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Glut 4 content in the plasma membrane of rat skeletal muscle: comparative studies of the subcellular fractionation method and the exofacial photolabelling technique using ATB-BMPA

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Employing subcellular membrane fractionation methods it has been shown that insulin induces a 2-fold increase in the Glut 4 protein content in the plasma membrane of skeletal muscle from rats. Data based upon this technique are, however, impeded by poor plasma membrane recovery and cross-contamination with intracellular membrane vesicles. The present study was undertaken to compare the subcellular fractionation technique with the technique using [^{3}H]ATB-BMPA exofacial photolabelling and immunoprecipitation of Glut 4 on soleus muscles from 3-week-old Wistar rats. Maximal insulin stimulation resulted in a 6-fold increase in 3-*O*-methylglucose uptake, and studies based on the subcellular fractionation method showed a 2-fold increase in Glut 4 content in the plasma membrane, whereas the exofacial photolabelling demonstrated a 6- to 7-fold rise in cell surface associated Glut 4 protein. Glucose transport activity was positively correlated with cell surface Glut 4 content as estimated by exofacial labelling. In conclusion: (1) the increase in glucose uptake in muscle after insulin exposure is caused by an augmented concentration of Glut 4 protein on the cell surface membrane, (2) at maximal insulin stimulation (20 mU/ml) approximately 40% of the muscle cell content of Glut 4 is at the cell surface, and (3) the exofacial labelling technique is more sensitive than the subcellular fractionation technique in measuring the amount of glucose transporters on muscle cell surface.

Glucose transporter; Exofacial photolabelling; Subcellular fractionation; Skeletal muscle; ATB-BMPA

1. INTRODUCTION

An increasing amount of data indicate that glucose uptake into skeletal muscles is the rate-limiting step in glucose metabolism [1]. Knowledge of its regulation is therefore important in understanding both glucose homeostasis in healthy man as well as the mechanisms underlying insulin resistant conditions, e.g. non-insulin-dependent diabetes mellitus, obesity and essential hypertension.

In insulin-sensitive tissues such as adipocytes and skeletal muscle cells insulin exposure results in an increased glucose transport into the cells. With the application of the subcellular membrane fractionation technique to adipocytes it has been demonstrated that the increased glucose transport is ascribable to translocation of glucose transporter proteins (primarily Glut 4) from an intracellular pool to the plasma membrane [2,3]. When a similar technique was applied in skeletal muscle from rats, insulin stimulation was shown to promote only a modest (2-fold) enrichment in the number of cytochalasin B binding sites or Glut 4 proteins in the plasma membrane fraction [4,5] and a modest decrease of Glut 4 (~40%) in the intracellular microsomal fraction. However, following maximal insulin stimulation 3-*O*-methylglucose transport into rat soleus muscle is increased 5- to 7-fold [6]. Thus, it might be assumed that translocation of the glucose transporters per se is only partly responsible for the increased glucose uptake, implying that the glucose transporter proteins, in addition to their ability to translocate to the cell surface, also are modified in their intrinsic activity. However, the subcellular fractionation technique is more difficult to apply to skeletal muscle than to adipocytes, and interpretation of data must be balanced by poor recovery of membrane markers (~10%), a high degree of cross-contamination among membrane subfractions, and a lack of specific markers for the intracellular pools of glucose transporters. Recent studies in rat adipose cells applying a new technique, namely exofacial photolabelling with the bis-mannose derivative (ATB-BMPA) and subsequent immunoprecipitation with Glut 4 antibodies show that the Glut 4 transporter content on the cell surface increases 15- to 20-fold following insulin exposure which

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Abbreviations ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl) benzoyl-1, 3-bis-(D-mannose-4-yloxy)-2-propylamine; $C_{12}E_9$, nonaethyleneglycol dodecyl; DOC, deoxycholic acid ; GLUT 4, insulin-sensitive muscle/fat glucose transporter.

