Insulin stimulation of glucose transport activity in rat skeletal muscle: increase in cell surface GLUT4 as assessed by photolabelling

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We have used a photoaffinity label to quantify cell surface GLUT4 glucose transporters in isolated rat soleus muscles. In this system, insulin stimulated an 8.6-fold increase in 3-*O*-methylglucose glucose transport, while photolabelled GLUT4 increased 8-fold. These results demonstrate that the insulin-stimulated increase in glucose transport activity in skeletal muscle can be accounted for by an increase in surface-accessible GLUT4 content.

INTRODUCTION

Skeletal muscle is the tissue primarily responsible for whole-body insulin-stimulated glucose disposal [1–3]. Glucose transport is thought to be the rate-limiting step for glucose utilization in skeletal muscle [4–6]. GLUT4 represents the primary glucose transporter isoform expressed in the insulin-sensitive tissues skeletal muscle, heart and adipocytes, and is the major glucose transporter responsible for the rapid stimulation of glucose uptake by insulin in these tissues [7–12]. However, the mechanism of insulin action in skeletal muscle has not yet been clarified.

Immunocytochemical and biochemical techniques have demonstrated the possibility of GLUT4 translocation to the cell surface of heart and skeletal muscle in response to insulin stimulation [12–18], similar to that seen in adipocytes [19]. In the isolated rat soleus and in perfused hindlimb, insulin stimulated a 5–20-fold increase in glucose transport activity [20–23]. However, insulin stimulates only a 2–3-fold increase in GLUT4 concentration in plasma membranes when measured by the traditional cytochalasin B binding method, and/or by GLUT4 Western blotting [10,24,25]. This difference initially gave rise to, and now perpetuates, the concept that insulin stimulation in skeletal muscle results in both recruitment and activation of glucose transporters [11,25–27]. On the other hand, these data could also indicate that such *in vitro* techniques for assessing cell surface glucose transporter content are inadequate to quantify the translocation process seen *in vivo* [10,12,25], as has been noted previously for rat adipocytes [28]. Until now, only by using isolated plasma membrane vesicles could glucose transport activity and glucose transporter content (by Western blotting or cytochalasin B binding) be measured in the same preparation [29,30]. However, no method exists to make similar measurements in intact skeletal muscle.

Recently an impermeant glucose transporter photoaffinity reagent, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA), has been used to quantify glucose transporter content and to study subcellular distribution in both isolated adipocytes [9,31–33] and cell cultures [7]. In the present study we quantified the cell surface GLUT4 in isolated rat soleus muscle in response to insulin stimulation by immunoprecipitation of ATB-[2-^{3}H]BMPA-labelled glucose transporters. This has allowed us, for the first time, to correlate the cell surface GLUT4 with glucose transport activity in order to answer the question of whether insulin-stimulated glucose transport in skeletal muscle occurs only by an increased surface-accessible content of GLUT4.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats (45–60 g) were purchased from Charles River Breeding Laboratories (Boston, MA, U.S.A.) and housed for 3–5 days prior to use. Fraction V BSA (Intergen, Purchase, NJ, U.S.A.) was dialysed against Krebs–Henseleit bicarbonate (KHB) buffer. Cytochalasin B and cytochalasin E were from Aldrich (Milwaukee, WI, U.S.A.). Insulin was a gift from Dr. R. B. Chance (Eli Lilly, Indianapolis, IN, U.S.A.). D-Glucose, L-glucose and 3-*O*-methylglucose were obtained from Sigma (St. Louis, MO, U.S.A.). Protein A–Sepharose was purchased from Pharmacia (Piscataway, NJ, U.S.A.). All radiolabelled compounds, other than the photolabel, were from Du Pont–New England Nuclear (Boston, MA, U.S.A.). A rabbit polyclonal antiserum, prepared against the 20-amino-acid peptide corresponding to the C-terminal sequence of GLUT4, was kindly supplied by Hoffmann–La Roche (Nutley, NJ, U.S.A.). ATB-[2-^{3}H]BMPA (specific radioactivity 10 Ci/mmol) was a gift from Dr. G. D. Holman (University of Bath, U.K.), and was prepared from ATB and [2-^{3}H]BMPA as previously described by Clark and Holman [34].

