Phorbol ester only partially mimics the effects of insulin on glucose transport and glucose-transporter distribution in 3T3-L1 adipocytes

E. Michael GIBBS,*§ David M. CALDERHEAD,* Geoffrey D. HOLMAN† and Gwyn W. GOULD‡

*Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756, U.S.A., †Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, U.K., and ‡Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

We examined the effects of the phorbol ester phorbol 12-myristate 13-acetate (PMA) on the rate of hexose transport into 3T3-L1 adipocytes. Exposure of adipocytes to PMA (1 μ M) for 60 min results in a 1.7–2.5-fold increase in the rate of hexose transport. This effect was mediated by translocation of two isoforms of glucose transporters to the plasma membrane, as determined by labelling *in situ*, photoaffinity labelling with a membrane-impermeant glucose analogue, and by immunoblotting of subcellular fractions. The PMA-induced stimulation of both transport and transporter translocation was substantially less than that induced by insulin in this cell line; the PMA-induced increase in plasma-membrane GLUT 1 and GLUT 4 transporter isoforms was only about 40 % and 10 % respectively of that induced by insulin. We suggest that the stimulation of transport by insulin and PMA occurs via different mechanisms, which is manifested by the ability of insulin to induce a much greater increase in the plasma-membrane content of GLUT 4 compared with the phorbol ester.

INTRODUCTION

Glucose transport into mammalian cells has been shown to be regulated by insulin, as well as a variety of other agents. In responsive cell types such as adipocytes, insulin induces the translocation of glucose transporters from an intracellular pool to the plasma membrane, resulting in an increased rate of glucose uptake into the cell (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Blok *et al.*, 1988). Translocation has also been demonstrated in Swiss 3T3 fibroblasts in response to epidermal growth factor, fibroblast growth factor, platelet-derived growth factor and the tumour promoter phorbol 12-myristate 13-acetate (PMA) (Kitagawa *et al.*, 1986; Kitagawa, 1987).

We have shown that 3T3-L1 adipocytes contain a glucose transporter that reacts with antibodies against a synthetic peptide corresponding to the C-terminal 16 amino acids of the human erythrocyte transporter (hereafter referred to as GLUT 1), and that in response to insulin there is a 3-fold increase in the amount of this transporter at the cell surface as the result of translocation from an intracellular site (Blok *et al.*, 1988; Calderhead & Lienhard, 1988). James *et al.* (1988) have obtained evidence for the existence of a novel transporter, immunologically distinct from GLUT 1, which is expressed in insulin-responsive tissues (hereafter referred to as GLUT 4). The amino acid sequence of this transporter has been deduced from cDNA cloning and sequencing (James *et al.*, 1989; Birnbaum, 1989; Kaestner *et al.*, 1989; Charron *et al.*, 1989; Fukumoto *et al.*, 1989), and the presence of this transporter in addition to GLUT 1 has been demonstrated in 3T3-L1 adipocytes (James *et al.*, 1989; Birnbaum, 1989; Kaestner *et al.*, 1989). Furthermore, translocation of GLUT 4 to the plasma membrane in response to insulin has been demonstrated in 3T3-L1 adipocytes (James *et al.*, 1989; Birnbaum, 1989). The present study examines the stimulation of glucose transport in 3T3-L1 adipocytes in response to PMA, and compares the characteristics of this stimulation with that induced by insulin.

The results herein show that PMA causes a modest translocation of both transporter species, but that its effects on translocation and stimulation of hexose transport are substantially less than those induced by insulin. Furthermore, it has been reported that in isolated rat adipocytes PMA mimics quantitatively the insulin effect on total transporter translocation, but not the effect on hexose transport (Muhlbacher *et al.*, 1988; Obermaier-Kusser *et al.*, 1989). In contrast with these findings, we report here that PMA does not mimic fully the insulin stimulation of transport or transporter translocation in 3T3-L1 adipocytes, and we suggest that the stimulation of transport by these two agents occurs via different mechanisms.

