

# Potentialiation of specific association of insulin with HepG2 cells by phorbol esters

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The effects of tumour-promoting phorbol esters on the receptor-mediated endocytosis of insulin were investigated in the human hepatoma cell line HepG2. Treatment of these cells with the biologically active phorbol 12-*O*-tetradecanoylphorbol 13-acetate (TPA), but not with the non-tumour-promoting analogue 4 $\alpha$ -phorbol 12,13-didecanoate, resulted in dramatic morphological changes, which were accompanied by a 1.5-2.5-fold increase in specific <sup>125</sup>I-insulin association with the cells at 37 °C. This increase in insulin binding was not observed when the binding reaction was performed at 4 °C. The potentiation of <sup>125</sup>I-insulin association with TPA-treated cells at 37 °C could be completely accounted for by an increase in the intracellular pool of internalized insulin; there was no concomitant increase in cell-surface insulin binding. Dissociation studies showed that the enhanced internalization of insulin by cells after treatment with TPA resulted from a decrease in the rate of intracellular processing of the insulin after receptor-mediated endocytosis. The phorbol-ester-induced enhancement of internalized insulin in HepG2 cells was additive with the potentiation of endocytosed insulin induced by both the lysosomotropic reagent chloroquine and the ionophore monensin; this indicates that TPA affects the intracellular processing of the insulin receptor at a point other than those disrupted by either of these two reagents. The potentiation of insulin receptor internalization by tumour-promoting phorbol esters could be completely mimicked by treatment with phospholipase C, but not with phospholipase A, and partially mimicked by treatment with the synthetic diacylglycerol 1-oleoyl-2-acetyl-glycerol. By these criteria, the effects of phorbol esters on the insulin receptor in HepG2 cells appear to be mediated through protein kinase C. These results support the concept that the activation of protein kinase C by treatment with phorbol esters causes a perturbation of the insulin-receptor-mediated endocytotic pathway in HepG2 cells, reflected in a long-term decreased rate of dissociation of internalized insulin by the phorbol-ester-treated cells.

## INTRODUCTION

Tumour-promoting phorbol esters have been shown to induce a multitude of effects, both morphological and biochemical, when added to mammalian cell lines (for reviews, see Diamond *et al.*, 1978; Blumberg, 1980). Although the direct mechanism by which phorbol esters induce cell transformation remains unclear, it is suggested that the biologically potent promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) mediates intracellular events which mimic those of endogenous polypeptide growth factors. There is evidence that these effects are due, at least in part, to the substitution of TPA for the putative intracellular mediator, diacylglycerol. In intact cells, diacylglycerol is produced as a result of phosphatidylinositol turnover, a metabolic pathway which has been implicated in the actions of several hormones (Michell, 1975; Berridge & Irvine, 1984). Work by Nishizuka and others suggest that a Ca<sup>2+</sup>, diacylglycerol- and phospholipid-dependent protein kinase is responsible for the transduction of the effects of both TPA and diacylglycerol, via an enzymic activation of the protein kinase, termed protein kinase C, and subsequent phosphorylation of cellular proteins (Kishimoto *et al.*, 1980; Castagna *et al.*, 1982; Lapetina *et al.*, 1985). Other studies have also shown that the receptor for TPA

co-purifies with protein kinase C, further reinforcing the hypothesis that this widely distributed enzyme plays a role in the intracellular mediation of tumour promotion or cell transformation (Niedel *et al.*, 1983).

A number of cellular proteins have been shown to undergo phosphorylation upon exposure of intact cells to TPA. Although the biological function of several of these proteins remains to be identified (Sano *et al.*, 1983; Seiss *et al.*, 1983; Lapetina *et al.*, 1985), the TPA-promoted phosphorylations which have been shown to occur at the receptors for epidermal growth factor (Cochet *et al.*, 1984; Iwashita & Fox, 1984; Friedman *et al.*, 1984), insulin-like growth factor (Jacobs *et al.*, 1983), transferrin (May *et al.*, 1984), catecholamines (Kelleher *et al.*, 1984; Sibley *et al.*, 1984) and insulin (Jacobs *et al.*, 1983; Takayama *et al.*, 1984) are frequently accompanied by changes in receptor functionality or subcellular distribution, implicating protein kinase C in the modification of receptor-mediated actions.

Studies aimed at determining the sites of action of the protein-kinase-C-mediated phosphorylation of the epidermal-growth-factor receptor and the insulin receptor have shown a specific increase in the phosphorylation of serine and threonine residues (Jacobs *et al.*, 1983; Cochet *et al.*, 1984; Iwashita & Fox, 1984; Friedman *et al.*, 1984). In many cases, this increased receptor phosphorylation

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; OAG, 1-oleoyl-2-acetyl-glycerol; MEM, Modified Eagle's Medium.

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has been shown to be accompanied by decreases in either high-affinity hormone binding or receptor-mediated physiological function; these effects appear to vary significantly from one cell line to the next. These changes may be due, in part, to the attenuation of the receptor-specific tyrosine kinase activity, which has been shown to be associated with both of these receptors; however, a direct correlation between both effects has yet to be shown.

In the present paper, we demonstrate that TPA treatment of the human hepatoma cell line HepG2 results in an increase in specific  $^{125}\text{I}$ -insulin association with intact cells at physiological temperatures. This increase in cell-associated hormone arises not from a change in either the number or the affinity of cell-surface insulin receptors, but rather from an increase in the pool of internalized insulin in the TPA-treated cells, mediated via a decrease in the rate of intracellular processing of insulin. Furthermore, it is possible to mimic the effects of TPA in these cells through the addition of the diacylglycerol derivative 1-oleoyl-2-acetyl-glycerol (OAG) and through the production of diacylglycerol by the addition of exogenous phospholipase C, demonstrating that this perturbation of the pathway of insulin-receptor-mediated endocytosis in HepG2 cells is mediated through protein kinase C.