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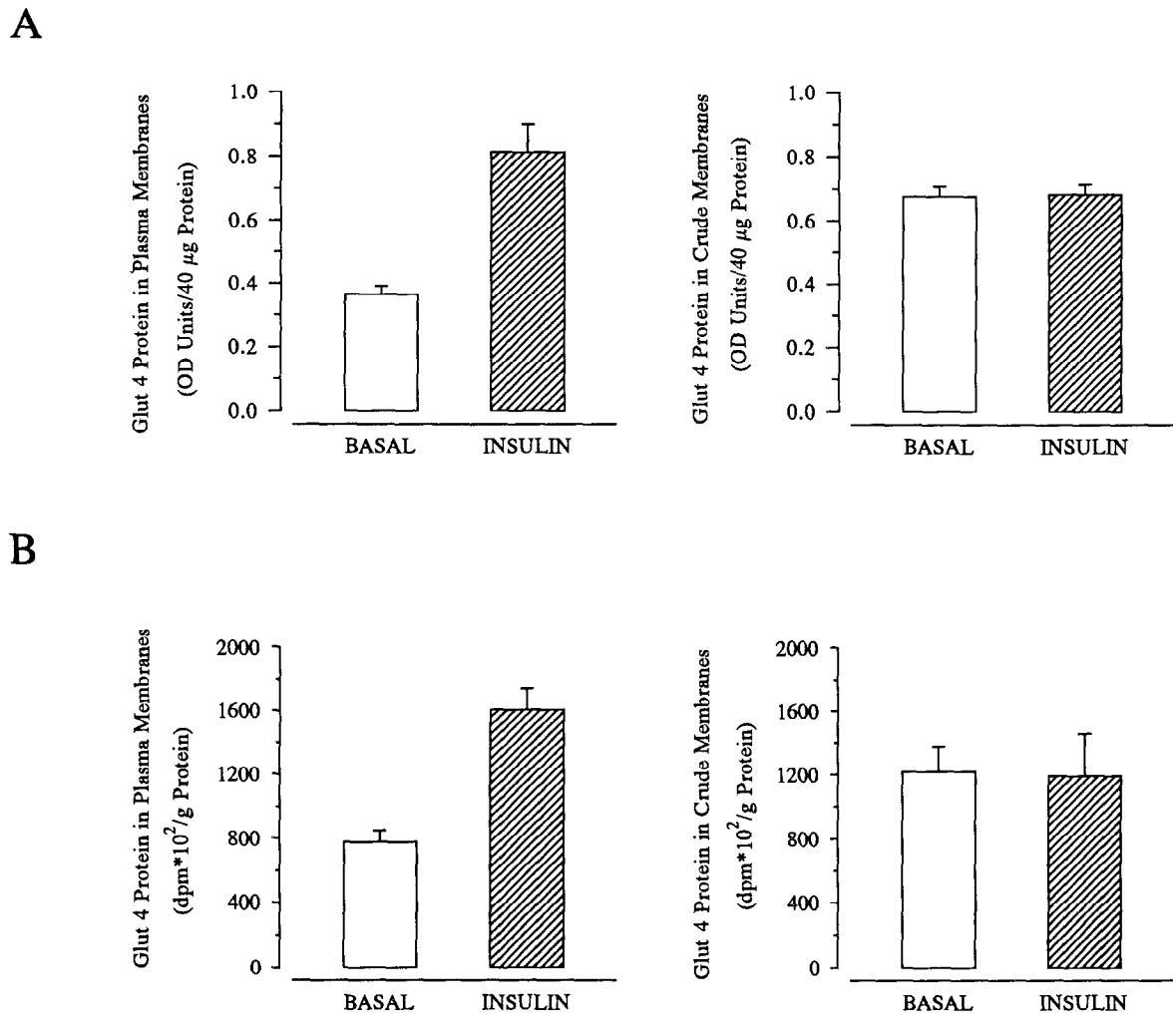

Fig. 1. Effect of insulin treatment on the distribution of Glut 4 in plasma membranes and crude membranes isolated from soleus muscles from 3-week-old rats. (A) Western blotting analysis: membrane protein (40 μg) was subjected to a 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 5% BSA, then incubated for 1 h at 37°C with 1F8, monoclonal anti-Glut 4 antibody (1:10.000 dilution), followed by 1 h incubation with HRP-labelled rabbit anti-mouse immunoglobulin (1:25.000 dilution). Labelled bands were visualised by the enhanced chemiluminescence technique (ECL-technique, Amersham). Values are mean ± S.E.M. (n = 6) . (B) ATB-BMPA photolabelling: plasma and crude membranes were photolabelled by incubating 180 μl membranes with 20 μl ATB-[^{3}H]BMPA (10 mCi/ml). The samples were irradiated for 2 min and solubilized for immunoprecipitation as described in section 2. Values are mean ± S.E.M. (n = 3).

is quite equivalent to the increment in 3-*O*-methylglucose transport [7]. Adipose tissue is, however, quantitatively (5–20%) of minor importance for whole-body glucose uptake and utilization in man during hyperinsulinemia [8]. The dominant tissue for glucose clearance during insulin stimulation is skeletal muscle, and the cellular characteristics of the glucose transport system are not necessarily similar in these two target tissues.