Photoaffinity labelling of isolated rat soleus muscles

Intact soleus muscles (approx. 25 mg) were isolated from rats following anaesthesia with a gas mixture of CO_2/O_2 (7:3) and decapitation. Muscles were equilibrated at 29 °C for 30 min in continuously gassed (O_2/CO_2, 19:1) KHB buffer containing 1 mM pyruvic acid, 0.1 % dialysed BSA and 1.8 mM calcium (basal buffer). The buffer was exchanged for fresh buffer for an additional 20 min incubation with one of the following additions: no addition for basal; 130 nM insulin, 130 nM insulin plus 80 μM cytochalasin B and 2.5 μM cytochalasin E; 130 nM insulin plus 300 mM D-glucose; or 130 nM insulin plus 300 mM

Abbreviations used: ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannos-4-yloxy)-2-propylamine.

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L-glucose. Individual soleus muscles were transferred to 1 ml of buffer, with the same additions, containing 1 mCi/ml ATB-[2-^{3}H]BMPA for another 5 min (in the dark at 24 °C) unless otherwise stated. Each muscle was then irradiated twice for 1 min intervals with an 80 W PhotoMax mercury arc lamp (Oriel, Stratford, CT, U.S.A.) employing a 2 cm aqueous 20% $CuSO_4$ filter. Muscles were manually turned over between irradiation intervals. Following irradiation, the muscles were rapidly blotted on wet 1 M Whatman filter paper, trimmed of their tendons and immediately frozen between liquid-nitrogen-cooled clamps. Muscles were kept at −70 °C until further analysis.

Preparation of total crude muscle membranes

Frozen muscles were individually weighed, and then homogenized with a Tekmar Polytron for 15 s in 2 ml of ice-cold buffer containing 25 mM Hepes, 1 mM Na-EDTA, 250 mM sucrose, 2 mM phenylmethanesulphonyl fluoride and 1 μM each of leupeptin, pepstatin and aprotinin. Following homogenization, muscles were centrifuged at 150000 $g_{max.}$ at 4 °C for 90 min. Cytosolic protein supernatants were discarded and the pellets were rinsed twice with 1 ml of ice-cold buffer. The total crude particulate pellets were then resuspended in approx. 400 μl of homogenization buffer and solubilized for immunoprecipitation in an equal volume of 4% Thesit ($C_{30}H_{62}O_{10}$) detergent (Boehringer Mannheim, Indianapolis, IN, U.S.A.). The samples were allowed to solubilize for 60 min at room temperature, then centrifuged (150000 $g_{max.}$ at 4 °C for 90 min) to pellet non-solubilized, glucose transporter-devoid material. The solubilized total crude membranes were then immunoprecipitated.

Immunoprecipitation of GLUT4

Immunoprecipitation was carried out as described by Calderhead and Lienhard [35], with the following modifications. An immunocomplex was made by incubating 22.5 μl of the anti-(GLUT4 C-terminus) antiserum in 500 μl of PBS with 100 μl of 50% Protein A–Sepharose for 90 min at 4 °C. Uncomplexed antibody was then washed off. Thesit-solubilized total crude muscle membranes were added to the immunocomplex and mixed at 4 °C overnight. The pellets were washed twice with 1 ml of 0.2% Thesit in PBS followed by 3 × 1 ml washes in PBS containing 1 mM EDTA. Finally, the antibody conjugates were released from the Protein A–Sepharose matrix in sample buffer containing 2.8% SDS, 4.6 M urea and 100 mM dithiothreitol (final concentrations).

Quantification of ATB-[2-^{3}H]BMPA-labelled GLUT4 glucose transporters

The immunoprecipitated GLUT4 glucose transporters were subjected to electrophoresis on 16 cm × 3 mm 10% SDS/polyacrylamide gels run in the Laemmli discontinuous buffer system [36]. Gels were stained with Coomassie Blue, destained, and then sliced by lane into 8 mm slices. The gel slices were dried at 80 °C for 3 h, dissolved in 500 μl of alkaline 30% H_2O_2 at 80 °C for 2 h; radioactivity was counted in a liquid scintillation counter. The positions of the photolabelled peaks were compared with those of prestained molecular mass markers run in adjacent lanes. The level of radioactivity associated with each peak was obtained by integrating the radioactivity under the peak and subtracting the average background activity of slices on either side of the peak. Total radioactivity was standardized by expression as c.p.m./g wet weight of tissue.