## EXPERIMENTAL

### Materials

$^{125}\text{I}$ -insulin (2200 Ci/mmol) was obtained from New England Nuclear. TPA, 4 $\alpha$ -phorbol 12,13-didecanoate, cycloheximide, chloroquine, phospholipase A and bovine serum albumin were from Sigma. Insulin was obtained from Elanco. Tissue-culture media and supplies were from Gibco. Hepes, monensin and phospholipase C were purchased from Calbiochem, and trypsin was from Worthington.

HepG2 cells were provided by Dr B. B. Knowles, Wistar Institute, Philadelphia (Knowles *et al.*, 1980). OAG was a gift from Dr. Y. Nishizuka, Kobe University, Japan (Kaibuchi *et al.*, 1983).

### Cell culture

HepG2 cells were grown as monolayer cultures to a final density of  $10^6$  cells/well in 24-well culture dishes in Modified Eagle's Medium with Earle's balanced salt solution (MEM; Gibco), supplemented with 1 mM non-essential amino acids, 10% (v/v) fetal-calf serum, 2 mM-glutamine, 0.1 mg of streptomycin/ml and 100 units of penicillin/ml. A humidified atmosphere of  $\text{CO}_2$ /air (2:23) was maintained for cell growth at 37 °C. Cells were passaged at 96 h after being dispersed with 0.25% trypsin/1 mM-EDTA. Confluent monolayers were utilized in all experiments.

### $^{125}\text{I}$ -insulin binding

Insulin binding was performed as described in the Figure legends on cell monolayers in 13 mm wells in 0.25 ml of MEM with no bicarbonate, including 20 mM-Hepes and 0.1% albumin ('MEM/Hepes buffer'), pH 7.4, in air, in the presence of 0.12 nM- $^{125}\text{I}$ -insulin. Non-specific binding, determined with the addition of 10  $\mu\text{M}$  unlabelled insulin, was less than 5% of the total bound. After binding, cells were quickly washed three times in cold Hanks buffer (Hanks balanced salt solution

containing 20  $\mu\text{M}$ -Hepes and 0.1% albumin, pH 7.4), removed from the dish by treatment with 0.5 mg of trypsin/ml for 30 min at 25 °C, or with 0.1 M-NaOH, and counted for radioactivity in a Beckman gamma counter. For Scatchard analysis, binding was performed at 4 °C overnight and data were analysed by the method of Munsen & Rodbard (1980), by using the LIGAND computer program.

For internalization of the receptor, cell monolayers were incubated with  $^{125}\text{I}$ -insulin at 37 °C for various times as indicated in the Figure legends. At the end of the incubation, cells were washed three times with cold Hanks buffer and the cell-surface receptor binding was inactivated by trypsin (0.5 mg/ml for 30 min at 25 °C). The cells were then removed from the plate, washed twice with cold Hanks buffer, and the  $^{125}\text{I}$  present in the cell pellet was counted to quantify internalized receptor. These trypsin-treatment conditions were adjusted such that 95% of the cell-surface-bound insulin was removed when the binding was performed at 4 °C to inhibit internalization. Total cellular receptor was measured in parallel plates by counting cells for radioactivity directly after removal from the plate, with no further washing. In experiments designed to measure the effects of various reagents on receptor binding and internalization, cells were first incubated with the reagent as described in the Figure legends before the addition of  $^{125}\text{I}$ -insulin to measure binding. Protein concentration was determined by the method of Lowry *et al.* (1951).

### Dissociation of bound $^{125}\text{I}$ -insulin

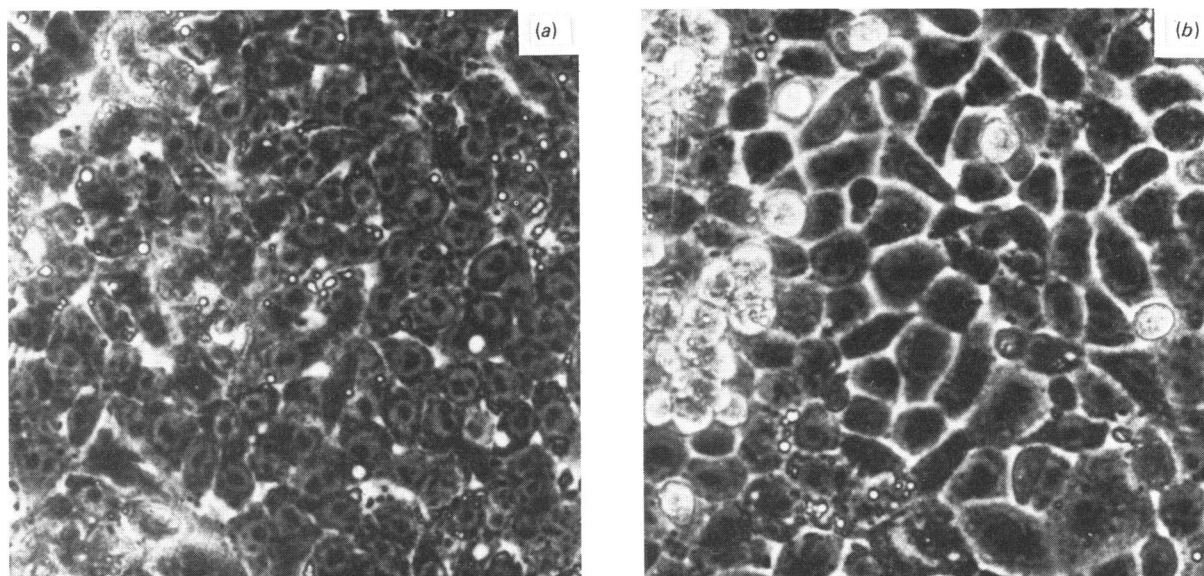
$^{125}\text{I}$ -insulin was bound to HepG2 monolayers at 37 °C for 30 min as described above. After the removal of free  $^{125}\text{I}$ -insulin, 0.25 ml of fresh MEM/Hepes buffer was added. At various times, samples of the supernatant were removed from the monolayer and the dissociated  $^{125}\text{I}$  present in the supernatant was measured. The percentage of radioactivity present as protein was determined by precipitation with 10% (v/v) trichloroacetic acid with 1% albumin (Fraction V) as the carrier. The amount of cell-associated radioactivity remaining at various times was determined by quickly washing the monolayers with cold Hanks buffer before removing them from the plate with 0.1 M-NaOH and counting for radioactivity.

