Biochem. J. (1988) 249, 865–870 (Printed in Great Britain)

Phorbol esters imitate in rat fat-cells the full effect of insulin on glucose-carrier translocation, but not on 3-*O*-methylglucose-transport activity

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Tumour-promoting phorbol esters have insulin-like effects on glucose transport and lipogenesis in adipocytes and myocytes. It is believed that insulin activates the glucose-transport system through translocation of glucose transporters from subcellular membranes to the plasma membrane. The aim of the present study was to investigate if phorbol esters act through the same mechanism as insulin on glucose-transport activity of rat adipocytes. We compared the effects of the tumour-promoting phorbol ester tetradecanoylphorbol acetate (TPA) and of insulin on 3-*O*-methylglucose transport and on the distribution of D-glucose-inhibitable cytochalasin-B binding sites in isolated rat adipocytes. Insulin (100 μunits/ml) stimulated 3-*O*-methylglucose uptake 9-fold, whereas TPA (1 nM) stimulated the uptake only 3-fold (mean values of five experiments, given as percentage of equilibrium reached after 4 s: basal $7 \pm 1.3\%$, insulin $60 \pm 3.1\%$, TPA $22 \pm 2.3\%$). In contrast, both agents stimulated glucose-transporter translocation to the same extent [cytochalasin B-binding sites (pmol/mg of protein; $n = 7$): plasma membranes, basal 6.2 ± 1.0, insulin 13.4 ± 2.0, TPA 12.7 ± 2.7; low-density membranes, basal 12.8 ± 2.1, insulin 6.3 ± 0.9, TPA 8.9 ± 0.7; high-density membranes, 6.9 ± 1.1; insulin 12.5 ± 1.0, TPA 8.1 ± 0.9]. We conclude from these data: (1) TPA stimulates glucose transport in fat-cells by stimulation of glucose-carrier translocation; (2) insulin and TPA stimulate the carrier translocation to the same extent, whereas the stimulation of glucose uptake is 3-fold higher with insulin, suggesting that the stimulatory effect of insulin on glucose-transport activity involves other mechanisms in addition to carrier translocation.

INTRODUCTION

Insulin binding to its receptor in the plasma membrane of adipocytes leads within 1 min to activation of the glucose-transport system, reaching the maximal stimulatory effect within 4–10 min (Häring *et al.*, 1981). The molecular events linking the initial binding step to the final response of increased glucose-transport activity are not fully understood. It is believed that they include activation of the intrinsic insulin-receptor kinase (Kasuga *et al.*, 1983) and phosphorylation of cellular proteins (Häring *et al.*, 1987; White *et al.*, 1985; Machicao *et al.*, 1987), followed by as yet unknown intermediary steps, finally resulting in a translocation of glucose carrier sites from intracellular membranes to the plasma membrane (Wardzala *et al.*, 1978; Cushman & Wardzala, 1980; Karnieli *et al.*, 1981). Several lines of evidence have suggested that the steps linking insulin-receptor kinase and activation of the glucose-transport system might involve the phosphatidylinositol cycle (Köpfer-Hobelsberger & Wieland, 1984; Kirsch *et al.*, 1985; Van de Werve *et al.*, 1985; Nishizuka, 1986), release of diacylglycerol (Farese *et al.*, 1985) and activation of protein kinase C (Farese *et al.*, 1986). This speculation is partially based on the observation that tumour-promoting phorbol esters, which can substitute for diacylglycerol as stimulators of protein kinase C, are able to elicit insulin-like effects on glucose transport by isolated rat fat-cells and of myocytes in culture (Kirsch *et al.*, 1985; Farese *et al.*, 1985; Van de Werve *et al.*, 1985). To obtain further information whether an activation of protein kinase C could possibly be one step in the signal-transmission chain between the insulin receptor and the glucose-transport system in the fat-cell, we studied whether phorbol esters exert their insulin-like effects on glucose-transport activity in fat-cells through the same mechanism which is believed to underly the insulin-induced increase in glucose-transport activity, namely through translocation of glucose carriers (Wardzala *et al.*, 1978; Cushman & Wardzala, 1980; Karnieli *et al.*, 1981).

