The translocation of the glucose transporter sub-types GLUT1 and GLUT4 in isolated fat cells is differently regulated by phorbol esters

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Insulin stimulates glucose transport in isolated fat cells by activation of glucose transporters in the plasma membranes and through translocation of the glucose transporter sub-types GLUT4 (insulin-regulatable) and GLUT1 (HepG2 transporter). The protein kinase C-stimulating phorbol ester phorbol 12-myristate 13-acetate (PMA) is able to mimic partially the effect of insulin on glucose transport, apparently through stimulation of carrier translocation. In order to ascertain whether protein kinase C is involved in the translocation signal to both carrier sub-types, we determined the effect of PMA on the subcellular distribution of GLUT1 and GLUT4 by immunoblotting with specific antibodies directed against these transporters. Isolated rat fat cells (4×10^8 cells/ml) were stimulated for 20 min with insulin (6 nM) or PMA (1 nM). 3-O-Methylglucose transport was determined and plasma membranes and low-density microsomes were prepared for Western blotting. 3-O-Methylglucose transport was stimulated 8–9-fold by insulin, and 3–4-fold by PMA (basal, 5.6 ± 2.3 %; insulin, 43.6 ± 7.3 %; PMA, 18.4 ± 4.9 %, $n = 9$). PMA was able to increase the amount of GLUT4 in the plasma membrane fraction by $2.5 (\pm 0.9)$ -fold ($n = 6$) whereas insulin stimulation was $4.4 (\pm 1.7)$ -fold ($n = 6$), paralleled by a corresponding decrease of transport in the low-density microsomes (insulin, 50 ± 5 % of basal; PMA, 63 ± 11 % of basal, $n = 6$). Although PMA regulates the translocation of GLUT4, it has no effect on GLUT1 in the same cell fractions (increase in plasma membranes: insulin, 1.7 ± 0.5 -fold; PMA, 0.91 ± 0.1 -fold, $n = 4$; decrease in low-density microsomes: insulin, 53 ± 11 % of basal; PMA, 101 ± 5 % of basal, $n = 4$). These data are in favour of a role for protein kinase C in signal transduction to GLUT4 but not to GLUT1 in fat cells.

INTRODUCTION

Insulin rapidly activates glucose uptake in isolated fat cells. The underlying mechanism is a translocation of glucose carriers from subcellular membranes to the plasma membrane [1,2] and a modulation of the intrinsic activity of glucose carriers [3–6]. The signal-transmitting steps connecting the insulin receptor and the glucose transport system are so far incompletely understood. A role for protein kinase C in signal transmission is suggested by the fact that phorbol esters are able to mimic partially the effect of insulin on glucose transport activity in fat cells [7] and muscle [6]. We have demonstrated previously that this insulin-like effect of phorbol esters in fat cells is paralleled by a translocation of cytochalasin B-binding sites [3], suggesting a specific function of protein kinase C in insulin signal transduction on glucose carrier translocation [3]. More recently it became evident that glucose carriers represent a family of several sub-types which differ with respect to tissue expression and insulin-sensitivity [8–12]. The carrier subtypes which are important for glucose transport in fat cells are GLUT4 (insulin regulatable transporter) and GLUT1 (HepG2 transporter) [8,11,13]. In a preliminary study using the monoclonal antibody 1F8 against GLUT4, we showed that phorbol 12-myristate 13-acetate (PMA) induced GLUT4 translocation [14]. The aim of the present study was to determine whether stimulation of protein kinase C mimics the insulin signal equally to both carrier subtypes involved in the insulin effect. Therefore we compared the effects of PMA on the subcellular distribution of GLUT1 and GLUT4 in parallel experiments. Using monoclonal and polyclonal antibodies against GLUT4 and GLUT1, we found that in contrast with the translocation

effect on GLUT4, PMA did not mimic the effect of insulin on the translocation of GLUT1. This suggests that protein kinase C might be involved in the regulation of GLUT4 but not in the signalling chain of GLUT1. On the other hand, it could be that protein kinase C merely modulates GLUT4 translocation by some insulin-independent mechanism.

MATERIALS AND METHODS

Materials

Pig insulin was purchased from Novo Industrie (Bagsvaerd, Denmark). The monoclonal antibody 1F8 was kindly donated by Dr. P. F. Pilch, Department of Biochemistry, Boston University School of Medicine, Boston, MA, U.S.A. The polyclonal serum directed against the C-terminal end of GLUT4 was kindly donated by Dr. T. Ploug, Panum Institute, University of Copenhagen, Copenhagen, Denmark. The polyclonal serum against GLUT1 was obtained from WAK-Chemie, Bad-Homburg, Germany. 125 I-labelled goat anti-mouse IgG was obtained from Du Pont–New England Nuclear, nitrocellulose was from Schleicher & Schuell, and electrophoresis chemicals were from Bio-Rad. A chemiluminescence detection system was purchased from Amersham. 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 [15] from male Sprague–Dawley rats (180–220 g body wt.). Incubations were carried out at 37 °C in the absence (basal) or the presence of

Abbreviations used: GLUT4, insulin-regulatable glucose transporter; GLUT1, HepG2 glucose transporter; PBS, phosphate-buffered saline (pH 7.4); DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate.