MATERIALS AND METHODS

Materials

The affinity-purified rabbit antibodies against the purified human erythrocyte glucose transporter (anti-GLUT 1 antibodies) and against the C-terminal peptide (residues 477–492) of the human/rat glucose transporter (anti-GLUT 1 C-peptide antibodies) were preparations that have been described (Schroer *et al.*, 1986; Davies *et al.*, 1987). Experiments that required antibodies to the muscle/fat-specific glucose transporter were performed with either mouse monoclonal (1F8) or rabbit polyclonal (R820) antibodies generously provided by Dr. David James (Department of Cell Biology, Washington University School of Medicine, St. Louis, MO, U.S.A.). 2-Deoxy-D-[1,2-³H]glucose and [³²P]P_i were obtained from ICN, and sodium boro[³H]hydride (20 Ci/mmol) was from Amersham. PMA and 4 α -phorbol didecanoate (4 α PDD) were supplied by Pharmacia P-L Biochemicals. Octaethylene glycol dodecyl ether (C₁₂E₈) was from Nikkol Chemical Co. (Chuoku, Tokyo, Japan). All other reagents were as previously described (Gibbs *et al.*, 1988).

Cell culture

3T3-L1 cells were carried as fibroblasts and differentiated into

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; 4 α PDD, 4 α -phorbol didecanoate; DMEM, Dulbecco's modified Eagle's medium; C₁₂E₈, octaethylene glycol dodecyl ether; KRP, Krebs–Ringer phosphate buffer; KRH, Krebs–Ringer Hepes buffer; PBS, phosphate-buffered saline.

§ To whom correspondence should be sent. Present address: Pfizer Central Research, Groton, CT 06340, U.S.A.

adipocytes as described by Frost & Lane (1985). The adipocytes were used between days 7 and 11 after initiation of differentiation. Insulin stimulation of 2-deoxyglucose uptake by the adipocytes during this period was routinely about 10–15-fold. Plates were incubated at 37 °C in serum-free Dulbecco's modified Eagle's medium (DMEM) for 2 h immediately before use.

Hexose-transport assays

Hexose transport was assayed by the uptake of [1,2-³H]-deoxyglucose (Gibbs *et al.*, 1988). Monolayers were washed with 3 × 3 ml of Krebs–Ringer phosphate (KRP; 136 mM-NaCl / 4.7 mM-KCl / 1.25 mM-MgSO₄ / 1.25 mM-CaCl₂ / 5 mM-sodium phosphate, pH 7.4) or Krebs–Ringer Hepes (KRH; 136 mM-NaCl / 4.7 mM-KCl / 1.25 mM-MgSO₄ / 1.25 mM-CaCl₂ / 10 mM-Hepes, pH 7.4) at 37 °C and then covered with 1 ml of KRP (or KRH). Insulin and phorbol ester were present for the times indicated in the Figure legends. PMA was dissolved in neat dimethyl sulphoxide and added to the cells such that the dimethyl sulphoxide concentration was ≤ 0.5 %, which had no effect on hexose transport. Uptake was initiated by the addition of [³H]deoxyglucose (final concn. 50 μM and 0.5 μCi/ml). After incubation for the times indicated in the Figure and Table legends, uptake was terminated by aspirating the medium and washing the monolayers three times with ice-cold phosphate-buffered saline (PBS; 150 mM-NaCl / 10 mM-NaH₂PO₄, pH 7.4). The cells were solubilized with 1.0 ml of 1 % Triton X-100, and the radioactivity in a sample was measured by liquid-scintillation spectrometry. Uptake values were corrected for the non-specific association of [³H]deoxyglucose with the cells by subtracting the uptake in the presence of 25 μM-cytochalasin B, a potent inhibitor of transport (Bloch, 1973). This correction typically amounted to about 12 % of the uptake by basal cells.

Labelling of glucose transporters at the cell surface

Method I. Adipocytes on 35 mm plates were treated with insulin or phorbol ester for the times indicated in the Figure legends. Glycoproteins at the cell surface, including the glucose transporter, were then labelled by treating the intact cells with galactose oxidase followed by [³H]borohydride at 0 °C. GLUT 1 was subsequently immunoprecipitated by using affinity-purified antibodies against the C-terminal peptide (Davies *et al.*, 1987). The immunoprecipitates were electrophoresed and the radioactivity in the transporter band was determined. The details of this method have been reported elsewhere (Calderhead & Lienhard, 1988; Gibbs *et al.*, 1988). The method provides a measure of the relative amounts of GLUT 1 at the cell surface.