We therefore studied the effect of the tumour-promoting phorbol ester TPA and in parallel also the effect of insulin on glucose-transport activity of isolated rat adipocytes as well as on the distribution of D-glucose-inhibitable cytochalasin B-binding sites.

MATERIALS AND METHODS

Materials

Pig insulin was purchased from Novo Industrie (Bagsvaerd, Denmark), [^{3}H]cytochalasin B and [^{14}C]urea were from New England Nuclear (Dreieich, Germany),

Abbreviation used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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and TPA, cytochalasin E and the 5'-nucleotidase enzyme kit were from Sigma (Munich, Germany). All other reagents were of the best grade commercially available.

Cell isolation and determination of 3-*O*-methylglucose transport

Rat adipocytes were prepared as described by Rodbell (1964) from male Sprague–Dawley rats fed *ad libitum* (180–220 g body wt.). Krebs–Ringer–Hepes buffer (131 mM-NaCl/4.8 mM-KCl/2.5 mM-$CaCl_2$/1.2 mM-KH_2PO_4/1.2 mM-$MgSO_4$/25 mM-Hepes) (pH 7.4, 37 °C; containing 25 mg of crystalline bovine serum albumin/ml) was used for all incubations. The phorbol esters were diluted in pure ethanol, dried with N_2, dissolved in buffer and then sonicated. Portions of the solution were frozen and the final concentrations were prepared shortly before the experiments. After isolation, the packed fat-cells [3(–3.5) × 10^6 cells/ml] were incubated in polypropylene tubes with gentle shaking. Incubation was carried out at 37 °C for 20 min in the absence (basal) or presence of insulin or TPA in the concentrations given in the Figure legends. 3-*O*-Methylglucose transport was measured as described by Häring *et al.* (1981).

Subcellular fractionation of adipose cells

Plasma- and microsomal-membrane fractions were prepared from incubated cells by a differential-centrifugation method described by McKeel & Jarret (1970), with the modifications described by Karnieli *et al.* (1981).

The reproducibility of the fractionation procedure was assessed by measuring the specific 5'-nucleotidase activity (enriched in plasma-membrane fraction), rotenone-insensitive NADH–cytochrome *c* reductase activity (enriched in endoplasmic-reticulum fraction) and UDP-galactosyltransferase activity (enriched in Golgi apparatus) of each fraction and the original homogenates. These assays were done by the methods of Avruch & Wallach (1971), Dallner *et al.* (1966) and Verdon & Berger (1983) respectively. Protein concentration was determined by the Bio-Rad assay. Lipocrit was determined by measuring the impedance in a haematocrit–electrolyte combination apparatus.