### Phorbol ester treatment

TPA and 4 $\alpha$ -phorbol 12,13-didecanoate were each dissolved in ethanol and stored in the dark at -20 °C. HepG2 monolayers were washed twice in MEM/20 mM-Hepes/0.1% albumin, pH 7.4, before the addition of the phorbol ester, diluted in this same buffer. All incubations with TPA were performed for 2 h at 37 °C, unless otherwise noted in Figure legends. At the end of the incubation time, the cells were washed three times in cold MEM buffer before  $^{125}\text{I}$ -insulin binding and internalization were measured as described above. The ethanol added with the TPA had no effect on the experimental results. The results presented here were also independent of whether or not the excess TPA was removed by washing before  $^{125}\text{I}$ -insulin binding to the cells.

### Phospholipase treatment

Washed HepG2 monolayers were incubated in MEM/Hepes buffer for 30 min at 37 °C with either phospholipase C or phospholipase A at a final concentration of 10 units/ml. After this time,  $^{125}\text{I}$ -insulin was added to



**Fig. 1. Morphological changes induced in HepG2 cells by treatment with phorbol esters**

Cells were incubated for 2 h at 37 °C with (a) 1  $\mu\text{M}$ -4 $\alpha$ -phorbol didecanoate or (b) 1  $\mu\text{M}$ -TPA.

the cells, and binding and internalization were measured for an additional 30 min at 37 °C as described above.

#### Treatment with OAG

OAG was stored as a chloroform solution of 100 mg/ml at -20 °C. For use with HepG2 cells, the chloroform was evaporated from a sample of OAG under a stream of N<sub>2</sub> before the addition of MEM/Hepes buffer. The mixture was sonicated on ice to the point of minimum turbidity (approx. 10 min). Cells were preincubated for various times with OAG concentrations of 0.01–0.2 mg/ml before the addition of <sup>125</sup>I-insulin for cell-binding measurements.

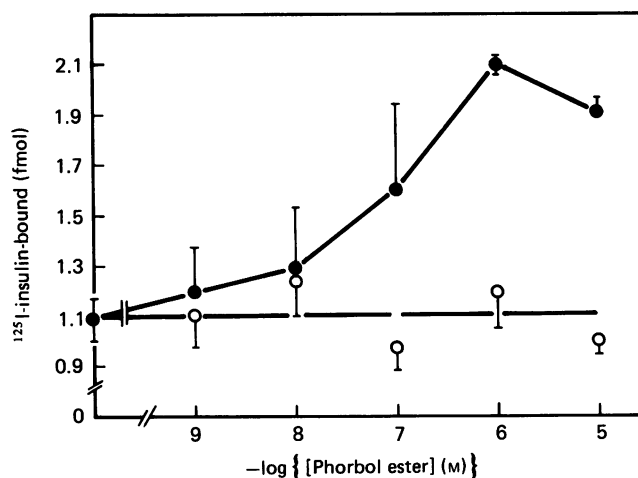
#### Photography

HepG2 cells were grown in 60 mm culture dishes to a stage of early confluence. Incubations with phorbol esters were performed as described in Fig. 1 legend in 2 ml of MEM/Hepes buffer. Cells were photographed from a Zeiss microscope equipped with a Nikon camera and Kodak PX-135 film.

## RESULTS

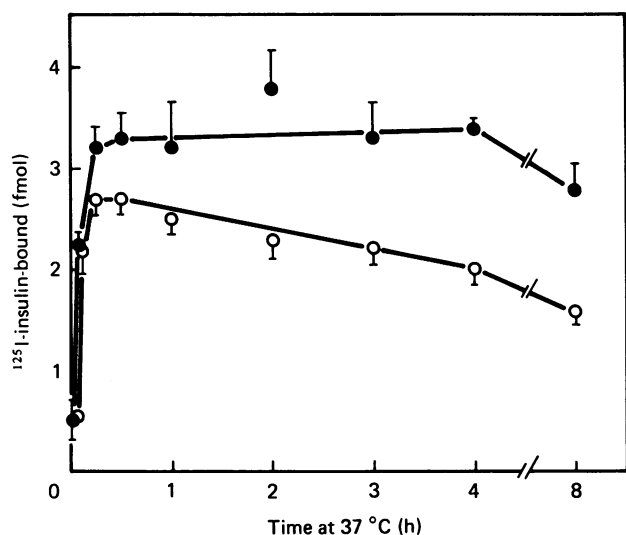
Treatment of various cells with tumour-promoting phorbol esters results in characteristic morphological changes, primarily reflecting changes in the cell membrane compartments (Diamond *et al.*, 1978; Blumberg, 1980, 1981). As shown in Fig. 1, the treatment of the HepG2 hepatoma cell line with TPA, a tumour-promoting phorbol ester, resulted in dramatic changes in the cell morphology. The TPA-treated cells in monolayer culture maintained a rounder shape, with membranes that were visually more sharply defined by phase-contrast light microscopy than the relatively flat, spread-out, control cells. These morphological changes were dependent on the time of incubation with TPA and on TPA concentration, with the half-maximal effects of 1  $\mu\text{M}$ -TPA observed at approx. 1 h. In contrast, treatment of these cells with