The present study was undertaken to compare the classic subcellular fractionation technique with the exofacial photolabelling technique in terms of measuring translocation of Glut 4 in skeletal muscle. We examined the in vitro effect of an insulin challenge on glucose transport as measured by 3-*O*-methylglucose and on the Glut 4 content in the plasma membrane using both techniques on the soleus muscle from 3-week-old Wistar rats.

Our results indicate that translocation of the Glut 4 glucose transporter to the cell surface is responsible for the increased glucose transport activity of skeletal muscle following insulin stimulation. In addition, the exofacial labelling technique appears to be considerably more sensitive to detect changes in the cell surface content of glucose transporters than the subcellular fractionation technique previously used.

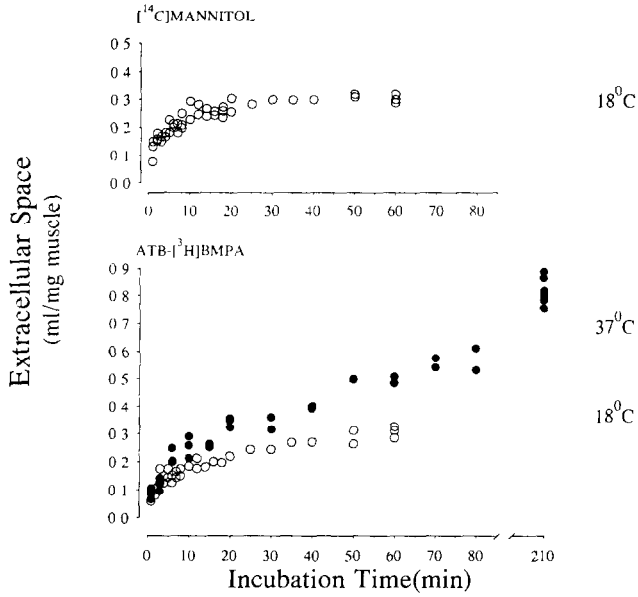

Fig. 2. Time course of ATB-BMPA and mannitol equilibration in intact soleus muscles. (a) [^{14}C]mannitol equilibration. Muscles were preincubated as described in Materials and Methods and then incubated at 18°C for the times indicated (○) in glucose-free medium containing 40 mM mannitol. (b) [^{3}H]ATB-BMPA equilibration. Muscles were incubated as described above at either 18°C (○) or 37°C (●).

2. MATERIALS AND METHODS

2.1. *Preparation of ATB-BMPA*

ATB-[2-^{3}H]BMPA (specific activity ≈ 10 Ci/mmol) was prepared as described previously [9]

2.2. *Animals and muscle preparation*

Three-week-old male Wistar rats (Mollegaards Breeding Laboratory, Denmark) weighing 50–70 g were examined. Animals were overnight fasted prior to the experiments. The rats used for measurements of 3-*O*-methylglucose uptake and ATB-BMPA labelling were killed by a blow to the neck followed by cervical dislocation. Soleus muscles were rapidly but carefully dissected out avoiding stretching of the muscle fibers, and the muscles were constantly flouted with saline.

The animals studied for plasma membrane preparation (subcellular fractionation) were injected subcutanously with either 4 units of soluble insulin (Actrapid, Novo-Nordisk, Denmark) dissolved in 250 μl of isotonic saline or with an equivalent volume of saline. Thirty minutes after the injections the rats were killed by a blow to the neck followed by cervical dislocation. At this time blood was collected for analysis of plasma insulin Soleus muscles from both legs were rapidly dissected, trimmed free of fat and connective tissue and immediately used for plasma membrane preparation.

