Phosphorylation of the adipose/muscle-type glucose transporter (GLUT4) and its relationship to glucose transport activity

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The effects of protein phosphorylation and dephosphorylation on glucose transport activity reconstituted from adipocyte membrane fractions and its relationship to the phosphorylation state of the adipose/muscle-type glucose transporter (GLUT4) were studied. *In vitro* phosphorylation of membranes in the presence of ATP and protein kinase A produced a stimulation of the reconstituted glucose transport activity in plasma membranes and low-density microsomes (51 % and 65 % stimulation respectively), provided that the cells had been treated with insulin prior to isolation of the membranes. Conversely, treatment of membrane fractions with alkaline phosphatase produced an inhibition of reconstituted transport activity. However, *in vitro* phosphorylation catalysed by protein kinase C failed to alter reconstituted glucose transport activity in membrane fractions from both basal and insulin-treated cells. In experiments run under identical conditions, the phosphorylation state of GLUT4 was investigated by immunoprecipitation of glucose transporters from membrane fractions incubated with [³²P]ATP and protein kinases A and C. Protein kinase C stimulated a marked phosphate incorporation into GLUT4 in both plasma membranes and low-density microsomes. Protein kinase A, in contrast to its effect on reconstituted glucose transport activity, produced a much smaller phosphorylation of the GLUT4 in plasma membranes than in low-density microsomes. The present data suggest that glucose transport activity can be modified by protein phosphorylation via an insulin-dependent mechanism. However, the phosphorylation of the GLUT4 itself was not correlated with changes in its reconstituted transport activity.

INTRODUCTION

The current concept of insulin action comprises a phosphorylation cascade mediating and amplifying the intracellular actions of the hormone (Denton *et al.*, 1981; Avruch *et al.*, 1982). Binding of insulin to the extracellular α -subunit of the insulin receptor activates an intrinsic tyrosine kinase at its intracellular β -subunit (Kasuga *et al.*, 1982). Subsequently, several proteins in plasma membranes, intracellular microsomes and cytosol are phosphorylated on tyrosine and serine residues (White *et al.*, 1985; Rees-Jones & Taylor, 1985; Bernier *et al.*, 1987; Weber *et al.*, 1988b). Furthermore, the activities of the insulin-regulated enzymes acetyl-CoA carboxylase (Brownsey & Denton, 1982), triacylglycerol lipase (Stralfors & Belfrage, 1983) and glycogen synthase (Parker *et al.*, 1983) are modified by phosphorylation and dephosphorylation of serine residues. Thus it appears reasonable to assume that most, if not all, metabolic effects of the hormone are conferred by changes in the phosphorylation state of insulin-regulated proteins.

Insulin produces a rapid and reversible stimulation of glucose transport activity in muscle and adipose tissue (for a review, see Joost & Weber, 1989). Furthermore, the transport activity is altered by other agents which activate serine kinases, e.g. isoprenaline (Joost *et al.*, 1986) and phorbol esters (Gibbs *et al.*, 1986; Mühlbacher *et al.*, 1988; Saltis *et al.*, 1988). Therefore the effects of insulin, isoprenaline and phorbol ester on the phosphorylation of glucose transporters have previously been studied in adipocytes (Joost *et al.*, 1987) and in an insulin-sensitive cell line, 3T3-L1 (Gibbs *et al.*, 1986). These studies showed that glucose transporters isolated with antiserum against the human erythrocyte glucose transporter contained no detectable amounts of phosphate in the basal state, after treatment with insulin or after treatment with isoprenaline. In contrast, the

transporters were phosphorylated in response to phorbol ester which produces a small stimulation of glucose transport in the absence of insulin, but does not alter the maximal effect of insulin (Gibbs *et al.*, 1986; Joost *et al.*, 1987). It was concluded from these data that phosphorylation or dephosphorylation of the glucose transporter is not involved in the regulation of glucose transport activity in adipocytes. Later, however, it became apparent that adipose tissue expresses not only the erythrocyte-type transporter (GLUT1) but also an adipose/muscle-type transporter (GLUT4) (Birnbaum, 1989; James *et al.*, 1989b) which is not immunoprecipitated by antiserum against GLUT1. Unlike GLUT1, GLUT4 contains phosphate in the basal state; its phosphorylation is further stimulated by isoprenaline (James *et al.*, 1989a). Consequently, it has been suggested that the phosphorylation of GLUT4, catalysed by the cyclic AMP-dependent protein kinase, mediates the inhibition of glucose transport activity by isoprenaline (Lawrence *et al.*, 1990).