Method II. Adipocytes on 35 mm plates were treated in serum-free DMEM with insulin or phorbol ester for the times indicated in the Figure legends. Cells were then washed with KRP (3 × 3 ml, 22 °C). KRP (0.25 ml) containing 200 μCi of the membrane-impermeant glucose-transporter photoaffinity-labelling agent 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine ('ATB-BMPA') (sp. radioactivity ~ 10 Ci/mmol), was added to each plate (Calderhead *et al.*, 1990; Clark & Holman, 1990). The cells were incubated for 2 min in a Rayonet reactor (RPR-3000 Å), and irradiated for a further 3 min. After irradiation, the cells were washed with KRP (3 × 3 ml), and then solubilized in 1.5 ml of 2 % C₁₂E₈ in PBS containing protease inhibitors (5 mM-*N*-ethylmaleimide, 1 μg of pepstatin A/ml, 10 μM-*L*-trans-epoxysuccinyl-leucylamido-3-methylbutane and 200 μM-di-isopropyl fluorophosphate). The lysate was centrifuged at 180 000 *g*_{max} for 1 h. The supernatant (1.4 ml) was incubated for 2 h with 7.5 μg of anti-GLUT 4 antibodies (R820) and Protein A–Sepharose (10 μl) for 90 min.

After brief centrifugation (6 s at 14 000 rev./min in an Eppendorf microfuge), the GLUT 4-depleted supernatant (1.2 ml) was treated with 3 μg of anti-GLUT 1 antibody and Protein A–Sepharose for 1.5 h. Both Protein A pellets were washed three times with 1 % C₁₂E₈ in PBS and once with 0.1 % C₁₂E₈ in PBS, and then the transporters were eluted as described by Gibbs *et al.* (1986). Samples of the immunoprecipitates (equivalent to 1.5 × 10⁶ cells) in SDS sample buffer were electrophoresed on 10 %-polyacrylamide gels; the gels were sliced and the slices were incubated in 6 % Protosol in Econofluor (National Diagnostics) at 37 °C with agitation overnight. The radioactivity in the slices was measured by liquid-scintillation spectrometry to determine the total radioactivity in the transporter peak.

Subcellular fractionation and immunoblotting

A method for obtaining a subcellular fraction containing most of the intracellular GLUT 1 has been described (Gibbs *et al.*, 1988; Brown *et al.*, 1988; Gould *et al.*, 1989). In brief, this method involves centrifugation of a cell homogenate at 16 000 *g*_{max} for 20 min at 4 °C, which pellets 95 % of the plasma membrane. The supernatant contains 50 % of the total GLUT 1 as determined by immunoblotting; the amount of GLUT 1 in this fraction decreases by 50 % in response to insulin (Brown *et al.*, 1988; Gould *et al.*, 1989). Further centrifugation of this supernatant at 180 000 *g*_{max} results in a pellet containing all of the membranes from the original supernatant. GLUT 4 can readily be detected in this membrane fraction by immunoblotting; insulin decreased the amount of GLUT 4 in this fraction by 50 %. Relative levels of both GLUT 1 and GLUT 4 in the 180 000 *g*_{max} pellet were quantified by immunoblotting with appropriate first antibodies, followed by detection with ¹²⁵I-labelled goat anti-rabbit or anti-mouse second antibody. Electrophoresis and immunoblotting were performed as described by Gibbs *et al.* (1988).

Down-regulation of protein kinase C

This was done by the method of Blackshear *et al.* (1985). Briefly, 3T3-L1 adipocytes were incubated for 16 h with 16 μM-PMA in DMEM/1 % BSA and then washed five times with DMEM/1 % BSA over a 30–45 min period. Cells were then either incubated in serum-free DMEM for 2 h and assayed for deoxyglucose uptake or incubated overnight in DMEM/1 % BSA plus insulin (1 μM) or PMA (1 μM) before the uptake assay.

Other assays

Protein was measured by a modification of the Lowry method (Peterson, 1977).

RESULTS

Effect of PMA and insulin on deoxyglucose transport

We have previously shown that in 3T3-L1 adipocytes PMA stimulates hexose transport 1.7–2.5-fold over a 1 h period, with a detectable increase becoming apparent within 10 min (Gibbs *et al.*, 1986). Furthermore, this effect is not additive with that of maximal stimulatory concentrations of insulin (Gibbs *et al.*, 1986). This result was confirmed in the present study (Fig. 1). However, sub-maximal insulin concentrations showed effects on transport that were additive with those of PMA (Fig. 1). The insulin dose–response curve for transport stimulation was not shifted by PMA pretreatment.

