect of TPA, like that of insulin, is blocked by wortmannin ([17]; also confirmed in our laboratory). This explanation, however, is not applicable to the rat adipocyte, wherein TPA effects on glycose transport are not inhibited by wortmannin [18], and PKC, apparently in its own right, activates glucose transport. With respect to glycogen synthase, the failure of TPA to stimulate its activity suggests that simple increases in membrane PI 3-kinase activity may not be sufficient to activate glycogen synthase.

The mechanism whereby TPA activates PI 3-kinase in adipocyte membranes is presently unclear; however, it is unlikely that PKC contributes importantly to insulin effects on membrane PI 3-kinase. Indeed, in the rat adipocyte, the major effects of insulin on PKC appear to be downstream of PI 3-kinase, which seems to be important for activation of a phospholipase D that hydrolyzes phosphatidylcholine in the plasma membrane [18], and increases D 3-phosphate derivatives of PI, which in turn, activate PKC. Further studies will be

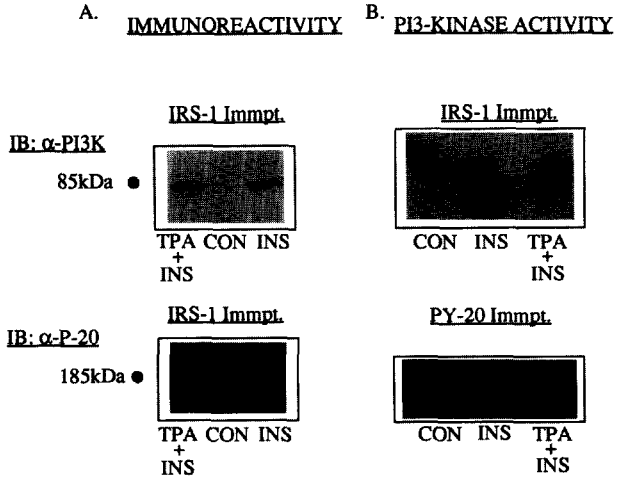

Fig. 2. (A) Effects of TPA on insulin-induced increases in PI 3-kinase levels (A, top) and PY content of IRS-1 (A, bottom) in α-IRS-1 immunoprecipitates, and (B) PI 3-kinase enzyme activity in α-IRS-1 immunoprecipitates (B, top) and α-PY immunoprecipitates (B, bottom). 1B designates immunoblots developed with α-PI-3 kinase and α-PY antibodies.

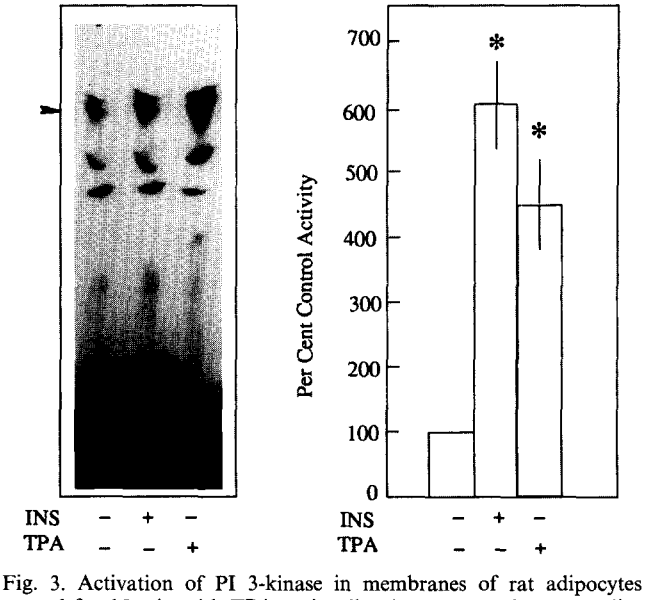

Fig. 3. Activation of PI 3-kinase in membranes of rat adipocytes treated for 15 min with TPA or insulin. A representative autoradiogram is shown at the left, and mean ± S.E.M. results of 5 experiments are shown to the right. Asterisk indicates $p < 0.05$ (paired t-test).

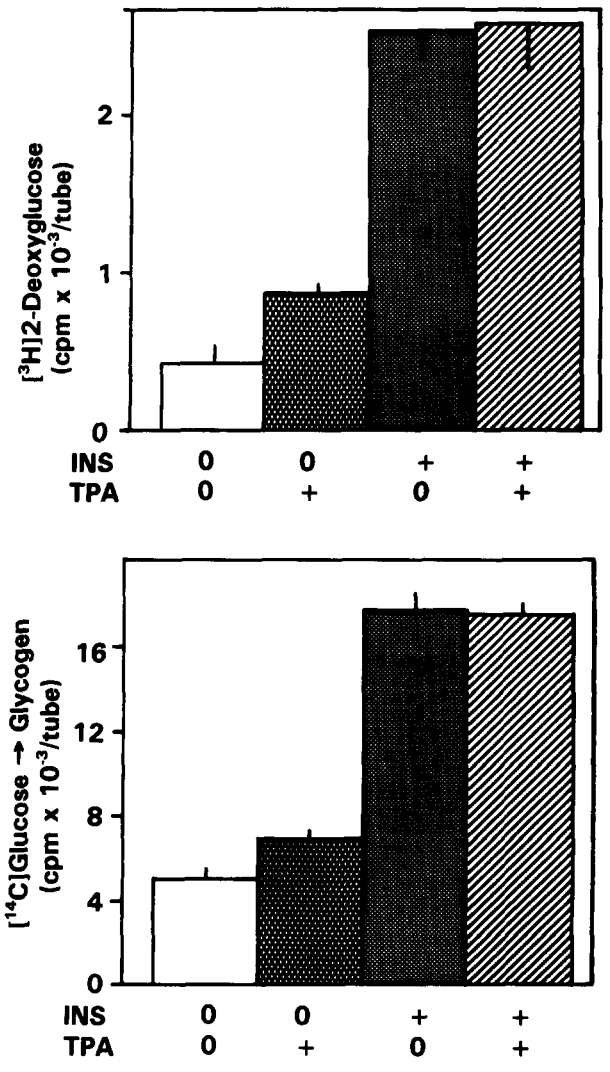

Fig. 4. Effects of TPA on control and insulin-stimulated 2-[^{3}H]deoxyglucose uptake (top) and [^{14}C]glucose incorporation into glycogen (bottom). Values are mean ± S.E.M. of 4 determinations.

needed to determine how TPA increases membrane PI 3-kinase activity in rat adipocytes.

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