The present study was designed in order to further correlate or dissociate changes in the phosphorylation state of the glucose transporter with changes in its glucose transport activity. We studied the effects of the *in vitro* phosphorylation of glucose transporters by protein kinase A and protein kinase C, and of dephosphorylation by alkaline phosphatase, on the reconstituted glucose transport activity. In addition, we investigated the effect of phorbol ester on the phosphorylation of GLUT4 under conditions which do not alter glucose transport activity. These experiments revealed several dissociations between the phosphorylation state of GLUT4 and glucose transport activity.

MATERIALS AND METHODS

Cell preparation and fractionation

Male Wistar rats, weighing 160–220 g, bred in our institute

Abbreviations used: KRBH, Krebs–Ringer bicarbonate/Hepes buffer; PMSF, phenylmethanesulphonyl fluoride; GLUT1, erythrocyte/brain-type glucose transporter; GLUT4, muscle/adipose-type glucose transporter.

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were used throughout. Adipose cells were isolated from epididymal adipose tissue by collagenase digestion as described (Rodbell, 1964) with minor variations (Weber *et al.*, 1988a). All incubations were carried out at 37 °C in a Krebs–Ringer bicarbonate buffer (Weber *et al.*, 1988a), pH 7.4, containing 4% BSA (Fraction V; Serva Chemicals, Heidelberg, Germany), 1 mM-glucose and 200 nM-adenosine (Honnor *et al.*, 1985).

Membrane fractions were isolated as previously described in detail (Simpson *et al.*, 1983; Joost *et al.*, 1988a; Weber *et al.*, 1988a). Briefly, adipocytes were incubated with the agents under investigation, and were thereafter washed and homogenized in a buffer containing Tris (20 mM), sucrose (255 mM) and EDTA (1 mM). The homogenate was centrifuged for 15 min at 17000 *g*. The resulting pellet, consisting of plasma membranes, mitochondria and nuclei, was resuspended in homogenization buffer, layered on to a sucrose cushion (38%), and centrifuged for 65 min at 23000 rev./min in a Beckman SW27 rotor (100000 *g*). Plasma membranes were collected from the top of the sucrose cushion; the pellet of the cushion contained both nuclei and mitochondria. The supernatant of the first centrifugation was centrifuged at 200000 *g* for 75 min to bring down the low-density microsomes. Membrane fractions were resuspended in the homogenization buffer, and were stored in liquid nitrogen.

Reconstitution of glucose transport activity from membrane fractions

Reconstituted glucose transport activity was assayed essentially as described by Robinson *et al.* (1982) with modifications described previously (Schürmann *et al.*, 1989). Briefly, membrane fractions were solubilized with 20 mM-sodium cholate in Tris buffer (20 mM, pH 7.4). Excess detergent was removed by gel filtration, and the transporter solution was combined with lecithin liposomes which had been prepared by two 3 min sonications of a 16% solution of egg lecithin (Sigma, Deisenhofen, Germany). The samples were sonicated for 5 s, frozen at –80 °C, thawed and sonicated again for 12 s. Transport was assayed at 37 °C with D-[U-¹⁴C]glucose, and rates were corrected for non-carrier-mediated uptake using L-[1-³H]glucose. The assay was stopped after 10 s, and samples were filtered on ME 24 membrane filters (Schleicher and Schuell, Dassel, Germany; cat. no. 401780). The dried filters were immersed in a water-compatible scintillation cocktail, and shaken for 2 h before liquid scintillation counting.

Preparation of protein kinase C

Protein kinase C was prepared as described (Walton *et al.*, 1987) from bovine brain with the aid of a phosphatidylserine column (Uchida & Filburn, 1984). The kinase was stored at –20 °C in 50% glycerol, which was removed by dialysis immediately before the experiments.