Cytochalasin B-binding assay

The concentration of D-glucose transporters in the membrane fractions was measured by a specific assay of D-glucose-inhibitable cytochalasin B binding, developed by Wardzala *et al.* (1978) and modified by Karnieli *et al.* (1981). In each assay we used 11 different cytochalasin B concentrations, in the range 17–400 nM-cytochalasin B in test. [^{14}C]Urea was used to determine trapped unbound [^{3}H]cytochalasin B in the pelleted membranes obtained during the binding assay. Therefore it was added, so that the final concentrations of ^{14}C and ^{3}H were the same. From the resulting 11-point cytochalasin B-binding curves, the number of glucose-inhibitable binding sites (R_0) and the affinity constants (K_d) were determined by Scatchard (1949) analysis. The CBB assay was carried out as follows: 500 μl of each membrane protein suspension was mixed with 40 μl of cytochalasin E solution (test concn. 2.3 μM) and vortex-mixed. Two samples (375 μl and 125 μl) were removed and added to 300 μl and 100 μl of 20 mM-Tris/1 mM-EDTA/255 mM-sucrose buffer respectively, either containing or not containing 500 mM-D-glucose. After vortex-mixing, samples (50 μl) were removed from the two suspensions and mixed with 10 μl of buffer, containing 11 different concentrations of [^{3}H]-cytochalasin B and tracer [^{14}C]urea without D-glucose and five different concentrations for the buffer containing D-glucose. These suspensions were vortex-mixed again and sedimented in 350 μl centrifuge tubes. For plasma membranes, glass tubes were used, and centrifugation was carried out in a SS 34 rotor with adapters at 48000 $g_{max.}$ for 30 min. Microsomal membranes were centrifuged in 350 μl cellulose propionate tubes, in a 42.2 Ti rotor at 220000 $g_{max.}$ for 120 min. Then 25 μl of each supernatant was transferred into scintillation vials, together with 110 μl of mixture containing 0.09 M-NaOH and 0.09% acetic acid, for measurement of the free [^{3}H]cytochalasin B concentration. The remaining supernatant of each tube was aspirated and discarded. The centrifuge tubes containing the pelleted membranes were incubated with 50 μl of 0.2 M-NaOH each to digest the pellet. Incubation was carried out at 50 °C for 60 min. All other steps are performed at 24 °C. After incubation, 60 μl of 1.17% (v/v) acetic acid was added to each sample for neutralization, the tubes were vortex-mixed and transferred to a scintillation vial for measuring bound [^{3}H]cytochalasin B. The concentration of the D-glucose-inhibitable cytochalasin B-binding sites (net binding) was determined by subtracting each curve obtained in the presence of 500 mM-D-glucose from its respective curve obtained in the absence of D-glucose along radial axes originating at $x/y = 0$ (Cushman & Wardzala, 1980). This subtraction yields the derived Scatchard plot forming a linear graph. Calculations were done by linear-regression analysis on an IBM AT personal computer.

RESULTS

Insulin as well as the tumour-promoting phorbol esters stimulate the glucose-transport activity of isolated

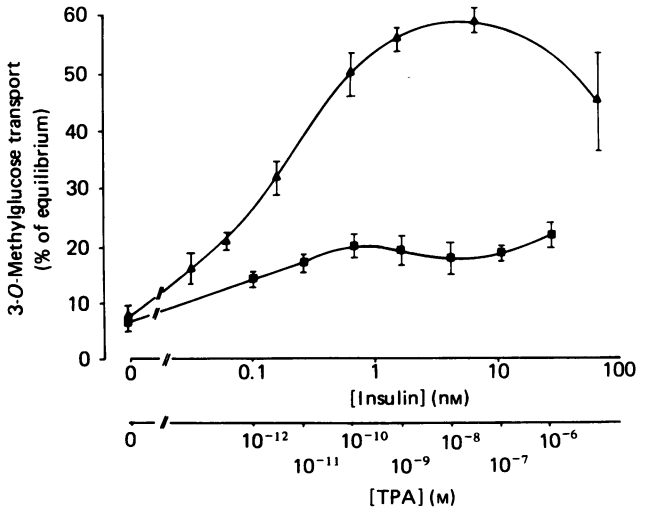

Fig. 1. Dose/response curves of 3-*O*-methylglucose-transport activation by insulin (▲) and TPA (■)

3-*O*-Methylglucose transport activity in isolated rat adipocytes is expressed as percentage of equilibrium uptake reached in 4 s. Mean values ± S.E.M. are shown for five experiments.

rat fat-cells within 1 min and reach the maximal effect after approx. 4–10 min (Häring *et al.*, 1981; Kirsch *et al.*, 1985). Fig. 1 shows the dose-dependent increase in glucose-transport activity after stimulation of fat-cells with insulin or TPA for 20 min. The maximal effect of insulin is reached at approx. 1000 μunits/ml, and TPA reaches its maximal effect at 1 nM. At the maximal effective insulin concentrations, an approx. 9–10-fold stimulation of glucose transport occurs, whereas TPA only reaches an approx. 2–3-fold maximal stimulation.