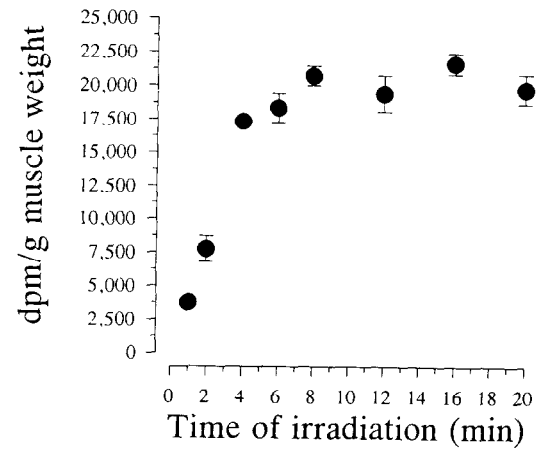

Fig. 3. Time course of irradiation for the effect on incorporation of the photolabel ATB-BMPA into the glucose transporter region. Muscles were irradiated in a Rayonet RPR photochemical reactor (RPR 3000 lamps). Values are mean ± S.E.M. (n = 4)

2.3. *Muscle incubations*

Intact soleus muscles were incubated twice for 30 min in 4 ml Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 2 mM pyruvate, 38 mM mannitol and 0.1% BSA (radioimmunoassay grade) in absence or presence of insulin in a 30°C shaking water bath. All the buffers used for the muscle incubation were continuously gassed with 95% O_2/5% CO_2.

2.4. *Measurement of glucose transport activity and extracellular water space*

Glucose transport activity was measured using the nonmetabolizable glucose analogue 3-*O*-methylglucose and a modification [10] of the procedure which previously has been described for frog muscles [11,12]. After incubation with (or without) insulin, muscles were blotted and rinsed for 10 min by shaking at 30°C in 4 ml KHB containing 40 mM mannitol, 0.1% BSA and insulin at the same concentration as during the preceding incubation The muscles were then blotted on filter paper and incubated in 3 ml KHB containing 8 mM 3-*O*-[^{3}H]methylglucose (437 μCi/mmol) and 32 mM [^{14}C]mannitol (8 μCi/mmol) for a period of 10 min, during which time the 3-*O*-methylglucose accumulation rate was linear Following incubation, the muscles were blotted briefly on filter paper dampened with incubation medium, trimmed and frozen in liquid N_2. The muscle samples were weighed, homogenized in 10% trichloroacetic acid, and centrifuged at 1,000 × g. Aliquots of the muscle extracts and of the incubation media were counted with channels preset for simultaneous ^{3}H and ^{14}C counting. The amount of each isotope present in the samples was determined and the extracellular space and the intracellular concentration of 3-*O*-[^{3}H]methylglucose were calculated The intracellular water content of the muscle was determined by subtracting the measured extracellular water space from total muscle water.

Table I

Weight, marker enzyme specific activity, recovery and fold enrichment, of soleus muscles from control and insulin treated rats

	Control	Insulin
Weight of muscle (mg)	415 ± 5	411 ± 11
Homogenate protein (mg)	28.5 ± 2.0	29.5 ± 3.2
Plasma membrane protein (μg)	214 ± 20	203 ± 19
Homogenate specific K_{pNPP}-ase activity (nmol/30 min/mg prot.)	158.2 ± 12.7	156.2 ± 8.8
Plasma membrane specific K_{pNPP}-ase activity (nmol/30 min/mg prot.)	5,564 ± 174	5,669 ± 391
Plasma membrane recovery (%)	13.1 ± 1.7	12.2 ± 1.4
Plasma membrane fold enrichment	38.8 ± 5.4	37.7 ± 3.4

Results are means ± S.E.M., n = 6 Total K_{pNPP}-ase activity of the homogenate and plasma membrane fraction were determined by multiplying their specific activities with their respective protein recoveries. Percent recovery was the result of dividing total enzyme activity of the plasma membrane by the total enzyme activity of the homogenate multiplied by 100. Plasma membrane fold enrichment is an indication of purity of the plasma membrane marker enzyme specific activity relative to that of the homogenate