Phosphorylation of GLUT4 in membrane fractions (*in vitro*)

Samples of membrane fractions (100 µg of protein) were incubated with buffer containing (mM): sodium cholate (20), Hepes (10), EDTA (1), EGTA (0.1) and sucrose (250), pH 7.4, for 10 min on ice. [³²P]ATP (60 µCi/sample; final concentration 0.1 mM) and either (1) protein kinase A [20 µg; specific activity as given by the supplier (Sigma); 0.2 pmol of phosphate/min per µg of protein incorporated into casein] plus MgCl₂ (16 mM), or (2) protein kinase C [phosphorylating activity of 9 pmol of phosphate/min incorporated into histone H1S, assayed as described (Machado de Domenech & Söling, 1987) and reconstituted with 50 µg of phosphatidylserine/ml and 10 µg of dioleoin/ml] plus MgCl₂ (10 mM) and CaCl₂ (2 mM) were added, and the phosphorylation was allowed to proceed for 20 min at room temperature. NaF (100 mM), EDTA (5 mM), Hepes

(50 mM), phenylmethanesulphonyl fluoride (PMSF; 2 mM), NaCl (150 mM) and Triton X-100 (1%) were added, and the samples were kept for 30 min on ice for further solubilization. The samples were centrifuged for 45 min at 20000 *g* in a refrigerated Microfuge, and the supernatants were saved for immunoprecipitation of GLUT4 as described below.

Phosphorylation of GLUT4 in intact adipocytes (*in vivo*)

Equilibration of cells with tracer phosphate was carried out essentially as described previously (Joost *et al.*, 1989). Isolated adipocytes were washed five times with phosphate-free Krebs–Ringer bicarbonate/Hepes buffer (KRBH), and were resuspended in buffer containing a lowered phosphate concentration (0.1 mM). Sodium [³²P]phosphate was added (0.2–0.4 mCi/ml), and cells were allowed to equilibrate with the tracer for 90 min. Insulin (8 nM) was then added, and the experiment was terminated after an additional incubation period of 30 min. Where indicated, phorbol ester (1 µM) or a combination of isoprenaline (1 µM) plus adenosine deaminase (2.5 µg/ml) was added 15 min after the addition of insulin, and the incubation was terminated after another 15 min. The adipocytes were separated from the incubation medium by a rapid spin (3000 *g*, 2 min) through silicone oil, and the tubes were immediately frozen in a solid CO₂/methanol bath. The frozen cells were scraped off the top of the silicone layer and were lysed with 3 ml of a buffer (pH 7.4) containing (mM): NaCl 150, Hepes 50, NaF 20, vanadate 0.4, PP_i 40, PMSF 4, glycerophosphate 30 and EDTA 1, plus 1% Triton X-100. Lysates were centrifuged (15000 *g*) for 45 min in a refrigerated Microfuge in order to remove fat and insoluble material. The resulting cell extract was used immediately for immunoprecipitation without further storage. In some experiments, cells were washed and homogenized and membrane fractions were prepared as described above. As independent controls of the effects of insulin and isoprenaline, proteins from the cell extracts or from low-density microsomes were separated by SDS/PAGE, and phosphate incorporation into the 115 kDa ATP-citrate lyase, the 32 kDa ribosomal protein S6 and a 64 kDa protein was monitored by autoradiography.

Immunoprecipitation of GLUT4

Aliquots of the cell extracts (800 µl) or solubilized membrane fractions were incubated with antiserum against GLUT4 (Weiland *et al.*, 1990; code α-CT3) at a 1:100 dilution for 60 min on ice. Samples were centrifuged (15000 *g*, 30 min), and the antiserum α-CT3 (1:100) was added to the supernatants. After incubation at 4 °C for 60 min, the immunocomplexes were adsorbed to protein A–Sepharose and washed four times with buffer containing 50 mM-Hepes and 0.1% (w/v) Triton X-100, three times with the same buffer supplemented with 300 mM-NaCl, and twice with buffer containing 300 mM-NaCl and 0.1% SDS. The immunocomplexes were eluted with electrophoresis sample buffer containing 4% (w/v) SDS, 20% (w/v) glycerol, 0.05% Bromophenol Blue, 125 mM-Tris, 5 mM-EDTA and 200 µM-dithioerythrol. After elution, immunocomplexes were separated by SDS/PAGE on 10% gels. Dried gels were subjected to autoradiography on Kodak AR1 films for 1–10 days. Control experiments with pre-immune serum and immune serum blocked with the immunizing peptide (results not shown) were run in order to ascertain that the 48 kDa phosphoprotein immunoprecipitated by the immune serum indeed represented GLUT4.