To study the effect of insulin and TPA on glucose-carrier distribution, freshly isolated rat adipocytes were stimulated with insulin (1000 μunits/ml) or TPA (1 nM) for 20 min. 3-*O*-Methylglucose-transport activity was then determined in a sample of these cells. Another sample of these cells was used for the determination of the glucose carrier distribution as described in the Materials and methods section. Membrane fractions, namely plasma membranes, high-density membranes and low-density membranes were prepared as described in the Materials and methods section. To assess the purity of the membrane preparations, marker enzymes were determined and the results are shown in Table 1. 5′-Nucleotidase was determined as a marker enzyme for plasma membranes, NADH–cytochrome *c* reductase as marker for the high-density membrane fraction and UDP-galactosyltransferase as marker for the low-density membrane fraction. The purification quality of the membrane fractions is within the ranges reported previously (Simpson & Cushman, 1985). Enzyme concentrations were not affected by incubation with insulin or TPA. Fig. 2 shows the results of the cytochalasin B-binding studies in these membrane fractions. In Figs. 2(*a*), 2(*c*) and 2(*e*), Scatchard plots of cytochalasin B binding to plasma membranes are shown. From these data the derived Scatchard plots (Figs. 2*b*, 2*d* and 2*f*) were calculated as described. The slopes of the linear Scatchard plots represent the affinity of the cytochalasin B-binding site for its substrate, and the intercept with the abscissa represents the concentration of cytochalasin B-binding sites. Fig. 2(*b*) shows binding data from plasma membranes of untreated adipocytes (●), insulin-treated adipocytes (▲) and TPA-treated adipocytes (■); insulin and TPA treatments increase the number of glucose-transport proteins approx. 2-fold. No effect on affinity of the glucose-carrier protein for cytochalasin B is evident. Figs. 2(*c*) and 2(*d*) show the same experiments for low-density membranes; Fig. 2(*c*) shows the raw binding data for labelled cytochalasin B, and Fig. 2(*d*) the derived Scatchard plots. TPA and insulin both decrease the number of cytochalasin B-binding sites in these subcellular membrane compartments. Figs. 2(*e*) and 2(*f*) finally show these data for high-density membranes, again the raw binding data in Fig. 2(*e*), and derived Scatchard plots for D-glucose-inhibitable cytochalasin B binding in Fig. 2(*f*). In this fraction only insulin caused an increase in carrier sites.

All cytochalasin B-binding data are summarized in Table 2. Glucose transporter numbers for equal amounts of cells were also calculated. We assume that the observed changes in glucose-transporter numbers reflect the previously proposed translocation process (Cushman & Wardzala, 1980). We cannot demonstrate an exact stoichiometry of a translocation process, as our low-density-membrane and plasma-membrane fractions did not contain equal amounts of protein. However, previous studies have shown that, from the calculation of cross-contamination of the membrane fractions by calculating the marker enzyme distribution, a stoichiometric translocation process can be assumed (Simpson & Cushman, 1985; Simpson *et al.*, 1983; Kono *et al.*, 1982). The distribution of marker enzymes found in our membrane preparations is in good agreement with the earlier reported values. Therefore we think that it is warranted to assume that the changes in cytochalasin B-binding capacities indeed reflect the translocation of glucose carriers. Fig. 3 shows a comparison of insulin and TPA effects on 3-*O*-methylglucose-transport activity of intact adipocytes and the effect of these substances on the distribution of cytochalasin B-binding proteins at the same time point. In five experiments, insulin at the maximal effective concentration stimulated the 3-*O*-methylglucose transport after 20 min incubation 8–10-fold. The effect of TPA in these five experiments was a 2–3-fold stimulation. The effect of insulin on the glucose carrier concentration in plasma membranes under these conditions was only 2-fold. TPA also caused a 2-fold increase in glucose carrier number in the plasma-

Table 1. Protein recovery and distribution of marker enzyme specific activities and activity ratios in subcellular membrane fractions from rat adipocyte cells

Results are expressed as the means of seven experiments. Activity ratios are calculated by setting the specific activity of the most enriched membrane fraction at 100% and those in other membrane fractions relative to that specific value.