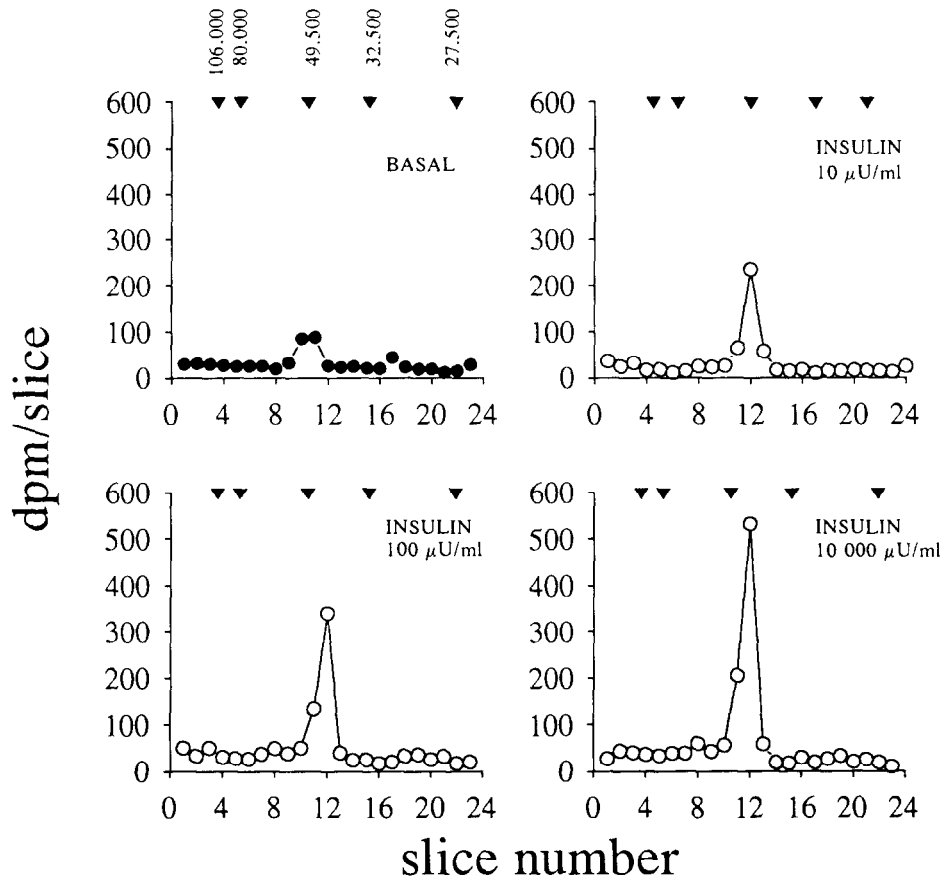

Fig. 4. Gel profile of exofacial photolabelling of intact rat soleus muscle with ATB-BMPA. Isolated rat soleus muscles were incubated for 8 min with 1 mCi ATB-BMPA/ml and irradiated twice for 3 min. Total membranes were prepared and solubilized, and photolabelled Glut 4 was immunoprecipitated and analyzed as described in section 2 Location of molecular weight markers are indicated with arrows. Basal (●) and insulin stimulated (○) muscles. Insulin contractions as indicated in the figures.

2.5. *Photolabelling of rat soleus muscles*

Muscles were incubated as described above and transferred to a dark room and further incubated at 18°C for 8 min in KHB buffer containing 1 mCi/ml ATB-[^{3}H]BMPA Muscles were then irradiated twice for 3 min in a Rayonet RPR 100 photochemical reactor (RPR 3000 lamps), with manual turning over of the muscles after 3 min Following irradiation, muscles were immediately blotted, trimmed and frozen in liquid N_2. The frozen muscles were weighed and homogenized in 5 ml ice-cold 25 mM HEPES, 1 mM NaEDTA, 250 mM sucrose buffer (pH 7.4) containing the following protease inhibitors: 1.0 mM pefabloc (AEBSF), 1.0 mM benzanidine and a mixture of leupeptin, pepstatin, aprotinin, antipain each at a final concentration of 10 μg/ml. The homogenate was centrifuged for 90 min at $230.000 \times g_{max}$ (4°C), the supernatant was discarded and the pellet resuspended and solubilized in buffer containing 2% $C_{12}E_9$, 0 5% DOC and 0.1% SDS (plus proteinase inhibitors) for 60 min at room temperature Subsequently, the suspension was centrifuged for 30 min at $80.000 \times g_{max}$ (4°C). The resulting supernatant was subjected to immunoprecipitation.