3-*O*-Methylglucose transport

Isolated soleus muscles were initially incubated for 30 min at 29 °C in continuously gassed (O_2/CO_2, 19:1) basal KHB buffer, then changed to fresh buffer in the presence or the absence of 130 nM insulin and incubated for an additional 20 min (as described for photolabelling). Sugar uptake incubation medium was then added (final concentrations: 40 mM 3-*O*-methylglucose, 9.44 μCi/mmol 3-*O*-[^{14}C]methylglucose, 0.625 μCi/ml L-[^{3}H]glucose) and the muscles were incubated for an additional 15 min (insulated-stimulated) or 25 min (basal). 3-*O*-Methylglucose uptake was calculated as μmol/5 min per g wet weight of tissue after correcting for extracellular space as calculated from the L-glucose counts [37,38].

Statistics

Data are expressed as means ± S.E.M. for the numbers of muscles as indicated. Significance was tested with a one-way analysis of variance or, if multiple conditions were compared, by factorial analysis of variance with a *post hoc* Scheffé *F* test. Differences were accepted as significant at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Conditions for optimal GLUT4 labelling with ATB-[2-^{3}H]BMPA in isolated soleus muscles

The incubation time and temperature experiments illustrated in Figure 1 were performed to: (1) determine the optimal incubation time for diffusion of ATB-[2-^{3}H]BMPA into isolated soleus muscles and (2) address the possibility that cell surface labelling might be overestimated due to the endocytosis of ATB-[2-^{3}H]BMPA bound to GLUT4 prior to irradiation. The appropriate u.v. exposure conditions (intensity and duration) for

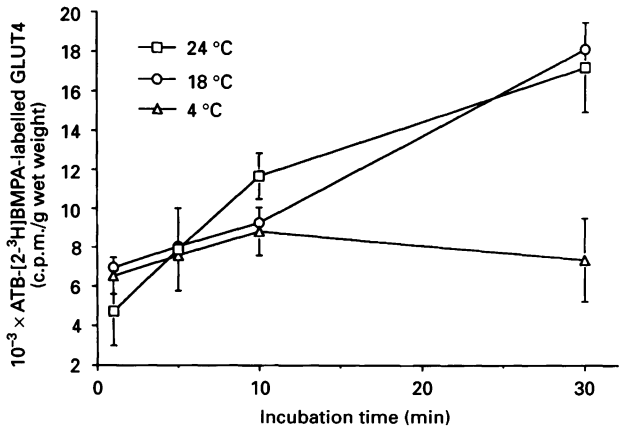

Figure 1 Determination of conditions for photolabelling of surface-accessible GLUT4 in isolated soleus muscles

To determine whether ATB-[2-^{3}H]BMPA was internalized over time, soleus muscles were equilibrated and insulin-stimulated as described in the Experimental section. Muscles were then incubated in the presence of ATB-[2-^{3}H]BMPA for 1, 5, 10 or 30 min at 24 °C (□), 18 °C (○) or 4 °C (△), and photolabelled GLUT4 was recovered. The lack of difference with temperature indicates adequate diffusion rather than internalization, up to 10 min. A 5 min incubation time at 24 °C was chosen for the subsequent experiments. Values are means ± S.D. ($n = 3$ soleus muscles for each data point).

maximal label incorporation following incubation of isolated soleus muscles with ATB-[2-^{3}H]BMPA for 5 min at 24 °C were initially determined (results not shown).

For these experiments, isolated soleus muscles were incubated with ATB-[2-^{3}H]BMPA for increasing time periods at room temperature (~ 24 °C), 18 °C or 4 °C. Clark and Holman have shown that incubation at 18 °C markedly inhibited endocytotic activity [39], while 4 °C slowed membrane movement. We found that the amount of photolabel incorporated into recovered GLUT4 increased when the incubation period was extended from 1 to 5 min at all three incubation temperatures, presumably due to diffusion into the muscle fibre layers, and remained relatively constant for up to 20 min. Only with 30 min of incubation was an additional increase in labelling observed at 24 °C as compared with continued constant labelling at 4 °C, suggesting uptake of photolabel into intracellular compartments (Figure 1). Thus GLUT4 that is photolabelled following incubation for 5–10 min at all three temperatures and for up to 30 min at 4 °C is likely to be localized to the cell surface membrane.