Membrane fractions ...	Plasma membranes	Low-density membranes	High-density membranes	Homogenate
Protein recovery (mg/10^9 cells)	35.7±8.2	58.5±4.0	41.5±3.6	826±138
Protein recovery (% of homogenate)	4.3	7.1	5.0	100
5′-Nucleotidase (μmol/h per mg of protein)	2.10±0.29	0.29±0.07	0.63±0.14	0.38±0.07
Activity ratio (%)	100	14	30	–
NADH-cytochrome *c* reductase (μmol/min per mg of protein)	1.26±0.10	1.37±0.20	4.62±0.29	0.45±0.14
Activity ratio (%)	27	30	100	–
UDP-galactosyltransferase (nmol/2 h per mg of protein)	24.93±3.70	67.84±28.10	33.55±9.60	14.59±3.20
Activity ratio (%)	36.8	100	49.5	21.5

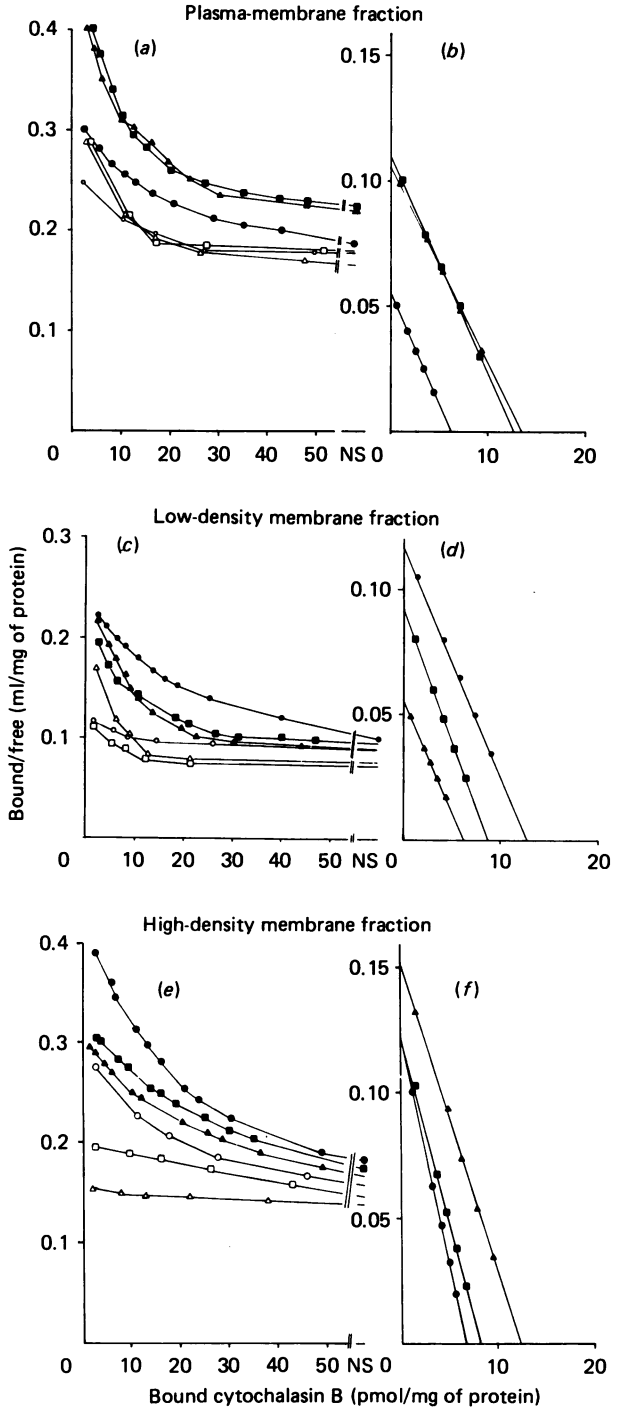

Fig. 2. Scatchard analyses of [^{3}H]cytochalasin B binding to rat adipocyte membranes isolated from basal (○, ●), insulin-stimulated (△, ▲) and TPA-stimulated (□, ■) adipocytes (*a*, *c* and *e*)

The results are the means of seven experiments. Derived Scatchard plots are shown in (*b*), (*d*) and (*f*) respectively. The resulting values were analysed by linear regression (NS = non-saturable). Cytochalasin B binding was measured in the absence (●, ▲, ■) or the presence (○, △, □) of 500 mM-D-glucose. The data represent typical profiles.

membrane fractions. Both substances caused a similar decrease in glucose carrier proteins in the low-density membrane fraction.