Western blotting of GLUT4

Samples of plasma membranes (40 µg of protein) or aliquots of the immunoprecipitates were separated by SDS/PAGE and transferred to nitrocellulose sheets (Schleicher & Schuell) using a semi-dry blotting apparatus (Sartoblot II; Sartorius, Göttingen,

Germany). Sheets were blocked for at least 60 min by incubation with buffer containing 0.05% (w/v) Tween 20, and were then incubated with antiserum α -CT3 (1:200) for 2 h at room temperature. After three washes with buffer containing (1%) Triton X-100 and (1%) albumin, sheets were incubated with ^{125}I -Protein A (0.1 $\mu\text{Ci/ml}$) for 2 h, thoroughly washed, and autoradiographed for 1–4 days.

RESULTS

Effect of protein kinase A on reconstituted glucose transport activity

Solubilized membrane fractions from basal and insulin-treated adipocytes were reconstituted into lecithin liposomes by the freeze-thaw-sonication procedure developed by Robinson *et al.* (1982). The combination of protein kinase A and ATP produced a 40% increase in glucose transport activity reconstituted from plasma membranes or low-density microsomes, provided that the cells had been treated with insulin before preparation of membranes (Table 1, IPM and ILD). In contrast, the combination

Table 1. Effect of protein kinase A on glucose transport activity reconstituted from adipocyte membrane fractions

Samples of 30 μg of plasma membranes or low-density microsomes obtained from basal or insulin-treated adipocytes were solubilized and reconstituted into lecithin liposomes as described in the Materials and methods section in the presence or absence of protein kinase A (phosphorylating activity 2 pmol of phosphate/min incorporated into casein) and ATP (1 mM) in a total volume of 100 μl . BPM, plasma membranes from basal cells; IPM, plasma membranes from insulin-treated cells; BLD, low-density microsomes from basal cells; ILD, low-density microsomes from insulin-treated cells. Means $\pm$ S.E.M. are given of triplicate samples from a representative experiment.

	Glucose transport activity (nmol/10 s per mg of protein)	
	Control	Protein kinase A
BPM	0.21 $\pm$ 0.05	0.21 $\pm$ 0.02
IPM	1.46 $\pm$ 0.03	2.21 $\pm$ 0.23
BLD	1.8 $\pm$ 0.2	1.92 $\pm$ 0.23
ILD	0.81 $\pm$ 0.15	1.34 $\pm$ 0.02

Table 2. Effects of alkaline phosphatase on glucose transport activity reconstituted from adipocyte membrane fractions

Samples of 30 μg of plasma membranes or low-density microsomes obtained from basal or insulin-treated adipocytes as indicated were solubilized and reconstituted into lecithin liposomes as described in the Materials and methods section in the presence or absence of alkaline phosphatase (5 units/ml) in a total volume of 100 μl . BPM, plasma membranes from basal cells; IPM, plasma membranes from insulin-treated cells; BLD, low-density microsomes from basal cells; ILD, low-density microsomes from insulin-treated cells. Results are means $\pm$ S.E.M. of triplicate samples from a representative experiment.

	Glucose transport activity (nmol/10 s per mg of protein)	
	Control	Alkaline phosphatase
BPM	1.04 $\pm$ 0.17	0.88 $\pm$ 0.22
IPM	7.49 $\pm$ 0.62	4.76 $\pm$ 0.26
BLD	6.50 $\pm$ 0.45	5.91 $\pm$ 0.25
ILD	3.71 $\pm$ 0.11	2.54 $\pm$ 0.15

of ATP and protein kinase A failed to alter the activity of glucose transporters reconstituted from basal cells (Table 1, BPM and BLD). ATP or cyclic AMP-dependent protein kinase alone failed to alter the reconstituted glucose transport activity (results not shown; and Schürmann *et al.*, 1989).