This interpretation was further supported by autoradiographic studies at the light microscope level, which demonstrated that ATB-[2-^{3}H]BMPA reached the innermost fibres with 1 min of incubation at 24 °C and appeared to be uniformly distributed throughout the intact soleus muscle within 5 min of incubation (R. W. Dudek, G. L. Dohm, G. D. Holman, S. W. Cushman and C. M. Wilson, unpublished work). Based on these results, we chose a 5 min incubation time with ATB-[2-^{3}H]BMPA at 24 °C to maximize cell surface labelling and minimize the possibility of internalization.

We have also compared the PhotoMax mercury arc lamp with the Rayonet lamp used in other photolabelling studies [9]. The exposure time for maximal ATB-[2-^{3}H]BMPA labelling of GLUT4 was 2–3 times less with the mercury lamp compared with the Rayonet (2 min versus 6 min). We used the mercury arc lamp for all studies, since it was important to minimize the total incubation time (incubation + irradiation) to prevent photolabel internalization.

Quantification of GLUT4 in photolabelled soleus muscles

Figure 2 illustrates typical gel profiles of photolabelled GLUT4 immunoprecipitated from crude membranes of basal and insulin-stimulated soleus muscles. A distinct single peak of ^{3}H counts was observed running in the molecular mass range that corresponds to that of GLUT4 as detected by Western blotting. Photolabelling in the presence of insulin plus cytochalasin B abolished the GLUT4 immunoprecipitated peak. The inset shows a gel profile of total crude membranes from insulin- and insulin + cytochalasin B-stimulated muscles. There appeared to be other photolabelled proteins, as indicated by the multiple peaks. However, only the 45 kDa peak was affected by cytochalasin B, indicating that this peak represents photolabelled glucose transporters. This demonstrates the importance of the immunoprecipitation procedure to eliminate non-specific background for better quantification.

Several criteria were met which ensured full recovery of photolabelled GLUT4. No glucose transporter 45 kDa protein was observed in the supernatant remaining after immunoprecipitation, as determined either by cutting and counting or by Western blotting for GLUT4. The efficiency of immunoprecipitation for GLUT4 used in these studies, as determined by Western blotting, was found to be greater than 85%. This efficiency enables reliable quantification of the amount of photolabelled GLUT4 from intact soleus muscles.

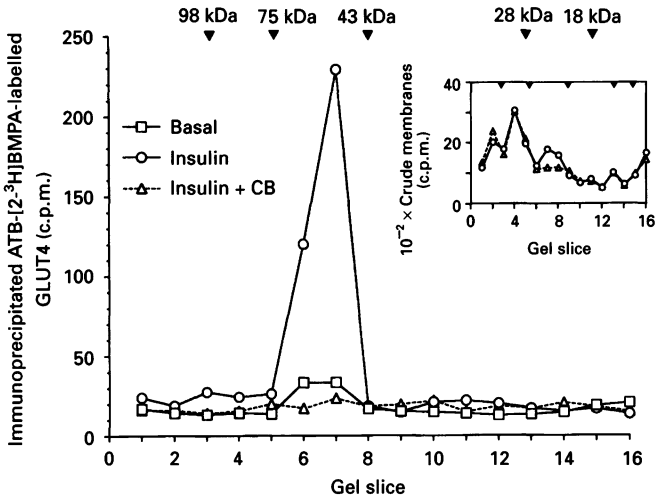

Figure 2 Identification of GLUT4 in photolabelled soleus muscle

The SDS/10%-PAGE profiles of the ^{3}H recovered from GLUT4 immunoprecipitated crude total membranes are shown with corresponding molecular mass positions. The three profiles are of single soleus muscles in the basal (□), insulin-stimulated (○), or insulin-stimulated plus cytochalasin B (CB) (△) condition. The competitive inhibitor eliminates the 45 kDa peak. The inset shows two gel profiles [muscles from insulin-stimulated (○), and insulin-stimulated plus cytochalasin B (△) muscles] of non-immunoprecipitated crude total membranes. There are multiple peaks, but only the 45 kDa peak disappears with cytochalasin B competition. The high background and multiple peaks make immunoprecipitation necessary for accurate quantification.