2.6. *Immunoprecipitation*

Immunoprecipitation of the photolabelled glucose transporter using rabbit antiserum against GLUT 4 was carried out as described elsewhere [13] except that immunoprecipitation was performed at room temperature.

2.7. *Electrophoresis*

The immunoprecipitated glucose transporters were mixed with electrophoresis buffer (10% SDS, 6 M urea and 10% β-mercaptoethanol) and run overnight on a 20 cm long 10% SDS-PAGE gel in Laemmli buffer. Bio-Rad's prestained low molecular weight standards were used as molecular weight markers. Gels were briefly stained and destained using Coomassie blue, and then cut into 6 mm slices. The slices were dried at 80°C for 3 h and then dissolved and incubated for 3 h in 1 ml of 30% H_2O_2 containing 2% NH_2OH at 80°C. The levels of radioactivity associated with each peak were obtained by summing the radioactivity in all slices comprising the peak and subtracting the background radioactivity which was based on the average radioactivity of the slices on either side of the peak The values were finally normalized per gram of frozen muscle.

2.8. *Preparations of crude membranes and plasma membranes*

Isolation of plasma membranes from skeletal muscle tissue was performed as described previously by Grimditch et al. [14] and as modified by Hirshman et al. [15].

Crude membranes (plasma membrane and microsome) were prepared as described by Garvey et al. [16]

2.9. *Western blotting and photolabelling of crude and plasma membrane fractions*

Muscle membrane preparations were subjected to SDS-PAGE, fol-

lowed by electrophoretical transfer to nitrocellulose filter membranes (20–50 μg/lane). The content of Glut 4 protein was determined by immunoblotting with the monoclonal antibody 1F8 (Genzyme Corporation, Cambridge, USA) Immunolabeled bands were visualized with the ECL-technique (Amersham). Autoradiograms were quantitated by scanning densitometry (Shimadzu CS 9001PC, flying spot scanner). Plasma and crude membranes were photolabelled by incubating 180 μl membranes with 20 μl ATB-[^{3}H]BMPA (10 mCi/ml). The samples were irradiated for 2 min and solubilized for immunoprecipitation as described above.

2 10. *Protein and marker enzyme determinations*

Muscle homogenate and membrane proteins were determined in duplicate by the Coomassie brilliant blue method (Bio-Rad protein assay) as described by Bradford [17] using bovine serum albumin as a standard. The specific activity of potassium stimulated-*p*-nitrophenol phosphatase (K_{pNPP}-ase) was assayed in duplicate with and without 20 mM K^+ [18]

3. RESULTS AND DISCUSSION

3.1. *Subcellular fractionation technique (Fig. 1)*

Both Western blotting analysis and ATB-BMPA photolabelling showed that in vivo insulin stimulation was associated with a 2-fold increase in the Glut 4 content in the plasma membrane. In contrast, the amount of Glut 4 in the crude membrane fraction was not influenced by insulin administration. The activity of the plasma membrane marker (K_{pNPP}ase-activity) was similar in the insulin- and the saline-injected animals, and showed a high degree of enrichment (38.3 ± 3.1)fold compared to the homogenate (Table I). In accordance with previous studies [15,19] the plasma membrane recovery was low (12.6 ± 1.1%). The increased abundance of glucose transporters in the plasma membrane after insulin exposure did not represent newly synthesized