Effect of alkaline phosphatase on reconstituted glucose transport activity

Alkaline phosphatase decreased the reconstituted transport activity in membrane fractions (Table 2, IPM and ILD) solubilized from insulin-treated cells by approx. 40%. In contrast, the enzyme failed to alter the glucose transport activity reconstituted from basal cells (Table 2, BPM and BLD). The inhibitory effect of alkaline phosphatase on glucose transport activity was dependent on the concentration of the enzyme; 1 unit/ml produced half-maximal inhibition (results not shown).

Effect of protein kinase C on reconstituted glucose transport activity

Phorbol ester has been shown to stimulate glucose transport activity and to induce a translocation of glucose transporters in rat fat cells and 3T3-L1 adipocytes (Mühlbacher *et al.*, 1988; Saltis *et al.*, 1988; Holman *et al.*, 1990; Gibbs *et al.*, 1991). Furthermore, phorbol ester stimulates the phosphorylation of GLUT1 (Gibbs *et al.*, 1986; Joost *et al.*, 1987), and appears also to moderately increase the phosphorylation of GLUT4 (see below, Fig. 3). The magnitude of the phorbol-ester-induced translocation, as assessed by surface labelling of glucose transporters, was lower than that produced by insulin, and corresponded reasonably well with the 2–3-fold stimulation of glucose transport activity in cells (Holman *et al.*, 1990; Gibbs *et al.*, 1991). On the basis of these data, it appeared unlikely that phorbol ester alters the intrinsic activity of glucose transporters. In contrast, Mühlbacher *et al.* (1988) reported that phorbol ester fully mimics the effect of insulin on transporter translocation, but produces a much smaller stimulatory effect on glucose transport activity. Thus it might be argued that protein kinase C, while generating an insulin-like translocation, decreases the intrinsic activity of glucose transporters. Therefore we studied the effect of protein kinase C on glucose transport activity in the reconstituted membranes. The results indicate that the combination of protein kinase C and ATP failed to alter the glucose transport activity reconstituted from both plasma membranes and low-density microsomes (Table 3).

Effects of the protein kinases A and C on GLUT4 phosphorylation

In order to correlate the observed effects of the protein kinases on the reconstituted glucose transport activity with the phosphorylation state of the transporters, the phosphate content of GLUT4 was assessed after incubation of the membrane fractions with [^{32}P]ATP and the protein kinases, and immunoprecipitation of GLUT4 with specific antiserum. Both kinases stimulated phosphate incorporation into a number of membrane proteins, protein kinase C giving rise to the phosphorylation of somewhat more bands than protein kinase A (Fig. 1a). Protein kinase C stimulated a large increase in the phosphate content of a 48 kDa protein immunoprecipitated with anti-GLUT4 antiserum in both plasma membranes (Fig. 1b) and low-density microsomes (Fig. 1c). Control experiments with pre-immune serum (Fig. 1) or serum blocked with the peptide used for generation of the antiserum (results not shown) indicated that the 48 kDa protein is indeed GLUT4. Protein kinase A, in contrast, produced a much lower phosphorylation of GLUT4 in low-density microsomes, and a hardly detectable phosphorylation in the plasma membranes (Fig. 1).

Table 3. Effect of protein kinase C on glucose transport activity reconstituted from adipocyte membrane fractions

Samples of 30 µg of plasma membranes or low-density microsomes obtained from basal or insulin-treated adipocytes were solubilized and reconstituted into lecithin liposomes as described in the Materials and methods section in the presence or absence of protein kinase C (phosphorylating activity 9 pmol of phosphate/min incorporated into histone H1S) and ATP (1 mM) in a total volume of 100 µl. IPM, plasma membranes from insulin-treated cells; ILD, low-density microsomes from insulin-treated cells. Results are means ± S.E.M. of triplicate samples from a representative experiment.