Table 1 Specificity of GLUT4 photolabelling in isolated soleus muscles

Isolated soleus muscles were equilibrated, incubated with insulin alone or insulin plus either the competitive inhibitor cytochalasin B or D-glucose, photolabelled, immunoprecipitated and quantified (see the Experimental section). Values are the means ± S.E.M. of the quantified area under the GLUT4 peak for each condition (*n* is given in parentheses). ATB-[2-^{3}H]BMPA labelling of GLUT4 in insulin-stimulated soleus muscles in the presence of cytochalasin B or D-glucose decreased photolabelling of GLUT4 in insulin-stimulated muscle by 86%. The L-glucose, an osmotic control, did not compete with the label. * indicates significance at $P < 0.001$ compared with insulin-stimulated (or L-glucose) muscles. Glc, glucose; Cyto, cytochalasin.

Incubation conditions	ATB-[2-^{3}H]BMPA-labelled GLUT4 (c.p.m./g wet weight)
Insulin	5406 ± 408 (49)
Insulin + CytoB	759 ± 250* (13)
Insulin + D-Glc	974 ± 183* (12)
Insulin + L-Glc	4404 ± 465 (11)

Effects of competition on photoaffinity labelling by ATB-[2-^{3}H]BMPA

Table 1 shows the effects of specific competitive inhibitors, cytochalasin B and D-glucose, on ATB-[2-^{3}H]BMPA photolabelling. Both inhibited GLUT4 photolabelling in the insulin-stimulated state by 80–90%, whereas L-glucose had no effect. These data indicate the specific labelling of the glucose transporter by ATB-[2-^{3}H]BMPA in intact soleus muscles. Because photolabelling of GLUT4 was so low in the basal state, competitive inhibition studies of labelling were not carried out in this condition. It can be noted that the photolabelled GLUT4 in the basal condition (see Figure 3) was not significantly different from the insulin + cytochalasin B or the insulin + D-glucose condition. Thus the magnitude of the response to insulin may be slightly

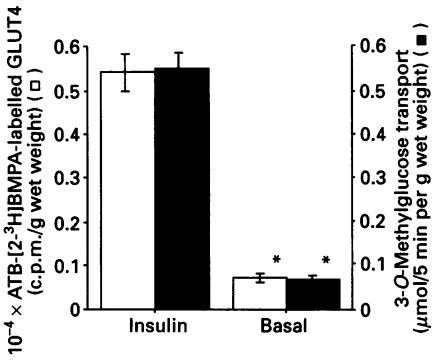

Figure 3 Comparison of surface-accessible GLUT4 with glucose transport activity

Isolated soleus muscles were equilibrated and incubated as basal or insulin-stimulated. They were then used for transport measurements; or were photolabelled, immunoprecipitated and GLUT4 was quantified as described (see the Experimental section). Surface-accessible GLUT4 (□) determined by photolabelling was 8-fold greater in insulin-stimulated compared with basal muscles ($n = 30$ for each). Glucose transport activity (■), determined using [^{14}C]3-*O*-methylglucose, showed an 8.6-fold greater transport activity in insulin-stimulated as compared with basal muscles ($n = 24$ for each condition). * indicates $P < 0.001$ versus insulin-stimulated.

underestimated in the absence of an appropriate correction for 'non-specific' basal labelling.

Isolated soleus muscle cell surface GLUT4 content compared with glucose transport

The results of a direct comparison, in isolated soleus muscles, of cell surface GLUT4 photolabelling with ATB-[2-^{3}H]BMPA in the basal and insulin-stimulated conditions (689 ± 91 versus 5406 ± 408 c.p.m./g wet weight) and 3-*O*-methylglucose transport (0.063 ± 0.009 versus 0.536 ± 0.04 μmol/5 min per g wet weight) are shown in Figure 3. Both parameters increased by approx. 8-fold with insulin stimulation over the basal condition. This clearly demonstrates that the stimulatory effect of insulin on glucose transport activity in an *in vitro* skeletal muscle preparation can be entirely accounted for by the appearance of surface membrane-accessible GLUT4. (The data reported here were first described at the 1992 Annual Meeting of the American Diabetes Association [40]. During the subsequent preparation of this paper similar data obtained using almost identical methods were reported by Lund et al. [41].)