DISCUSSION

Previous studies had suggested that products of the phosphatidylinositol cycle, in particular diacylglycerol, might participate in insulin signal transmission through activation of protein kinase C (Farese *et al.*, 1985). One piece of evidence supporting this hypothesis was the observation that phorbol esters, which can substitute for diacylglycerol as an activator of protein kinase C, show insulin-like effects in isolated adipocytes (Van de Werve *et al.*, 1985; Kirsch *et al.*, 1985) as well as in myocytes in culture (Farese *et al.*, 1985). Whereas in the myocyte culture diacylglycerol and phorbol esters are able to elicit the full insulin response (Farese *et al.*, 1986), only a partial insulin-like effect, usually not more than 30% of the maximal insulin effect, can be induced in isolated rat adipocytes (Kirsch *et al.*, 1985). The aim of our study was to elucidate whether the phorbol esters exert their insulin-like effects on glucose transport through the same mechanisms as described for insulin (Wardzala *et al.*, 1978; Karnieli *et al.*, 1981; Simpson & Cushman, 1985, 1986), namely through stimulation of glucose-carrier translocation. This information would allow a further differentiation if the insulin-like effects of phorbol esters may be interpreted as evidence for a role of protein kinase C in insulin signal transmission. If the phorbol effects on glucose-transport activity occur by a mechanism different from that used by insulin, this would rather argue against a role of protein kinase C in insulin signal transmission in the rat fat-cell. Our results show on the one hand that phorbol esters indeed induce carrier translocation like insulin does; on the other hand, they show that insulin uses other mechanisms in addition to carrier translocation to activate the glucose-transport system.

Our data show that TPA activates glucose-transport activity of the isolated rat adipocyte by stimulation of glucose-carrier translocation. From the Scatchard plot an approx. 2-fold increase in glucose-carrier proteins in the plasma membrane is evident. The same increase in carrier sites in the plasma membrane can be induced by insulin. Thus insulin and the protein kinase C activator both act through carrier translocation. Phorbol esters stimulate glucose-transport activity 2–3-fold, and it is likely that carrier translocation is the only mechanism which leads to the increased glucose-transport activity of the fat-cell after phorbol ester stimulation. In contrast, it is obvious that the insulin effect on the glucose-transport activity of the isolated fat-cell must involve mechanisms in addition to carrier translocation. Insulin induces the same increase in glucose carriers in the plasma membrane as is observed with phorbol ester. In contrast, the glucose-transport activity of the fat-cell is increased considerably more. Although both substances have the same effect on carrier translocation, the effect of insulin on glucose-transport activity is 3–4-fold higher. This strongly suggests that insulin action on the glucose-transport activity in fat-cell involves an additional pathway which cannot be activated by phorbol esters. The most likely interpretation is that insulin acts directly on the activity of the glucose carrier after the translocation process to the plasma membrane, as similarly proposed previously by Carter-Su & Czech (1980). Another possible explanation could be that insulin and phorbol esters might act on different types of glucose carriers, which were described by Matthaei *et al.* (1987).

Table 2. Cytochalasin B-binding data from subcellular membrane fractions

Results are means ± S.E.M. ($n = 7$).