the non-tumour-promoting phorbol esters 4 $\alpha$ -phorbol didecanoate and TPA 4-*O*-methyl ester resulted in no changes in the cell morphology, as assessed by phase-contrast light microscopy. These dose-dependent TPA-induced changes in the morphology of HepG2 cells were accompanied by an increase in specific insulin association with the cell monolayer at 37 °C, as shown in Fig. 2. Cells preincubated for 2 h with various concentrations of TPA showed a significant increase in cell-associated hormone after a subsequent 1 h incubation with 0.1 nM-<sup>125</sup>I-insulin. A maximal increase in <sup>125</sup>I-insulin



**Fig. 2. Effect of phorbol esters on <sup>125</sup>I-insulin binding to HepG2 cells**

Cells ( $10^6$ ) were incubated with increasing concentrations of 4 $\alpha$ -phorbol didecanoate (○) or TPA (●) for 2 h at 37 °C before <sup>125</sup>I-insulin binding was performed for 1 h at 37 °C as described in the Experimental section. <sup>125</sup>I-insulin was 3000 c.p.m./fmol. Data shown are means  $\pm$  s.d. for triplicate determinations, representative of three experiments.



**Fig. 3.** Time course of  $^{125}\text{I}$ -insulin binding of HepG2 cells

Cells ( $10^6$ ) were incubated with either  $4\alpha$ -phorbol didecanoate (○) or TPA (●) for 2 h at  $37^\circ\text{C}$ , then washed, and  $^{125}\text{I}$ -insulin binding was performed for the times shown at  $37^\circ\text{C}$ . The experiment shown is representative of four performed; data are means  $\pm$  S.D. for triplicate samples.

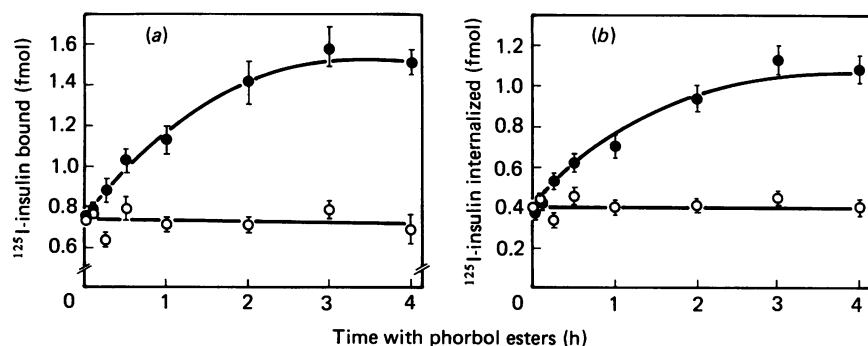
association to 1.9-fold ( $P < 0.01$ ) occurred in response to preincubation with  $1\ \mu\text{M}$ -TPA, with an  $\text{ED}_{50}$  of approx.  $0.1\ \mu\text{M}$ . This dose-response of the increase in  $^{125}\text{I}$ -insulin binding to HepG2 cells as a function of TPA concentration paralleled that of the TPA-induced morphological changes shown in Fig. 1. The increase in  $^{125}\text{I}$ -insulin association was not accompanied by any increase in total cellular protein (results not shown). Parallel incubations with the biologically inactive phorbol ester  $4\alpha$ -phorbol didecanoate at concentrations up to  $10\ \mu\text{M}$  resulted in no change in the subsequent association of  $^{125}\text{I}$ -insulin with HepG2 cells (Fig. 2).

The enhancement of specific  $^{125}\text{I}$ -insulin association with HepG2 cells at  $37^\circ\text{C}$  by preincubation with TPA

was not reversed by the removal of excess TPA from the monolayer. This is shown in Fig. 3, where cells were preincubated with  $1\ \mu\text{M}$ -TPA for 2 h before excess TPA was washed away and  $^{125}\text{I}$ -insulin was bound to the cells at  $37^\circ\text{C}$ . In control cells or in cells treated with the biologically inactive  $4\alpha$ -phorbol didecanoate, the binding of  $^{125}\text{I}$ -insulin to the cells increased rapidly for the first 15 min, after which it gradually fell to 63% of maximal binding after 8 h with  $^{125}\text{I}$ -insulin. In TPA-treated cells, the amount of  $^{125}\text{I}$ -insulin binding also rose to a maximal value within 15 min, but then remained at a plateau for the next several hours; the maximal binding was higher and the decrease in bound  $^{125}\text{I}$ -insulin with time was slower in TPA-treated cells than in controls. Both of these aspects of  $^{125}\text{I}$ -insulin association with TPA-treated cells contributed to the enhancement of binding observed under these conditions.