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insulin or the phorbol ester PMA, as detailed in the Figure legends. PMA was diluted in pure ethanol, dried with N_2 , taken up in incubation buffer and sonicated. Glucose transport activity was measured as described by Häring *et al.* [15].

Cell fractionation and Western blotting

Rat adipocytes were isolated and subcellular fractions were obtained using a differential centrifugation procedure [3,16,17]. The purity of the membrane fractions was assessed by the determination of marker enzyme activities as described earlier [3].

Membranes were subjected to SDS/PAGE on a 7.5 % gel in the presence of 10 mM-dithiothreitol (DTT) using the system of Laemmli [18]. Proteins were transferred to nitrocellulose by electroblotting (buffer: 192 mM-glycine, 25 mM-Tris and 20 % methanol, pH 8.3) for 3 hr at 200 mA.

Following transfer, the filters were blocked with 5 % non-fat dried milk in phosphate-buffered saline (PBS) for 1 h at 37 °C and subsequently incubated with the first antibody (dilution in PBS/1 % dried milk) overnight at 4 °C. Immunocomplexes were visualized by incubation with 125 I-goat anti-mouse antiserum or 125 I-Protein A (25000 c.p.m./ml) for 1 h at 37 °C or 5 h at 4 °C respectively followed by autoradiography. The immunolabelled bands of 44 kDa were excised and counted for radioactivity. Background correction was done by counting non-labelled areas of nitrocellulose. With the GLUT4 antibody, immunolabelled bands were also visualized by chemiluminescence. After incubation with the specific antibody, nitrocellulose was washed several times in TBS (20 mM-Tris/150 mM-NaCl/2.5 % non-fat dried milk/1 % Triton X-100). To the immunocomplexes, horseradish-peroxidase-labelled anti-rabbit IgG was bound, and visualization of the immunolabelled bands was carried out by addition of a chemiluminescence reagent.

RESULTS

Isolated fat cells were stimulated with insulin or PMA. Insulin stimulated the uptake of 3-*O*-methylglucose approx. 8–9-fold, whereas PMA induced only a 3–4-fold increase in glucose uptake ([3]; results not shown). After 10 min of stimulation the cells were homogenized and plasma membrane and low-density membrane fractions were prepared. The purity of the membrane preparations was assessed by measurement of marker enzyme activities; there was a similar marker enzyme distribution as reported in our earlier studies ([3]; results not shown). The amounts of GLUT1 and GLUT4 were determined after separation of membrane proteins by gel electrophoresis, Western blotting and immunoblotting. Fig. 1 shows a representative autoradiogram of an immunoblot with a polyclonal antibody against GLUT1. Proteins in lanes 1 and 4 are from unstimulated cells, in lanes 2 and 5 from insulin-stimulated cells and in lanes 3 and 6 from PMA-treated cells. As reported earlier [13], considerable amounts of GLUT1 are found in the plasma membrane fraction under basal conditions. It is clear that after insulin stimulation the amount of labelling in the low-density microsomal fraction decreases, whereas a concomitant increase in the label in the plasma membrane fraction is detectable. PMA did not alter the distribution of the GLUT1 label. Table 1 shows the quantification of immunoblots for four separate experiments. In contrast with insulin treatment, phorbol ester treatment did not consistently decrease the labelling in the low-density microsome fraction or increase the labelling in the plasma membrane fraction. The analogous experiments were performed with two different antibodies against GLUT4, i.e. the monoclonal antibody 1F8 and a polyclonal antiserum against the C-terminal

Fig. 1. Western blot analysis of GLUT1 in subcellular membrane fractions of rat adipocytes after stimulation with insulin (6 nM) or PMA (1 nM) for 20 min

Total protein (100 µg) from plasma membranes (PM) and low-density microsomes (LDM) was immunoblotted using a polyclonal serum directed against GLUT1. Immunolabelled bands were visualized by autoradiography after using 125 I-labelled Protein A. B, basal; Ins, insulin-stimulated.

Table 1. Effects of insulin and PMA on GLUT1 translocation

Radioactivity was measured in c.p.m. in immunolabelled bands of 44 kDa using anti-GLUT1 antiserum for immunoblotting and 125 I-labelled Protein A (25000 c.p.m./ml) for immunolabelling in different subcellular fractions. PM, plasma membranes; LDM, low-density microsomes. Background correction was made by counting non-labelled areas of nitrocellulose, and was 10–20 %. Results are expressed as percentages of basal (means $\pm$ S.E.M., $n = 4$). Actual c.p.m. values were between 500 and 2000.