Effect of PMA on reversal of insulin-stimulated deoxyglucose transport

We tested the hypothesis that PMA might prevent the reversal of insulin-stimulated hexose transport by first stimulating cells

Fig. 1. Deoxyglucose uptake: insulin dose-response with or without PMA

3T3-L1 adipocytes were incubated for 2 h in serum-free DMEM, washed once in KRH and then incubated in the same buffer with (○) or without (●) PMA (1 μ M) for 10 min. Insulin was then added at the indicated concentration for a further 20 min. Deoxyglucose uptake over a 3 min period was then measured as described in the Materials and methods section. Data from two experiments performed in duplicate are expressed as pmol/min per 10^6 cells (mean $\pm$ S.E.M., $n = 4$). Significant differences from cells that were not treated with PMA are indicated: * $P < 0.05$; ** $P < 0.01$.

Fig. 2. Reversal of insulin-stimulated 2-deoxyglucose transport with or without PMA

3T3-L1 adipocytes were incubated for 2 h in serum-free DMEM at 37 °C and then treated with (○, ●) or without (□) insulin for 10 min in the same medium. PMA (1 μ M) was added to half of the insulin-treated cells (○) and the incubation was continued for an additional 20 min. The cells were washed once with 3 ml of KRM (136 mM-NaCl, 4.7 mM-KCl, 1.25 mM-MgSO₄, 1.25 mM-CaCl₂, 1 mg of BSA/ml, 10 mM-Mes, pH 6.0 at 37 °C), and then incubated at 37 °C in 3 ml of KRM without insulin or PMA for the indicated times. The cells were then washed once with 3 ml of KRM and twice with 2.5 ml of KRH, and deoxyglucose uptake over a 1 min period was measured as described in the Materials and methods section. Data from two experiments performed in duplicate are expressed as pmol/min per 10^6 cells (mean $\pm$ S.E.M., $n = 4$). Significant differences from cells that were not treated with PMA are indicated: * $P < 0.05$; ** $P < 0.01$.

with insulin for 10 min, adding PMA for a further 20 min, and then subjecting the cells to a washing protocol that reverses the insulin stimulation (Gibbs *et al.*, 1986). PMA failed to prevent the return toward basal transport rates; the half-time for reversal was identical with that obtained in cells treated with insulin alone (Fig. 2). It is clear that the additive effect of PMA with sub-

maximal insulin stimulation was maintained at all time points (Fig. 2). The apparent discrepancy of additivity with maximal insulin stimulation (i.e. at zero time) in this experiment was probably the result of partial reversal of the insulin stimulation that had already occurred by the time the initial transport measurement was made; the five washes before initiating the transport assay took about 1 min to perform, and the assay itself required 1 min. Thus partial reversal of insulin stimulation occurred before we made our initial measurement.

Effect of PMA on glucose-transporter distribution

In order to determine whether PMA induced translocation of either type of transporter in these cells, we performed three different types of experiments to quantify transporter localization. The first series of experiments involved labelling of plasma-membrane glycoproteins with galactose oxidase and [³H]borohydride, followed by isolation of GLUT 1 by immunoprecipitation. These results are summarized in Table 1. This method failed to introduce sufficient label into GLUT 4 to allow quantification of this transporter isoform. GLUT 1 showed a time-dependent increase at the cell surface with PMA treatment that was not observed with treatment with the non-protein-kinase-C-stimulating phorbol, 4 α PDD. However, the increase in surface transporters by PMA was only one-quarter of that observed with insulin. Combination treatment of PMA plus insulin yielded the same number of surface transporters as seen by insulin treatment alone (Table 1).

Because it was not possible to quantify cell-surface GLUT 4 by the glycoprotein-labelling method, we also measured cell-surface transporters by photoaffinity labelling with a membrane-impermeant probe (Calderhead *et al.*, 1990). The results of a representative experiment are presented in Table 2. The results with GLUT 1 are in reasonable agreement with those obtained by the glycoprotein-labelling method, although the magnitudes of the increases are slightly larger for both insulin and PMA; the increase in surface transporters produced by PMA was about 40% of that produced by insulin. Insulin produced a dramatic increase (17-fold) in the cell-surface content of GLUT 4, in agreement with our recent results (Calderhead *et al.*, 1990). GLUT 4 also increased with PMA treatment, but to an extent only about 10% of that produced by insulin (Table 2; see the Discussion section).