Like the morphological changes depicted in Fig. 1, the TPA-induced increase in  $^{125}\text{I}$ -insulin association with HepG2 monolayers was dependent on the time of preincubation of the cells with TPA at  $37^\circ\text{C}$ , as shown in Fig. 4(a). The potentiation of specific cell-associated  $^{125}\text{I}$ -insulin induced by  $1\ \mu\text{M}$ -TPA continued to increase for approx. 2 h. Thereafter, the amount of  $^{125}\text{I}$ -insulin associated with the cell monolayer remained constant, with a maximal increase of 2.2-fold over control cells. The half-time of this increase was approx. 60 min with  $1\ \mu\text{M}$ -TPA. Comparable incubations with the biologically inactive  $4\alpha$ -phorbol didecanoate at  $1\ \mu\text{M}$  had no effect on the binding of  $^{125}\text{I}$ -insulin to HepG2 cells throughout the 4 h period (Fig. 4a).

Under the conditions in which the binding of  $^{125}\text{I}$ -insulin to these HepG2 cells was measured (i.e. at  $37^\circ\text{C}$ ), the insulin-receptor complex would rapidly be internalized in response to insulin binding ( $t_{1/2} = 2\ \text{min}$ ; Strader *et al.*, 1985), and the receptor recycled to the cell surface as a function of time. It was therefore decided to determine whether the TPA-induced increase in  $^{125}\text{I}$ -insulin association with the cells reflected an increase in the amount of cell-surface-bound insulin, an increase in internalized insulin, or a combination of the two possibilities. To approach this question,  $^{125}\text{I}$ -insulin was bound to TPA-treated cells and the cell-surface receptors were



**Fig. 4.** (a) Time course of TPA preincubation on  $^{125}\text{I}$ -insulin binding to HepG2 cells and (b) time-dependent effect of phorbol ester treatment on  $^{125}\text{I}$ -insulin internalization at  $37^\circ\text{C}$

(a) Cells ( $10^6$ ) were preincubated for various times at  $37^\circ\text{C}$  with either  $1\ \mu\text{M}$ -TPA (●) or  $1\ \mu\text{M}$ - $4\alpha$ -phorbol didecanoate (○).  $^{125}\text{I}$ -insulin binding was performed at  $37^\circ\text{C}$  for 1 h. The experiment shown is representative of three performed; data are means  $\pm$  S.D. for triplicate samples. (b) Internalized  $^{125}\text{I}$ -insulin was determined as described in the Experimental section. Data shown are from the same experiment shown in (a) and are representative of three experiments, with each point done in triplicate. Values are means  $\pm$  S.D.

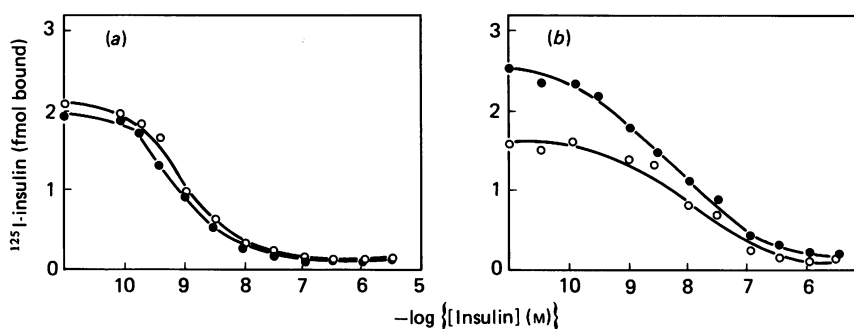


Fig. 5. Binding of insulin to HepG2 cells

Cells ( $10^6$ ) were treated with either 4 $\alpha$ -phorbol didecanoate (○) or TPA (●), then washed, and insulin was bound at (a) 4 °C for 16 h or (b) 37 °C for 1 h. The data shown are the means of triplicate determinations, representative of four similar experiments.

inactivated by trypsin before the internalized (trypsin-resistant)  $^{125}\text{I}$ -insulin was measured.

The effects of TPA on the internalization of  $^{125}\text{I}$ -insulin by HepG2 cells are shown in Fig. 4(b). Preincubation of the cells with 1  $\mu\text{M}$ -TPA caused a time-dependent increase in internalized  $^{125}\text{I}$ -insulin; the percentage of cell-associated  $^{125}\text{I}$ -insulin that was internalized by the cell within 4 h at 37 °C rose from 50% in control cells to a maximum of 72% after 4 h of preincubation with TPA, a 2.4-fold enhancement of the internal pool of insulin. Cells treated with the non-tumour-promoting 4 $\alpha$ -phorbol didecanoate, on the other hand, showed no such increase in the binding or internalization of  $^{125}\text{I}$ -insulin throughout the 4 h period. When the relative contributions of cell-surface-bound and internalized  $^{125}\text{I}$ -insulin to the increase in cell-associated insulin were assessed, it was determined that the increase in internalized  $^{125}\text{I}$ -insulin accounted for all of the observed increase in  $^{125}\text{I}$ -insulin binding to the cell. Thus the increase in  $^{125}\text{I}$ -insulin binding to HepG2 cells promoted by the phorbol ester TPA resulted entirely from an increase in the intracellular pool of  $^{125}\text{I}$ -insulin, with no increase in the binding of  $^{125}\text{I}$ -insulin to the cell surface receptors.