Addition	Relative 125 I-labelling of the GLUT1 band (%)	
	PM	LDM
None (basal)	100	100
Insulin (1000 µunits/ml)	170 $\pm$ 50	53 $\pm$ 11
PMA (1 nM)	91 $\pm$ 10	101 $\pm$ 5

end of GLUT4. Fig. 2 shows a representative autoradiogram of an immunoblot with the polyclonal antiserum. The lanes are shown in the same order as in Fig. 1. Again, insulin caused a clear decrease in the labelling in the low-density microsome fraction and a concomitant increase in labelling in the plasma membrane fraction. Immunoblots of six different experiments are quantified in Table 2. In contrast with the experiments shown in Fig. 1 and Table 1, PMA was able to induce translocation of GLUT4. As found in our preliminary study conducted with antibody 1F8, an approx. 35 % decrease in the amount of GLUT4 labelling in low-density microsomes and a 2.5-fold increase in the labelling in the plasma membrane occurred after stimulation with PMA. No significant differences could be detected between results obtained with the two anti-GLUT4 antibodies.

DISCUSSION

We have used in the present study an immunoblot technique to repeat translocation experiments we performed earlier with the cytochalasin B-binding technique. Two major conclusions

Fig. 2. Western blot analysis of GLUT4 in subcellular membrane fractions of rat adipocytes after stimulation with insulin (6 nM) and PMA (1 nM) for 20 min

Total protein (100 µg) from plasma membranes (PM) and low-density microsomes (LDM) was immunoblotted using anti-GLUT4 antiserum. Immunolabelled bands were visualized by horseradish peroxidase-labelled anti-rabbit Ig followed by chemiluminescence.

Table 2. Effects of insulin and PMA on GLUT4 translocation

Radioactivity was measured in c.p.m. in immunolabelled bands of 44 kDa using mAb1F8 for immunoblotting and ^{125}I -labelled goat anti-mouse antiserum (25000 c.p.m./ml) for immunolabelling in different subcellular fractions. PM, plasma membranes; LDM, low-density microsomes. Background correction was made by counting non-labelled areas of nitrocellulose, and was 10–20%. Results are expressed as percentages of basal (means $\pm$ S.E.M., $n = 6$). Actual c.p.m. values were between 500 and 2000.

Addition	Relative ^{125}I -labelling of the GLUT4 band (%)	
	PM	LDM
None (basal)	100	100
Insulin (1000 µunits/ml)	440 $\pm$ 170	50 $\pm$ 5
PMA (1 nM)	250 $\pm$ 90	63 $\pm$ 11

were drawn from the earlier reported data: (1) protein kinase C might be involved in carrier translocation, and (2) the stimulation by insulin of glucose transport has to occur as a two-step mechanism. Whereas the cytochalasin B-binding technique does not discriminate between carrier sub-types, the immunoblot technique used in the present paper allows us to differentiate between effects on the two carrier sub-types. The immunoblot data support in general the proposed role of protein kinase C in the translocation signal of glucose carriers and the proposed two-step mechanism described earlier. However, they also suggest that the mechanism of the effect of insulin on carrier translocation is more complex than originally thought.

Separate signalling chains to GLUT1 and GLUT4

The conclusion that protein kinase C is involved in the signal from the receptor to the glucose carrier translocation is only valid for GLUT4; it is not valid for the signal transduction affecting GLUT1. A possible explanation for this finding would be that two separate signal-transmitting chains regulate translocation of glucose carriers, but that only one of them includes protein kinase C as a signal-transducing or signal-modulating

element. An alternative explanation could be that it is not the signalling mechanism but the translocation machinery or the carrier-containing vesicle itself that determines the different susceptibilities to stimulation by PMA. It is known that GLUT1 and GLUT4 are localized in separate vesicles [13]. It is not known at present whether protein kinase C is connected somehow to GLUT4-containing vesicles, but it is known that phorbol esters induce translocation of protein kinase C to the plasma membrane. It seems conceivable that the translocation of GLUT4 is the result of a co-translocation of GLUT4 with protein kinase C.

Two-step mechanism of glucose transport stimulation: translocation and activation

Our earlier studies had shown that phorbol esters induce a translocation of cytochalasin B-binding sites into plasma membranes of rat adipocytes which is almost equal to the insulin-induced increase in cytochalasin B-binding sites in this membrane fraction. The insulin-induced stimulation of 3-*O*-methylglucose uptake was, however, 2–3-fold higher than the PMA-induced increase in 3-*O*-methylglucose uptake. We therefore concluded that the effect of insulin on glucose transport has to be the result of a two-step mechanism, including carrier translocation and also, as a second step, carrier activation [3]. The proposed model was later strengthened by the observation that a translocation-independent activation of glucose transport is possible [4,5]. With the immunoblot method we have confirmed for GLUT4 that phorbol esters induce carrier translocation. However, there is a quantitative difference between the results obtained with immunoblotting and the earlier-reported cytochalasin B-binding data. Western blotting, which is clearly a more accurate method with which to determine carrier numbers, shows that the effect of PMA reaches only approx. 70% of the effect of insulin. However, the difference between the insulin-induced increase and the PMA-induced increase in GLUT4 translocation is still too small to explain the large difference in the effects of insulin and PMA on 3-*O*-methylglucose uptake. The present data are therefore still consistent with the idea that translocation of transporters is not sufficient to explain the effect of insulin on glucose uptake. A second mechanism, i.e. the activation of glucose carriers by insulin, still has to be considered.

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