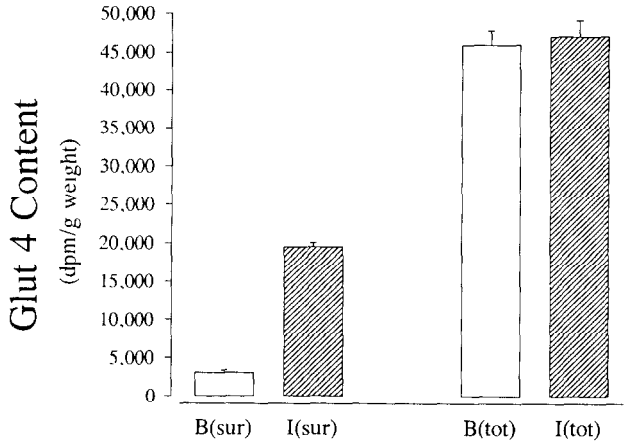

Fig. 5. Comparison of ATB-BMPA labelling of cell surface and total cellular pools of Glut 4 in rat soleus muscles. Values for cell surface labelling of Glut 4 proteins are taken from Fig. 6. The total cellular pool of Glut 4 was determined by incubating muscles for 210 min with 1 mCi ATB-[^{3}H]BMPA at 37°C in a dark room. Muscles were then irradiated, homogenated, solubilized and immunoprecipitated as described under Materials and Methods. Insulin (20 mU/ml) was added after 150 min of incubation. Values are mean ± S.E.M. (n = 4). (sur) cell surface, (tot) total cellular content, (B) basal and (I) insulin treated muscles (20 mU/ml).

transporters or was caused by differences in separation of subcellular membrane fraction after insulin stimulation, since the content in the crude membrane fraction as well as the plasma membrane marker activity were similar in the two groups. Consequently, the increment in plasma membrane content of Glut 4 most likely represents translocation of glucose transporters from an intracellular pool to the plasma membrane. The high level of labelling of basal membranes and the correlation between the Glut 4 content as detected by Western blotting and ATB-BMPA labelling suggests that plasma membrane fractions are highly contaminated by Glut 4 which is normally inaccessible to the photolabel in intact cells. Furthermore, since the total level of labelling in crude membranes is the same in the muscles in the basal and insulin stimulated state, the possibility that insulin may have altered the intrinsic reactivity of the muscle transporter for the photolabel seems unlikely. Plasma insulin levels measured 30 min after injection were 756 ± 53 μU/ml in insulin-injected rats and 2.1 ± 0.2 μU/ml in control rats.

3.2. *Time course of ATB-BMPA equilibrium in the extracellular water space (Fig. 2) and optimal irradiation time (Fig. 3)*

Because the diffusion distance in rat soleus muscles is crucial for the time necessary to obtain substrate equilibrium [6] only small rats (50–60 gram) were used. To minimize the risk of an internalization of ATB-BMPA due to recycling of the glucose transporter at the cell surface an incubation temperature of 18°C was chosen [13]. During elevation of the temperature to 37°C a constantly increasing amount of ATB-BMPA was found in the muscle, apparently due to fluid-phase endocytosis. After 210 min of incubation at 37°C ATB-BMPA was in equilibrium in both the extracellular and intracellular water space (similar results were achieved when mannitol equilibrium was examined at 37°C, (data not shown). An incubation time of 8 min was adequate for near-equilibrium of ATB-BMPA at 18°C. The short incubation period implied minimal likelihood of non-specific internalization. Six min of irradiation was sufficient to achieve maximal labelling of ATB-BMPA to the glucose transporters.

3.3. *Effect of insulin on Glut 4 content in the plasma membrane – exofacial photolabelling technique (Fig. 4).*

A gel profile of muscles labelled with ATB-BMPA and subsequently immunoprecipitated with Glut 4 antibody is shown in Fig. 4. The photolabelled glucose transporters run as a clear peak at a molecular weight ≈ 48 kDa. The specificity of ATB-BMPA labelling was examined by the addition of cytochalasin B (5 μM) or D-glucose (240 mM) which resulted in a complete inhibition of insulin stimulated incorporation of the photolabel into the glucose transporter region of the gel (data