The fact that the increase in the binding of  $^{125}\text{I}$ -insulin to HepG2 cells after preincubation with TPA did not result from an increase in cell-surface insulin receptors is also illustrated by a comparison of insulin binding to TPA-treated cells at 4 °C and at 37 °C, as shown in Fig. 5. In these experiments, all cells were treated with 1  $\mu\text{M}$ -phorbol ester for 2 h at 37 °C before the cells were washed and insulin was bound at either 4 °C (Fig. 5a) or 37 °C (Fig. 5b). Examination of the data in Fig. 5(a) shows that there was no change in the binding of  $^{125}\text{I}$ -insulin at 4 °C to cells that had been pretreated with either the tumour promoter TPA or the biologically inactive 4 $\alpha$ -phorbol didecanoate at 37 °C. Scatchard analysis of the high-affinity insulin binding to these cells at 4 °C revealed a  $K_D$  of  $5.5 (\pm 1.6) \times 10^{-10}$  M for control cells and  $5.0 (\pm 0.8) \times 10^{-10}$  M for TPA-treated cells ( $P > 0.05$ ,  $n = 8$ ). The receptor concentration for control cells was  $2.3 (\pm 0.7) \times 10^{-10}$  M, and that of the TPA-treated cells was  $1.9 (\pm 0.3) \times 10^{-10}$  M ( $P > 0.05$ ,  $n = 4$ ).

In contrast, insulin competition curves generated at 37 °C on identically treated cells showed an enhancement of specific  $^{125}\text{I}$ -insulin association with the cells after pretreatment with TPA, but no change after pretreatment with 4 $\alpha$ -phorbol didecanoate (Fig. 5b). Although the presence of receptor internalization and recycling during

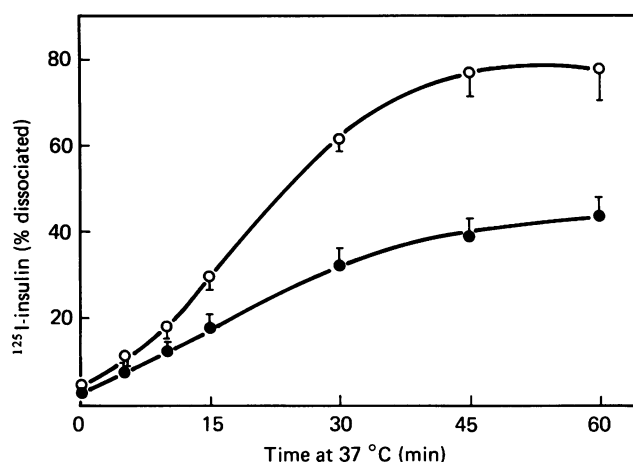
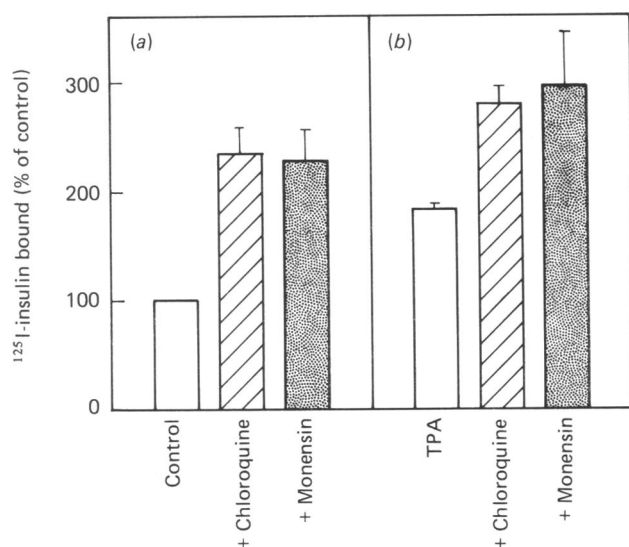


Fig. 6. Dissociation of  $^{125}\text{I}$ -insulin for HepG2 cells

Cells ( $10^6$ ) were incubated for 2 h at 37 °C with either 4 $\alpha$ -phorbol didecanoate (○) or TPA (●) before  $^{125}\text{I}$ -insulin was bound for 30 min; the excess was washed away, and the bound insulin allowed to dissociate for various lengths of time, as described in the Experimental section. Total cell-associated  $^{125}\text{I}$ -insulin was 1.2 fmol for control cells and 0.9 fmol for TPA-treated cells. Data shown are means  $\pm$  s.d. for triplicate determinations, representative of three similar experiments.

the binding at 37 °C makes rigorous Scatchard analysis of these binding curves invalid, it is clear from these data and from those discussed above in Figs. 2–4 that pretreatment with TPA does cause a significant enhancement of the amount of  $^{125}\text{I}$ -insulin bound to the cells at 37 °C ( $P < 0.01$ ,  $n = 24$ ).

Thus it has been determined that treatment with the tumour-promoting phorbol ester TPA enhances specific  $^{125}\text{I}$ -insulin association with HepG2 cells at 37 °C by increasing the amount of internalized  $^{125}\text{I}$ -insulin. This could result either from an increase in the rate of receptor internalization or from a decrease in the rate of processing of internalized  $^{125}\text{I}$ -insulin and the release of  $^{125}\text{I}$  into the media. Because the rate of internalization of  $^{125}\text{I}$ -insulin in control cells is fast compared with the rate of binding ( $t_{1/2} = 2$  min for internalization versus  $t_{1/2} = 6$  min for binding; Strader *et al.*, 1985), we were unable to measure reliably any increase in the rate of internalization in TPA-treated cells (results not shown).



**Fig. 7. Effects of chloroquine and monensin on insulin binding to HepG2 cells**

Cells were not preincubated (□), or preincubated with 0.1 mM-chloroquine (■), or pretreated with 10 μM-monensin (▨) before phorbol ester treatment and <sup>125</sup>I-insulin binding at 37 °C. In (a) the effects of these reagents in cells incubated with 1 μM-4α-phorbol didecanoate are shown, and (b) shows the effects of chloroquine and monensin on cells incubated with 1 μM-TPA. Bars show means ± S.E.M. for triplicate determinations from three separate experiments.