	Membrane fractions ...	Plasma membranes	Low-density membranes	High-density membranes	
[^{3}H]Cytochalasin B dissociation constant (nM)	Basal	110±16	109±20	56±3	
	Insulin	127±18	115±18	84±8	
	TPA	114±18	98±15	67±9	
[^{3}H]Cytochalasin B-binding sites (pmol/mg of protein)	Basal	6.2±1.0	12.8±2.1	6.9±1.1	
	Insulin	13.4±2.0	6.3±0.9	12.5±1.0	
	TPA	12.7±2.7	8.9±0.7	8.1±0.9	
					Total
Calculated [^{3}H]cytochalasin B-binding sites (pmol/10^7 cells)	Basal	2.2±0.04	7.5±0.12	2.9±0.05	12.6
	Insulin	4.8±0.07	3.7±0.05	5.2±0.04	13.7
	TPA	4.5±0.09	5.2±0.04	3.4±0.04	13.1

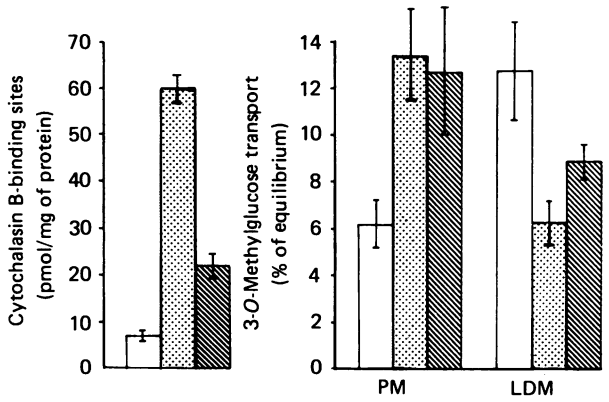

Fig. 3. Comparison of glucose-transport activity in isolated intact rat adipocytes and steady-state distribution of glucose transporters among subcellular membrane fractions

Preparations are from basal cells (□), insulin-stimulated cells (▨) and TPA-stimulated cells (▧). Key: PM, plasma membranes; LDM, low-density membranes. The data represent the means ± S.E.M. of the values generated in five (glucose transport) and seven (cytochalasin B-binding) experiments.

However, this study suggested that only one carrier subtype can undergo the translocation process. Summarizing these data, it appears most likely that insulin acts by a dual or a two-step mechanism on glucose transport in fat-cells, namely through carrier translocation and a subsequent additional, unknown, activating mechanism.

It is possible that the proposed two-step mechanism in the activation of the glucose-transport system by insulin might be specific for the fat-cell; in the rat fat-cell, phorbol esters are only able to induce a partial insulin-like effect (Kirsch *et al.*, 1985). In contrast, in myocytes phorbol esters are able to induce the full insulin response (Farese *et al.*, 1985) of the glucose-transport system, and it can be speculated that in this cell line glucose-transport activation occurs exclusively by carrier translocation, which can be initiated to the full extent also by the phorbol ester.

Two major questions remain: how convincing is the evidence for a role of protein kinase C in insulin signal transmission, and what could be the mechanism of insulin to activate the glucose carrier in the plasma membrane?

The present data add a further piece to the earlier indirect arguments for a role of protein kinase C in insulin action. On the other hand, Gibbs *et al.* (1986) showed that the glucose transporter in 3T3 adipocytes is phosphorylated on serine residues in response to phorbol ester, but not in response to insulin. This observation in 3T3 adipocytes would clearly argue against a role of protein kinase C. Thus further studies are needed to clarify this question. Concerning the mechanism how insulin might activate the glucose carrier, it is tempting to speculate that this occurs through direct phosphorylation of the glucose carrier by the insulin-receptor tyrosine kinase of the fat-cell. However, we ourselves were unable to phosphorylate *in vitro* the isolated glucose transporter (H. U. Häring, C. R. Kahn & M. F. White, unpublished work) or to precipitate a phosphorylated band in intact fat-cells by antibodies against glucose transporter (Häring *et al.*, 1987). Also, the results of Gibbs *et al.* (1986) argue against a tyrosine phosphorylation of the glucose carrier by the insulin-receptor kinase. Thus a direct phosphorylation of the carrier by the receptor tyrosine kinase, as proposed by Horuk *et al.* (1985), is unlikely. However, it remains to be clarified if one of the putative substrate proteins of the receptor kinase in the cell (White *et al.*, 1985; Häring *et al.*, 1987) might function as activators of the glucose-carrier protein.

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Received 20 May 1987/30 July 1987; accepted 30 September 1987