	Glucose transport activity (nmol/10 s per mg of protein)	
	Control	Protein kinase C
IPM	0.47 ± 0.04	0.44 ± 0.05
ILD	0.33 ± 0.05	0.38 ± 0.05

Effects of isoprenaline, insulin and phorbol ester on the phosphorylation of GLUT4 in intact adipocytes

The striking difference between basal and insulin-exposed glucose transporters in their sensitivity to cyclic AMP-dependent protein kinase and alkaline phosphatase raised the question as to whether insulin itself changes the phosphorylation state of GLUT4. A stimulatory effect of insulin on GLUT4 phosphorylation has been described in a preliminary report (Delvecchio & Pilch, 1989), whereas another study (James *et al.*, 1989a) failed to observe any effect of insulin. Therefore we re-examined the effects of the hormone on the incorporation of [³²P]phosphate into GLUT4 in isolated adipocytes. Cells were equilibrated with the labelled phosphate and subsequently treated or not with insulin. In order to preserve the phosphorylation state of glucose transporters during their isolation procedures, in the initial experiments the cells were centrifuged through silicone oil and immediately frozen. Lysates were then prepared from the frozen cells with solubilization buffer containing Triton X-100 and the phosphatase inhibitors vanadate, fluoride and EDTA.

Fig. 2. Effects of insulin and isoprenaline on the phosphorylation of total cellular GLUT4 in intact adipocytes

Immunoprecipitates of cell extracts from adipocytes pre-incubated with [³²P]phosphate and the agents under investigation were separated by electrophoresis, and subjected to autoradiography for 1 day at -70 °C. (a) Pre-immune serum; (b) α-CT3, immune serum against the tissue-specific glucose transporter. Lane 1, extracts from basal cells; lane 2, extracts from insulin-treated cells; lane 3, extracts from cells treated with insulin (8 nM) and subsequently with isoprenaline (1 µM) plus adenosine deaminase (2.5 µg/ml).

GLUT4 was immunoprecipitated from the lysates, and its [³²P]phosphate content was assessed by autoradiographic analysis. As is illustrated in Fig. 2, GLUT4 from basal cells incorporated detectable amounts of radioactive phosphate. Insulin failed to alter its phosphorylation state, whereas isoprenaline, as anticipated (James *et al.*, 1989a), gave rise to a moderate increase in [³²P]phosphate incorporation. Quantification of the effect of isoprenaline by determination of the radioactivity incorporated into the 48 kDa band indicated a 26.7 ± 9.6% (n = 3) increase compared with the control value.

The data presented in Fig. 2 indicate that insulin does not alter the phosphorylation state of the total cellular glucose

Fig. 1. Stimulation of *in vitro* phosphate incorporation into adipocyte GLUT4 by protein kinase C and cyclic AMP-dependent protein kinase

Samples of membrane fractions (100 µg of protein) were incubated with buffer containing (mM): sodium cholate (20), Hepes (10), EDTA (1), EGTA (0.1) and sucrose (250), pH 7.4, for 10 min on ice. In preliminary experiments the activities of the protein kinases producing a comparable phosphorylation of total membrane proteins were determined. [³²P]ATP (60 µCi/sample; final concentration 0.1 mM) and protein kinase A (phosphorylating activity 4 pmol of phosphate/min incorporated into casein) or protein kinase C (phosphorylating activity 9 nmol of phosphate/min incorporated into histone H1S) were added, and the phosphorylation was allowed to proceed for 20 min at room temperature. GLUT4 was immunoprecipitated as described in the Materials and method section, and immunoprecipitates were separated by SDS gel electrophoresis. (a) Phosphate incorporation into total plasma membranes; (b) immunoprecipitation of GLUT4 from plasma membranes; (c) immunoprecipitation of GLUT4 from low-density microsomes. Control samples (minus immune serum) in (b) and (c) were precipitated with pre-immune serum.

Fig. 3. Effects of insulin, isoprenaline and phorbol ester on *in vivo* phosphorylation of GLUT4 isolated from plasma membranes (PM) and low-density microsomes (LDM)

(a) Western blots of the glucose transporter GLUT4 in immunoprecipitates. Aliquots of the immunoprecipitations were separated by SDS/PAGE and blotted on to nitrocellulose membranes. The nitrocellulose membranes were incubated with the α -CT3 antiserum, and γ -globulins were labelled with ¹²⁵I-Protein A. The sheets were autoradiographed for 2 days at -70°C with the aid of an enhancing screen. (b) Autoradiograph of ³²P incorporation. Aliquots of the immunoprecipitates were separated by SDS/PAGE, and the dried gels were subjected to autoradiography for 5 days at -70°C with the aid of an enhancing screen. Lane 1, membrane fractions from basal cells; lane 2, membrane fractions from insulin-treated cells; lane 3, membrane fractions from cells treated with insulin (8 nM) and subsequently with isoprenaline (1 μM) plus adenosine deaminase (2.5 $\mu\text{g}/\text{ml}$); lane 4, membrane fractions from cells treated with insulin (8 nM) and subsequently with phorbol ester (1 μM).