Dissociation of cell-associated <sup>125</sup>I-insulin is a much slower process, however ( $t_{1/2} = 20$  min), and dramatic differences in this parameter between control cells and TPA-treated cells were detected, as shown in Fig. 6. In this experiment, cells were exposed to 1 μM-TPA and <sup>125</sup>I-insulin was bound at 37 °C. After washing to remove unbound insulin, the cells were returned at 37 °C to permit dissociation of prebound <sup>125</sup>I-insulin. After an initial lag period of 5 min, the rate of dissociation of <sup>125</sup>I-insulin from control cells, or from cells treated with 4α-phorbol didecanoate, was 2.3 times faster than that from cells treated with TPA. This faster rate of dissociation continued for approx. 45 minutes, after which time the release of radioactivity from both treated and control cells reached a plateau. It is noteworthy that, at all time points, the fraction of the radioactivity released from the cell which was present in the form of protein, as judged by precipitation with trichloroacetic acid, was identical in control and TPA-treated cells. After 5 min of dissociation, 56 ± 1% of the <sup>125</sup>I released from both control and TPA-treated cells could be precipitated by trichloroacetic acid, whereas after 1 h only 22 ± 1% of the <sup>125</sup>I dissociated from both sets of cells was in the form of protein.

Further evidence for the effects of TPA on the intracellular processing of the insulin-receptor complex came from experiments investigating the effects of chloroquine and monensin on <sup>125</sup>I-insulin binding and internalization by HepG2 cells. The lysosomotropic reagent chloroquine has been shown to enhance the size of the pool of internal insulin receptors (Marshall *et al.*, 1981; Green & Olefsky, 1982). This reagent, which acts by raising the pH of lysosomes and other intracellular

organelles, inhibits the dissociation of the receptor-insulin complex, thus inhibiting the recycling of receptors and enlarging the intracellular pool of internalized insulin. The ionophore monensin has also been shown to inhibit receptor recycling in various systems (Basu *et al.*, 1981). As shown in Fig. 7(a), preincubation of either 0.1 mM-chloroquine or 10 μM-monensin with HepG2 cells before treatment with 4α-phorbol didecanoate resulted in a 2.2-fold increase in subsequent <sup>125</sup>I-insulin binding to the cells in 1 h at 37 °C. Treatment of cells with TPA alone caused a 1.8-fold increase in <sup>125</sup>I-insulin bound, as shown in Fig. 7(b). When cells were preincubated with either 0.1 mM-chloroquine or 10 μM-monensin before treatment with TPA, there was a further enhancement of <sup>125</sup>I-insulin binding, to 2.7-fold that of the untreated control, or 1.5-fold over that of TPA-treated cells. Thus the effects of TPA and of monensin or chloroquine on <sup>125</sup>I-insulin binding were additive and not multiplicative. This provides further evidence that the effects of TPA, like those of chloroquine and monensin, are related to the endocytotic processing pathways of <sup>125</sup>I-insulin in the HepG2 cell rather than to changes in surface receptor number. If the TPA did act via an increase in the number of cell-surface receptors, then its effects would be expected to be multiplicative with those of chloroquine or monensin, which act within the cell. The fact that the effects of TPA and those of maximally effective concentrations of chloroquine and monensin are additive indicates that TPA effects the intracellular processing of insulin at some point other than that of either of these reagents. The exact location of its site of action is as yet undetermined.

A major intracellular target of the tumour-promoting phorbol esters is a Ca<sup>2+</sup>/phospholipid-dependent protein kinase, termed protein kinase C. In viewing the enhancement of the internalized pool of insulin which results from TPA treatment of HepG2 cells, it was decided to examine whether other activators of protein kinase C can mimic these effects of TPA. One such activator is the synthetic diacylglycerol, OAG, which has been shown to activate protein kinase C in platelets (Kaibuchi *et al.*, 1983). In preliminary experiments, we found a small (15%) but significant increase in <sup>125</sup>I-insulin binding to HepG2 cells at 37 °C after treatment with 0.2 mg of OAG/ml (results not shown). Further analysis showed that all of this increase in binding could be attributed to an increase in internalized <sup>125</sup>I-insulin, with no change in the cell-surface binding. We attribute the submaximal effects of OAG on HepG2 cells to either permeability problems or to rapid metabolic conversion of OAG within the cell. A study of the effects of phorbol esters on the epidermal growth factor receptor reported similar submaximal effects of OAG in A431 cells, providing support for this interpretation (Davis *et al.*, 1985).

Phospholipase C has also been shown to stimulate protein kinase C by raising intracellular concentrations of diacylglycerol (Allan *et al.*, 1978). When HepG2 cells were incubated with phospholipase C, there was a dose-dependent increase in subsequent <sup>125</sup>I-insulin binding to the cells, with the maximal effect being observed with 10 munits of phospholipase C/ml. As shown in Fig. 8(a), incubation of HepG2 cells with phospholipase C enhanced the binding of <sup>125</sup>I-insulin to the cells at 37 °C to 1.4-fold that of control cells, approximately the same stimulation as shown by TPA under these conditions. This effect was not mimicked by phospholipase A, which does not produce diacylglycerol and hence does not