This study demonstrates the novel use of the exofacial photolabel ATB-[2-^{3}H]BMPA to detect surface membrane-accessible GLUT4 glucose transporters in isolated rat soleus muscles. Using identical muscle preparations, we have carefully assessed the specificity of the labelling of GLUT4 in each step, in order to confirm the quantitative relationship between cell surface GLUT4 and glucose transport activity. This study directly demonstrates the change in number of surface-accessible GLUT4 in contact with the extracellular space in response to insulin in a model traditionally used to measure glucose transport activity.

The present study, however, does not answer the question of the cellular location of the glucose transporters that become accessible to ATB-[2-^{3}H]BMPA and glucose in response to insulin. ATB-[2-^{3}H]BMPA has been shown to bind to the functional glucose transporters, and not to those occluded or otherwise inactive [31,33,42]. Therefore the increase in photolabelled GLUT4 could be due either to translocation to the surface membrane and/or to unmasking of transporters already in the cell surface membrane, such that they become accessible to glucose and photolabel. Recent studies with ATB-[2-^{3}H]BMPA in isolated rat adipocytes have shown that an approx. 20-fold stimulation of glucose transport activity by insulin was accompanied by a similar steady-state increase in cell surface GLUT4. These transporters were ultimately redistributed to the intracellular pool [9,32], directly demonstrating translocation in adipocytes. Further studies are necessary in order to determine if one or both mechanisms are involved in the increase of surface-accessible GLUT4 by insulin stimulation in skeletal muscle.

We acknowledge the assistance of Dr. T. Ploug in the transport assay. We thank Dr. G. L. Dohm for his critical reading of the manuscript. We also thank Dr. G. D. Holman for generously providing the ATB-[2-^{3}H]BMPA.