In addition, the effect of PMA on the transporter content of the intracellular membrane fraction was examined by quantitative immunoblotting. After 60 min, PMA significantly decreased both intracellular GLUT 1 and GLUT 4 by 25%, but again the changes were less than the 50% decrease observed in response to insulin (Table 3). As was observed with the glycoprotein-labelling method for GLUT 1 (Table 1), the effect of combined treatment with PMA and insulin was essentially the same as with insulin alone. 4 α PDD did not cause a decrease in intracellular glucose transporters of either type.

Effect of protein kinase C down-regulation on acute stimulatory actions of PMA and insulin

Our results on the differences in magnitude of transport stimulation and transporter translocation induced by PMA or insulin argue strongly that the two agents stimulate transport by different mechanisms. In this regard, we down-regulated protein kinase C by pretreating cells for 16 h with 16 μ M-PMA as described by Blackshear *et al.* (1985) to determine whether the stimulatory effect of insulin on transport was affected. As a functional assessment of the effectiveness of the down-regulation protocol, we determined whether the phosphorylation of GLUT 1 that is known to occur with acute PMA treatment (Gibbs *et al.*,

Table 1. Effect of phorbol ester on the cell-surface GLUT 1 content as measured by galactose oxidase/[³H]borohydride labelling

Labelling of the surface glucose transporters by Method I was as described in the Materials and methods section. Data from three independent experiments are presented as percentages of the value for cell-surface transporters in basal cells (means $\pm$ S.E.M.). Insulin, PMA and 4 α PDD were all added at 1 μ M for the indicated times. The values in parentheses are actual c.p.m. values measured in the transporter band from one representative experiment. Transport rates were measured in parallel with each surface-labelling experiment. Typical values for the stimulation of transport (relative to basal cells) were 15.6-fold for insulin, 1.6- and 2.1-fold for PMA at 30 and 60 min respectively, and 0.9-fold for 4 α PDD after 60 min.

Condition	Cell surface transporters (% of basal)
Basal	100 (82)
4 α PDD for 60 min	85 $\pm$ 11 (65)
PMA for 1 min	99 $\pm$ 12 (70)
PMA for 30 min	123 $\pm$ 6 (111)
PMA for 60 min	156 $\pm$ 10 (144)
Insulin for 20 min	339 $\pm$ 50 (197)
PMA for 30 min plus insulin for final 20 min	274 $\pm$ 24 (214)

Table 2. Effect of phorbol ester on the cell-surface GLUT 1 and GLUT 4 contents measured by photoaffinity labelling

Photoaffinity labelling of the surface glucose transporters was as described in the Materials and methods section. The data represent mean values of two determinations and are presented as percentages of cell-surface transporters in basal cells; the values in parentheses are the actual c.p.m. values in the transporter bands. Quantitatively similar results were obtained in a second independent experiment. Transport rates were measured in parallel with each surface-labelling experiment. Typical values for the stimulation of transport (relative to basal cells) were 13.6-fold for insulin, 3.2-fold for PMA and 0.8-fold for 4 α PDD.

Condition	Cell surface transporters (% of basal)	
	GLUT 1	GLUT 4
Basal	100 (68, 82)	100 (19, 19)
4 α PDD for 60 min	105 (75, 82)	142 (25, 29)
PMA for 60 min	268 (206, 196)	174 (26, 40)
Insulin for 30 min	509 (420, 344)	1670 (252, 382)

1986; Joost *et al.*, 1987) would occur in down-regulated cells. PMA-induced phosphorylation of GLUT 1 was essentially abolished in down-regulated cells, which indicates that the protein kinase C isoform that mediates GLUT 1 phosphorylation was effectively down-regulated (E. M. Gibbs, G. E. Lienhard & G. W. Gould, unpublished work). Furthermore, PMA addition to protein kinase C down-regulated cells produced little or no stimulation of transport over a short (20 min) or long (24 h) incubation (Table 4). On the other hand, down-regulation of protein kinase C did not result in a diminished stimulatory response of hexose uptake to acute (10 min) or chronic (24 h) insulin treatment (Table 4). This finding implies that protein kinase C does not mediate the action of insulin to stimulate transport.

Table 3. Effect of phorbol ester on the glucose-transporter content of the intracellular membranes

These data were obtained from subcellular fractionation, followed by quantitative immunoblotting of the 180000 g_{max} membrane fraction, and represent means $\pm$ S.E.M. from three separate experiments with the phorbol esters and five experiments with insulin; nd, not determined. Samples in SDS sample buffer derived from 0.13×10^6 cells or 1×10^6 cells from the same fractionations were applied to each gel lane to quantify GLUT 1 or GLUT 4 respectively.