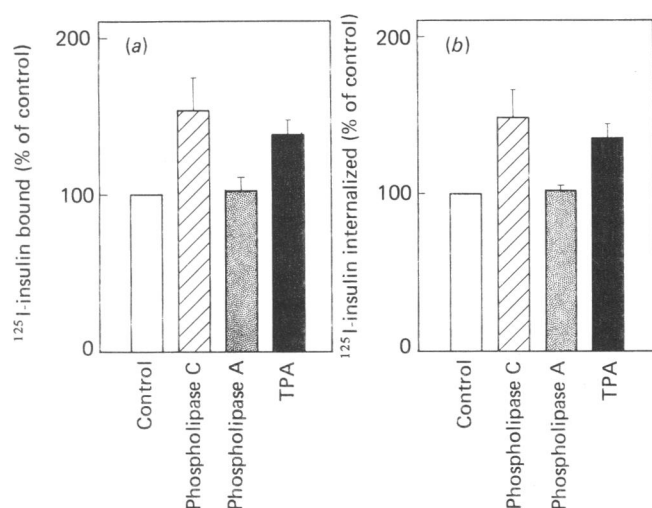


Fig. 8. Effects of phospholipases on insulin association with HepG2 cells

(a)  $^{125}\text{I}$ -insulin binding at  $37^\circ\text{C}$  and (b)  $^{125}\text{I}$ -insulin internalized were measured in cells which had been pretreated with  $1\ \mu\text{M}$ - $4\alpha$ -phorbol didecanoate ( $\square$ ), 10 munits of phospholipase C/ml ( $\text{▨}$ ), 10 munits of phospholipase A/ml ( $\text{▩}$ ), or  $1\ \mu\text{M}$ -TPA ( $\blacksquare$ ). Binding and internalization of  $^{125}\text{I}$ -insulin were performed for 30 min at  $37^\circ\text{C}$ . Results shown are means  $\pm$  S.D. of triplicate determinations from three separate experiments.

activate protein kinase C. Fig. 8(b) shows the effects of phospholipase C on the subsequent internalization of  $^{125}\text{I}$ -insulin by HepG2 cells at  $37^\circ\text{C}$ . Again, 10 munits of phospholipase C/ml mimicked the effects of TPA in enhancing this parameter, whereas phospholipase A was without effect. It is noteworthy that the effects of maximally stimulating concentrations of phospholipase C and TPA were not additive, indicating that they were acting through the same pathway (results not shown). Thus the effects of TPA on the receptor-mediated internalization of insulin by HepG2 cells appear to be mediated through protein kinase C.

## DISCUSSION

Treatment of HepG2 hepatoma cell monolayers with the tumour-promoting phorbol ester TPA resulted in dramatic morphological changes, visible at the light-microscopic level. These morphological changes correlated in a time- and dose-dependent fashion with an enhancement of the association of  $^{125}\text{I}$ -insulin with these cells at  $37^\circ\text{C}$ . The TPA-promoted potentiation of insulin binding to the HepG2 cells resulted solely from an increase in internalized insulin, and not from a change in cell-surface receptor number, as reflected both in its temperature-dependence and in its resistance to degradation by trypsin. The additivity of the effects of phorbol esters with those of chloroquine and monensin provides further evidence that the potentiation of insulin binding by TPA results from a change in the receptor-mediated endocytotic pathway, rather than from a change in insulin receptor numbers.

The enhancement of the pool of internalized insulin observed in TPA-treated HepG2 cells resulted from a

decrease in the rate of intracellular processing of the insulin. This was reflected in a decrease in the rate of dissociation of prebound insulin from cells which had been incubated with TPA, but not from cells incubated with the non-tumour-promoting  $4\alpha$ -phorbol didecanoate. Dissociation of prebound  $^{125}\text{I}$ -insulin from HepG2 cells at  $37^\circ\text{C}$  presumably could reflect two major components: a direct dissociation of  $^{125}\text{I}$ -insulin from cell-surface receptors, or a dissociation via the endocytotic pathway, whereby the insulin-receptor complex is internalized and the two components are separated in an intracellular compartment before the  $^{125}\text{I}$ -insulin is released into the medium and the receptors recycled to the cell surface. In HepG2 cells, as in many cell types, the former pathway takes place very slowly, the long incubation times and more acidic conditions being required for the dissociation of the cell-surface insulin-receptor complex to occur; dissociation by this pathway is negligible over a period of 2 h at pH 7.4 (Strader *et al.*, 1985). Therefore the decreased rate of dissociation of the cell-associated  $^{125}\text{I}$ -insulin in TPA-treated cells must reflect an effect of the phorbol ester on the release of internalized  $^{125}\text{I}$ -insulin from the cell.

Phorbol esters have been demonstrated to have a variety of effects on various hormone receptors in several different cell types. Grunberger & Gorden (1982) have reported that treatment of human U-937 macrophages or IM-9 lymphocytes with TPA decreased specific insulin binding at both  $15^\circ$  and  $37^\circ\text{C}$ , while having no effect on insulin binding to cultured human fibroblasts. Thomopoulos *et al.* (1982) also reported a decrease in insulin binding at  $15^\circ\text{C}$  to both U-937 cells and to the human leukaemic cell line HL-60, although with differential sensitivities. On the other hand, Takayama *et al.* (1984) have reported that insulin binding at  $15^\circ\text{C}$  to rat Fao hepatoma cells was unchanged by prior exposure of the cells to TPA. Since this binding was done at a non-permissive temperature for receptor internalization ( $15^\circ\text{C}$ ), it is not known whether the TPA-induced increase in internalized insulin that we report here for the human HepG2 hepatoma cell at  $37^\circ\text{C}$  would also be present in the rat Fao hepatoma cell at the higher temperature. However, it is clear that the HepG2 cell, the rat Fao hepatoma cell and the human fibroblasts did not show the TPA-induced loss of insulin binding observed in both the U-937 macrophages and IM-9 lymphocytes. Thus the effects of phorbol esters on receptor-mediated insulin binding vary from one cell type to another. The work presented here is the first report of an effect of phorbol esters on insulin-receptor endocytosis; the universality of this effect remains to be explored.

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