REFERENCES

1 Elbrink, J. and Bihler, I. (1975) Science **188**, 1177–1184
2 Yki-Jarvinen, H., Sahlin, K., Ren, J. M. and Koivisto, V. A. (1990) Diabetes **39**, 157–167
3 Furler, S. M., Jenkins, A. B., Storlien, L. H. and Kraegen, E. W. (1991) Am. J. Physiol. **261**, E337–E347
4 DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J. and Felber, J. P. (1981) Diabetes **30**, 1000–1007
5 James, D. E., Jenkins, A. B. and Kraegen, E. W. (1985) Am. J. Physiol. **248**, E567–E574
6 Koranyi, L. I., Bourey, R. E., Vuorinen-Makkola, H., Koivisto, V. A., Mueckler, M., Permutt, M. A. and Yki-Jarvinen, H. (1991) Diabetologia **34**, 763–765
7 Calderhead, D. M., Kitagawa, K., Tanner, L. I., Holman, G. D. and Lienhard, G. E. (1990) J. Biol. Chem. **265**, 13800–13808
8 Douen, A. G., Ramlal, T., Rastogi, S., Bilan, P. J., Cartee, G., Vranic, M., Holloszy, J. O. and Klip, A. (1990) J. Biol. Chem. **265**, 13427–13430
9 Holman, G. D., Kozka, I. J., Clark, A. E., Flower, C. J., Saltis, J., Habberfield, A. D., Simpson, I. A. and Cushman, S. W. (1990) J. Biol. Chem. **265**, 18172–18179
10 Klip, A. and Paquet, M. R. (1990) Diabetes Care **13**, 228–243
11 Rodnick, K. J., Piper, R. C., Slot, J. W. and James, D. E. (1992) Diabetes Care **15**, 1679–1689
12 Carruthers, A. (1990) Physiol. Rev. **70**, 1135–1176
13 Bornemann, A., Ploug, T. and Schmalbruch, H. (1992) Diabetes **41**, 215–221
14 Friedman, J. E., Dudek, R. W., Whitehead, D. S., Downes, D. L., Frisell, W. R., Caro, J. F. and Dohm, G. L. (1991) Diabetes **40**, 150–154
15 Slot, J. W., Guez, H. J., Gigengack, S. and James, D. E. (1991) J. Cell Biol. **113**, 123–135
16 Rodnick, K. J., Slot, J. W., Studelska, D. R., Hanpeter, D. E., Robinson, L. J., Guez, H. J. and James, D. E. (1992) J. Biol. Chem. **267**, 6278–6285
17 Watanabe, T., Smith, M. M., Robinson, F. W. and Kono, T. (1984) J. Biol. Chem. **259**, 13117–13122
18 Henriksen, E. J. and Holloszy, J. O. (1991) Acta. Physiol. Scand. **143**, 381–386
19 Simpson, I. A. and Cushman, S. W. (1986) Annu. Rev. Biochem. **55**, 1059–1089
20 Richter, E. A., Garetto, L. P., Goodman, M. N. and Ruderman, N. B. (1982) J. Clin. Invest. **69**, 785–793
21 Klip, A., Ramlal, T., Young, D. A. and Holloszy, J. O. (1987) FEBS Lett. **224**, 224–230
22 Ploug, T., Galbo, H., Vinten, J., Jorgensen, M. and Richter, E. (1987) Am. J. Physiol. **253**, E12–E20
23 Kraegen, E. W., Storlien, L. H., Jenkins, A. B. and James, D. E. (1989) Am. J. Physiol. **256**, E242–E249
24 Wardzala, L. J. and Jeanrenaud, B. (1983) Biochim. Biophys. Acta **730**, 49–56
25 Hirshman, M. F., Goodyear, L. J., Wardzala, L. J., Horton, E. D. and Horton, E. S. (1990) J. Biol. Chem. **265**, 987–991
26 Sternlicht, E., Barnard, R. J. and Grimditch, G. K. (1988) Am. J. Physiol. **254**, E633–E638
27 Goodyear, L. J., Hirshman, M. F., Smith, R. J. and Horton, E. S. (1991) Am. J. Physiol. **261**, E556–E561
28 Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karneili, E., Salans, L. B. and Cushman, S. W. (1984) Biochim. Biophys. Acta **763**, 393–407
29 Goodyear, L. J., Hirshman, M. F., Valyou, P. M. and Horton, E. S. (1992) Diabetes **41**, 1091–1099
30 Ploug, T., Wojtaszewski, J., Kristiansen, S. and Hespel, P. (1993) Am. J. Physiol. **264**, E270–E278

31 Czech, M. P. and Buxton, J. M. (1993) J. Biol. Chem. **268**, 9187–9190
32 Satoh, S., Nishimura, H., Clark, A. E., Kozka, I. J., Vannucci, S. J., Simpson, I. A., Quon, M. J., Cushman, S. W. and Holman, G. D. (1993) J. Biol. Chem. **268**, 17820–17829
33 Vannucci, S. J., Nishimura, H., Satoh, S., Cushman, S. W., Holman, G. D. and Simpson, I. A. (1992) Biochem. J. **288**, 325–330
34 Clark, A. E. and Holman, G. D. (1990) Biochem. J. **269**, 615–622
35 Calderhead, D. M. and Lienhard, G. E. (1988) J. Biol. Chem. **263**, 12171–12174
36 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
37 Narahara, H. T. and Ozand, P. (1963) J. Biol. Chem. **238**, 40–49
38 Zaninetti, D., Greco-Perotto, R., Assimacopoulos-Jeannet, F. and Jeanrenaud, B. (1988) Biochem. J. **250**, 277–283
39 Clark, A. E. and Holman, G. D. (1990) J. Biol. Chem. **265**, 13800–13808
40 Wilson, C. M. and Cushman, S. W. (1992) Diabetes **40**, 167
41 Lund, S., Holman, G. D., Schmitz, D. and Pederson, O. (1993) FEBS Lett. **330**, 312–318
42 Harrison, S. A., Clancy, B. M., Pessino, A. and Czech, M. P. (1992) J. Biol. Chem. **267**, 3783–3788

Received 4 November 1993; accepted 18 November 1993