Condition	Intracellular transporters (%)	
	GLUT 1	GLUT 4
Basal	100	100
4 α PDD for 20 min	104 $\pm$ 6	nd
for 60 min	104 $\pm$ 6	97 $\pm$ 3
PMA for 1 min	97 $\pm$ 4	nd
for 20 min	88 $\pm$ 8	nd
for 30 min	91 $\pm$ 9	101 $\pm$ 2
for 60 min	76 $\pm$ 8	74 $\pm$ 6
for 120 min	79 $\pm$ 6	nd
Insulin for 30 min	49 $\pm$ 4	59 $\pm$ 6
Insulin for 30 min plus PMA for 20 min	58 $\pm$ 5	nd

Table 4. Effect of protein kinase C down-regulation on hexose-transport stimulation by insulin and PMA

Control cells or cells that were subjected to protein kinase C down-regulation by treatment with PMA (16 μ M) for 16 h as described in the Materials and methods section were incubated for 2 h in serum-free DMEM and then assayed for 2-deoxyglucose uptake (Expt. I), or subjected to an additional 24 h incubation in DMEM/1% BSA with the indicated additions before the serum-free wash and uptake assay (Expt. II). Uptake was measured over a 10 min period, and the values represent means $\pm$ S.D. from triplicate observations.

Condition	Deoxyglucose transport (pmol/min per 10^6 cells)	
	Control	Protein kinase C down-regulated
Expt. I		
Basal	69 $\pm$ 6	104 $\pm$ 3
PMA for 20 min	248 $\pm$ 19	107 $\pm$ 5
Insulin for 30 min	642 $\pm$ 30	708 $\pm$ 28
Expt. II		
Basal	53 $\pm$ 7	88 $\pm$ 6
PMA for 24 h	186 $\pm$ 7	103 $\pm$ 3
Insulin for 10 min	402 $\pm$ 19	418 $\pm$ 7
Insulin for 24 h	481 $\pm$ 3	523 $\pm$ 4

The overnight PMA treatment resulted in a 75% increase in total cellular GLUT 1 expression (results not shown), which probably accounts for the small (50%) transport stimulation that was observed. However, GLUT 1 expression was unchanged after a 2 h incubation (results not shown), whereas deoxyglucose uptake was increased 2-fold, suggesting that the rapid uptake stimulation was independent of increased transporter expression. These findings on GLUT 1 expression are in agreement with other reports (Flier *et al.*, 1987; Kitagawa *et al.*, 1989). In

contrast with the effect on GLUT 1, GLUT 4 expression was unchanged with prolonged PMA treatment (results not shown).

DISCUSSION

In this work, we set out to characterize the molecular basis for the acute stimulation of glucose transport induced by PMA in 3T3-L1 adipocytes (Gibbs *et al.*, 1986). Exposure of these cells to PMA results in a small, but reproducible, stimulation of hexose transport, which represents a 1.7–2.5-fold stimulation over basal levels by 60 min. The phorbol analogue 4 α PDD, which does not activate protein kinase C, had no effect on transport. We (Gibbs *et al.*, 1986) previously reported that the effects of maximally stimulating concentrations of PMA and insulin on glucose transport in 3T3-L1 adipocytes are not additive, and here we demonstrate that at sub-maximal concentrations of insulin additive effects are reproducibly observed. The lack of additivity at maximally activating concentrations of insulin implies that the mechanisms by which the two agents stimulate transport converge at some stage, although the initial signal probably differs (see below). Furthermore, we have shown that PMA does not prevent the return toward basal transport rates observed when insulin-treated adipocytes are washed to remove bound insulin. Significantly, the kinetics of the reversal were identical in both control and PMA-treated cells. On the basis of these results, we propose that PMA does not inhibit the translocation from the plasma membrane when the insulin signal is reversed, since only the incremental increase in transport attributable to PMA remains after the reversal procedure.