transporters. Nevertheless, the overall phosphorylation of GLUT4 might be misleading, since insulin alters the subcellular distribution of glucose transporters between the plasma membrane and an intracellular pool (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). Thus the data presented in Fig. 2 do not exclude the possibility that an increase in transporter phosphorylation in plasma membranes was compensated by a dephosphorylation of transporters in the low-density microsomes. Therefore we studied [³²P]phosphate incorporation into the transporter protein after separation of plasma membranes and low-density microsomes (Fig. 3). Aliquots of the immunoprecipitates were used for assessment of the transporter concentration by immunoblotting (Fig. 3a). As anticipated, insulin increased the immunoreactivity of GLUT4 in plasma membranes. Conversely, the immunoreactivity of GLUT4 in low-density microsomes was decreased. In parallel with this re-distribution of glucose transporters, insulin altered the [³²P]phosphate content of GLUT4 in plasma membranes and low-density microsomes: the hormone produced a moderate increase in phosphorylation in the plasma membranes, and a comparable decrease in the low-density microsomes (Fig. 3b). Isoprenaline in combination with insulin, in contrast, failed to alter the insulin-induced re-distribution of GLUT4, but increased the phosphate content of GLUT4 in both plasma membranes and low-density microsomes (Fig. 3b). As also illustrated in Fig. 3, phorbol ester in combination with insulin failed to alter the insulin-induced re-distribution of GLUT4, but produced a small increase in the phosphate content of GLUT4 in plasma

membranes. It failed, however, to increase the phosphate content of GLUT4 from low-density microsomes (Fig. 3b). Similar results have been obtained previously with GLUT1: phorbol ester, when added to cells after a maximal stimulation by insulin, increased phosphate incorporation into GLUT1 in plasma membranes, but not in low-density microsomes (Joost *et al.*, 1988b).

DISCUSSION

The present data indicate that glucose transport activity reconstituted from adipocyte membrane fractions is stimulated by cyclic AMP-dependent protein kinase and inhibited by alkaline phosphatase. These findings suggest that glucose transport activity in adipocytes is specifically modulated by protein phosphorylation and dephosphorylation. However, the modifications of the reconstituted transport activity were not correlated with changes in the phosphorylation state of the GLUT4 glucose transporter. Firstly, protein kinase C failed to alter reconstituted glucose transport activity but stimulated the incorporation of [³²P]phosphate into GLUT4. Secondly, the stimulatory effect of protein kinase A on the phosphorylation of GLUT4 was much smaller than that of protein kinase C, and was much smaller in the plasma membranes than in low-density microsomes. In contrast, reconstituted glucose transport activity was similarly stimulated by protein kinase A in both membrane fractions. Therefore it appears unlikely that the observed changes in transport activity reflect changes in the phosphorylation state of GLUT4. It appears more likely that phosphorylation of another membrane protein, e.g. a regulatory protein, is responsible for the observed effects.

Catecholamines inhibit glucose transport activity in adipocytes provided that the endogenous adenosine is removed from the incubation by adenosine deaminase (Joost *et al.*, 1986; Kuroda *et al.*, 1987). It has been suggested that this effect is due to a phosphorylation of GLUT4 (Lawrence *et al.*, 1990), since catecholamines enhance the phosphorylation of this transporter, as was shown previously (James *et al.*, 1989a) and in the present study. However, protein kinase A did not inhibit but rather stimulated reconstituted glucose transport activity in adipocyte membrane fractions. Thus it is unlikely that the inhibitory effect of catecholamines is due to an increase in the phosphorylation of GLUT4 catalysed by the cyclic AMP-dependent protein kinase. Accordingly, previous studies have failed to establish a close correlation between cyclic AMP levels (Joost *et al.*, 1985) or the activity of the cyclic AMP-dependent protein kinase (Kuroda *et al.*, 1987) and the inhibitory effect of the catecholamines.