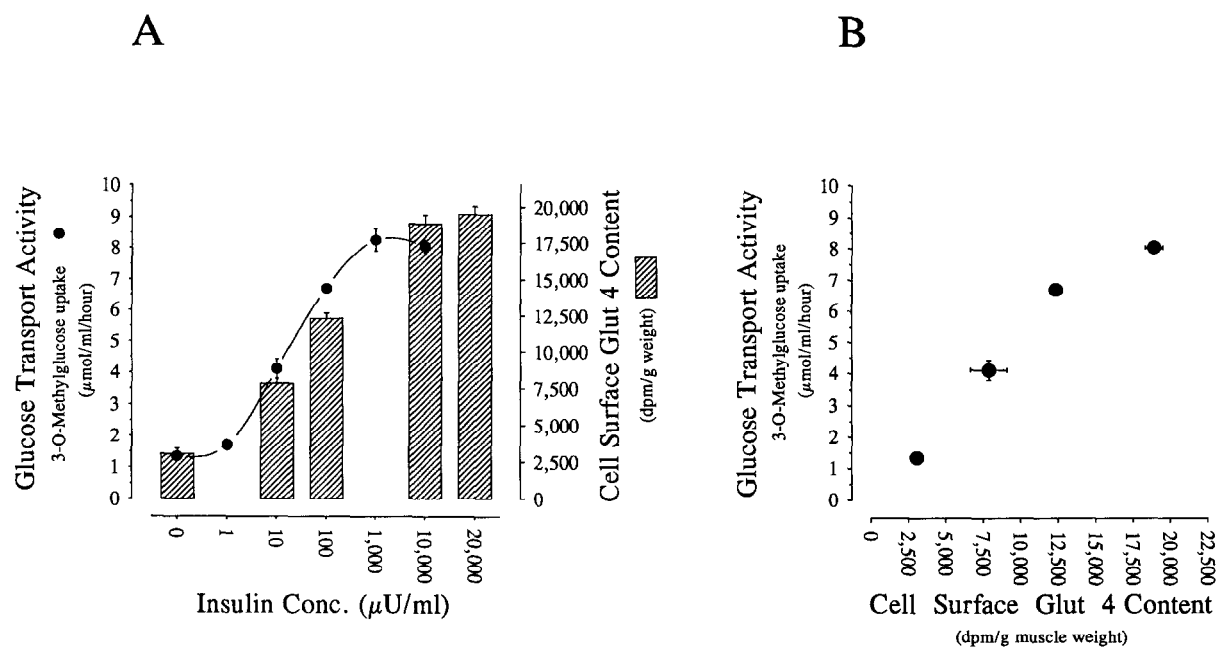

Fig. 6. (a) Effect of insulin on 3-*O*-methylglucose uptake and Glut 4 content in intact rat soleus muscles. Glucose transport activity (●) was measured using [^{3}H]3-*O*-methylglucose and [^{14}C]mannitol as described in section 2. Cell surface membrane Glut 4 content was determined by ATB-BMPA photolabelling (□). Muscles were incubated for 8 min with 1 mCi ATB-BMPA/ml at 18°C in the dark, then irradiated twice for 3 min in a Rayonet RPR 100 photochemical reactor (RPR 3000 lamps), with manual turning over of the muscles after 3 min. After irradiation muscles were homogenized, solubilized and immunoprecipitatied with anti-Glut 4 antibodies as described in section 2. Values are mean ± S.E.M. (n = 5–15). (b) Glucose transport activity vs. cell surface Glut 4 content detected by ATB-BMPA photolabelling (mean ± S.E.M.).

not shown). The labelling which was detected after maximal insulin stimulation (20,000 μU/ml) was 6- to 7-fold greater than in the basal state. This finding is in agreement with preliminary data reported from another group of investigators [20].

3.4. *Total muscle Glut 4 content determined by ATB-BMPA labelling (Fig. 5)*

210 min incubation of muscles with ATB-BMPA at 37°C resulted in equilibrium of ATB-BMPA in both the extracellular and intracellular water space thus making the intracellular located glucose transporters accessible to photolabelling. Labelling of the total cellular pools of Glut 4 were similar in the basal and insulin-treated condition. This finding supports the suggestion (see section 3.1) that insulin is unlikely to have altered the intrinsic reactivity of Glut 4 towards the photolabel. Comparison of the labelling of surface and the total cellular content of Glut 4 suggest that, at maximal insulin stimulation (20 mU/ml), approximately 40% of Glut 4 is at the cell surface. Similar results have been found in 3T3-L1 cells [21].

3.5. *Dose–response of insulin on 3-O-methylglucose uptake in rat soleus muscles (Fig. 6a)*

Applying the non-metabolizable glucose analogue, 3-*O*-methylglucose, the glucose transport process can be studied independently of glucose metabolism [11]. The glucose transport activity increased 6-fold (from 1.3 to 8.1 μmol/ml/h) following exposure to maximal insulin concentrations. Half-maximal stimulation was obtained at an insulin concentration of 20 μU/ml. Fig. 6b shows glucose transport activity as assessed by 3-*O*-methylglucose uptake was strongly positive correlated with cell surface Glut 4 content, suggesting that the increase in skeletal muscle glucose uptake after insulin exposure is caused by an augmented concentration of Glut 4 in the plasma membrane.

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