It has been demonstrated that adipocytes contain two immunologically distinct glucose transporters, both of which undergo insulin-stimulated translocation from an intracellular site to the plasma membrane (Blok *et al.*, 1988; Calderhead & Lienhard, 1988; Gibbs *et al.*, 1988; James *et al.*, 1988, 1989; Birnbaum, 1989; Kaestner *et al.*, 1989; Calderhead *et al.*, 1990). A previous study demonstrated that PMA treatment of rat adipocytes leads to the translocation of glucose transporters to an extent comparable with that induced by insulin (Muhlbacher *et al.*, 1988; Obermaier-Kusser *et al.*, 1989). We therefore used a number of techniques to compare the contribution of both GLUT 1 and GLUT 4 to the stimulation of transport by PMA and insulin in 3T3-L1 adipocytes.

Using a procedure for labelling *in situ* (Calderhead & Lienhard, 1988), we quantified the relative increases of GLUT 1 at the plasma membrane in response to PMA and insulin (Table 1). Insulin increased GLUT 1 at the plasma membrane about 3-fold. In contrast, PMA resulted in a smaller increase in cell-surface GLUT 1, corresponding to about 25% of that induced by insulin. As this method was not able to introduce sufficient label into GLUT 4 to produce reliable quantification, we used a membrane-impermeant photoaffinity probe to label both transporter isoforms at the plasma membrane (Table 2). The results obtained with GLUT 1 were quantitatively similar to those obtained by using the labelling procedure *in situ*; PMA increased cell-surface GLUT 1 to an extent of only about 40% of that with insulin. However, we found that in response to insulin GLUT 4 increased about 17-fold at the plasma membrane. PMA treatment resulted in a significantly smaller increase in cell-surface GLUT 4 than did insulin (only about a 2-fold increase). We do not wish to imply that these measurements are rigorous quantifications, as the low level of label incorporated into GLUT 4 in basal cells (19 c.p.m. above background; Table 2) precludes this. However, it is clear that the extent of translocation of GLUT 4 in response to PMA is significantly smaller than that induced by insulin. Additionally, the effect of PMA on GLUT 1 is greater than that on GLUT 4 when compared as a percentage of the translocation

induced by insulin (40% for GLUT 1 and 10% for GLUT 4). These data indicate that PMA does not induce the same extent of translocation as does insulin in 3T3-L1 adipocytes, and also suggest that PMA and insulin act via different mechanisms to stimulate hexose transport in this cell line.

To address this issue further, we examined the effect of protein kinase C down-regulation on insulin-stimulated hexose transport. Chronic exposure of cells to PMA (16 μ M) has been shown to down-regulate protein kinase C in this cell line (Blackshear *et al.*, 1985). Under conditions of down-regulation, PMA had no effect on transport when administered acutely (20 min) or chronically (24 h). However, protein kinase C down-regulation had no effect on acute or chronic insulin-stimulated hexose transport; these findings indicate that the PMA-sensitive protein kinase C isoform is not involved in hexose transport stimulation by insulin, and further argue that PMA- and insulin-induced stimulations of hexose transport occur by distinct mechanistic routes in 3T3-L1 adipocytes. We note that these data do not preclude a role of protein kinase C in the stimulation of hexose transport, as multiple isoforms of protein kinase C have been identified, and not all of them may be down-regulated by the treatment used in this study (Huang *et al.*, 1989). However, the isoform which mediates PMA-induced stimulation of hexose transport is clearly down-regulated by this treatment, and therefore cannot be involved in transport stimulation by insulin.

In conclusion, the PMA-induced stimulation of both glucose transport and glucose-transporter translocation in 3T3-L1 adipocytes was only a small fraction of that induced by insulin. The PMA-induced increase in plasma-membrane GLUT 1 and GLUT 4 transporter isoforms amounted to only about 40% and 10% of the insulin-induced increase respectively. These results suggest that most of the difference in transport stimulation by the two agents can therefore be explained by the inability of PMA to induce as large an increase in the plasma membrane content of GLUT 4 as that induced by insulin.

We are indebted to Dr. David James for providing us with both mouse monoclonal (1F8) and rabbit polyclonal (R820) antibodies to GLUT 4 and Dr. Steve Baldwin for the gift of antiserum against GLUT 1. This work was supported by the Juvenile Diabetes Foundation International (Postdoctoral Fellowship and Research Grant to G.W.G. and a Career Development Award to E.M.G.), National Institutes of Health Grant DK 25336 (G. E. Lienhard), and the M.R.C. and S.E.R.C. (grants to G.D.H.). We thank Susan J. Brown for performing the cell culture, Dr. Lee Witters for helpful discussions and especially Dr. Gustav Lienhard for his support and critical reading of the manuscript.