Insulin stimulates a rapid increase in glucose transport activity by translocation of transporters from an intracellular compartment of the plasma membrane (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). A stimulatory effect of insulin on the phosphorylation of GLUT4 has been reported in a preliminary paper (Delvecchio & Pilch, 1989), whereas others failed to observe such an effect (James *et al.*, 1989a). Confirming the latter findings, the present data failed to provide any indication that insulin might change the phosphorylation state of GLUT4 as a whole isolated from adipocytes. In addition, phosphate incorporation into GLUT4 isolated from plasma membranes and low-density microsomes after fractionation of cells appeared to correspond with the changes in the number of transporters in these cellular compartments. It has to be noted, however, that it is difficult to compare the phosphorylation state of basal and insulin-treated glucose transporters after fractionation of cells, because the change in the number of transporters in the two cellular compartments renders a comparison of the basal and insulin-treated states very inaccurate. Thus with the current

methods it is almost impossible to rule out the possibility that small changes in GLUT4 phosphorylation in plasma membranes were compensated by opposite changes in the low-density microsomes. In spite of these limitations, we feel that it is reasonable to conclude that phosphorylation or dephosphorylation of GLUT4 does not represent the signal for its insulin-induced translocation from the low-density microsomes to the plasma membrane. This conclusion is supported by the previous finding that GLUT1, like GLUT4, is translocated in response to insulin (Joost *et al.*, 1988a; Weiland *et al.*, 1990), but, unlike GLUT4, contains no detectable amount of phosphate in the basal state or after treatment of cells with insulin (Joost *et al.*, 1987).

Whereas phosphorylation of GLUT4 itself does not appear to be involved in the regulation of glucose transport activity, phosphorylation of other proteins might modulate the intrinsic activity of the glucose transporter. A crucial finding of the present study is that we had to treat the cells with insulin before homogenization and fractionation in order to demonstrate an effect of phosphorylation and dephosphorylation on the reconstituted glucose transport activity. Therefore the hormone must indirectly trigger this increase in intrinsic transporter activity. At present we can only speculate on the mechanism of this effect of insulin. It appears possible that insulin renders a regulatory protein susceptible to phosphorylation, which in turn increases the activity of the glucose transporter.

The present data indicate that protein kinase C stimulates a marked phosphorylation of GLUT4 *in vitro* in both membrane fractions, without any change in reconstituted glucose transport activity. Thus phosphorylation of GLUT4 catalysed by protein kinase C does not appear to alter the intrinsic activity of glucose transporters. This finding is in agreement with previously published data indicating that the stimulatory effect of phorbol ester on glucose transport in intact cells reflects a transporter translocation of similar magnitude (Holman *et al.*, 1990; Gibbs *et al.*, 1991). The present data do not fully exclude the possibility, however, that phosphorylation of glucose transporters catalysed by protein kinase C participates in the phorbol ester-mediated translocation of the transporters. The phorbol ester-induced translocation appears to be mediated via a different mechanism than that induced by insulin (Gibbs *et al.*, 1991). Unlike insulin, phorbol ester does not selectively translocate GLUT4, but gives rise to a somewhat more pronounced translocation of GLUT1 (Holman *et al.*, 1990; Gibbs *et al.*, 1991). It has previously been demonstrated in intact adipocytes that phorbol ester gives rise to a 3-fold increase in the phosphate content of GLUT1 in plasma membranes (Gibbs *et al.*, 1986; Joost *et al.*, 1987) but not in low-density microsomes (Joost *et al.*, 1988b). In the present study, treatment of adipocytes with phorbol ester produced a much smaller, or undetectable, increase in the phosphorylation of GLUT4 in plasma membranes and failed to phosphorylate GLUT4 in the low-density microsomes (Fig. 3).

In summary, the present data suggest that the intrinsic activity of GLUT4, as assayed in a reconstituted system of lecithin liposomes, can be modified by protein phosphorylation in an insulin-dependent manner. However, phosphorylation of GLUT4 itself does not appear to be responsible for these effects. In addition, the data suggest that phosphorylation of the GLUT4 is involved neither in the insulin-induced translocation of glucose transporters nor in the inhibitory effect of catecholamines on transporter activity.

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