REFERENCES

- Birnbaum, M. J. (1989) *Cell* **57**, 305–315
- Blackshear, P. J., Witters, L. A., Girard, P. R., Kuo, J. F. & Quamo, S. N. (1985) *J. Biol. Chem.* **260**, 13304–13315
- Bloch, R. (1973) *Biochemistry* **12**, 4799–4801
- Blok, J., Gibbs, E. M., Lienhard, G. E., Slot, J. W. & Guezee, H. J. (1988) *J. Cell Biol.* **106**, 69–76
- Brown, S. J., Gould, G. W., Davies, A., Baldwin, S. A., Lienhard, G. E. & Gibbs, E. M. (1988) *Biochim. Biophys. Acta* **971**, 339–350
- Calderhead, D. M. & Lienhard, G. E. (1988) *J. Biol. Chem.* **263**, 12171–12174
- Calderhead, D. M., Kitagawa, K., Tanner, L. I., Holman, G. D. & Lienhard, G. E. (1990) *J. Biol. Chem.* **265**, 13800–13808
- Charron, M. J., Brosius, F. C., Alper, S. L. & Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2535–2539
- Clark, A. E. & Holman, G. D. (1990) *Biochem. J.* **269**, 615–622
- Cushman, S. W. & Wardzala, L. J. (1980) *J. Biol. Chem.* **255**, 4758–4762
- Davies, A., Meeram, K., Cairns, M. T. & Baldwin, S. A. (1987) *J. Biol. Chem.* **262**, 9347–9352
- Flier, J. S., Mueckler, M. M., Usher, P. & Lodish, H. F. (1987) *Science* **235**, 1492–1495

- Frost, S. C. & Lane, M. D. (1985) *J. Biol. Chem.* **260**, 2646–2652
- Fukumoto, H., Kayano, T., Buse, J. B., Edwards, Y., Pilch, P. F., Bell, G. I. & Seino, S. (1989) *J. Biol. Chem.* **264**, 7776–7779
- Gibbs, E. M., Allard, W. J. & Lienhard, G. E. (1986) *J. Biol. Chem.* **261**, 16597–16603
- Gibbs, E. M., Lienhard, G. E. & Gould, G. W. (1988) *Biochemistry* **27**, 6681–6685
- Gould, G. W., Lienhard, G. E., Tanner, L. I. & Gibbs, E. M. (1989) *Arch. Biochem. Biophys.* **268**, 264–275
- Huang, F. L., Yoshida, Y., Cunha-Melo, J. R., Beaven, M. A. & Huang, K. P. (1989) *J. Biol. Chem.* **264**, 4238–4243
- James, D. E., Brown, R., Navarro, J. & Pilch, P. F. (1988) *Nature (London)* **333**, 183–185
- James, D. E., Strube, M. & Mueckler, M. (1989) *Nature (London)* **338**, 83–87
- Joost, H. G., Weber, T. M., Cushman, S. W. & Simpson, I. A. (1987) *J. Biol. Chem.* **262**, 11261–11267
- Kaestner, K. H., Christy, R. J., McLenithan, J. C., Braiterman, L. T., Cornelius, P., Pekala, P. H. & Lane, M. D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3150–3154
- Kitagawa, K. (1987) *Biochim. Biophys. Acta* **928**, 272–281
- Kitagawa, K., Nishino, H. & Iwashima, A. (1986) *Biochim. Biophys. Acta* **887**, 100–104
- Kitagawa, T., Tanaka, M. & Akamatsu, Y. (1989) *Biochim. Biophys. Acta* **980**, 100–108
- Muhlbacher, C., Karnieli, E., Schaff, P., Obermaier, B., Mushack, J., Rattenhuber, E. & Haring, H. U. (1988) *Biochem. J.* **249**, 865–870
- Obermaier-Kusser, B., Muhlbacher, C., Mushak, J., Seffer, E., Ermel, B., Machicao, F., Schmidt, F. & Haring, H. U. (1989) *Biochem. J.* **261**, 699–705
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Schroer, D. W., Frost, S. C., Kohanski, R. A., Lane, M. D. & Lienhard, G. E. (1986) *Biochim. Biophys. Acta* **885**, 317–326
- Suzuki, K. & Kono, T. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2542–2546

Received 22 October 1990/14 December 1990; accepted 17 December 1990