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Glycaemia regulates the glucose transporter number in the plasma membrane of rat skeletal muscle

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The number of glucose transporters was measured in isolated membranes from diabetic-rat skeletal muscle to determine the role of circulating blood glucose levels in the control of glucose uptake into skeletal muscle. Three experimental groups of animals were investigated in the post-absorptive state: normoglycaemic/normoinsulinaemic, hyperglycaemic/normoinsulinaemic and hyperglycaemic/normoinsulinaemic made normoglycaemic/normoinsulinaemic by phlorizin treatment. Hyperglycaemia caused a reversible decrease in total transporter number, as measured by cytochalasin B binding, in both plasma membranes and internal membranes of skeletal muscle. Changes in GLUT4 glucose transporter protein mirrored changes in cytochalasin B binding in plasma membranes. However, there was no recovery of GLUT4 levels in intracellular membranes with correction of glycaemia. GLUT4 mRNA levels decreased with hyperglycaemia and recovered only partially with correction of glycaemia. Conversely, GLUT1 glucose transporters were only detectable in the plasma membranes; the levels of this protein varied directly with glycaemia, i.e. in the opposite direction to GLUT4 glucose transporters. This study demonstrates that hyperglycaemia, in the absence of hypoinsulinaemia, is capable of down-regulating the glucose transport system in skeletal muscle, the major site of peripheral resistance to insulin-stimulated glucose transport in diabetes. Furthermore, correction of hyperglycaemia causes a complete restoration of the transport system in the basal state (determined by the transporter number in the plasma membrane), but possibly only an incomplete recovery of the transport system's ability to respond to insulin (since there is no recovery of GLUT4 levels in the intracellular membrane insulin-responsive transporter pool). Finally, the effect of hyperglycaemia is specific for glucose transporter isoforms, with GLUT1 and GLUT4 proteins varying respectively in parallel and opposite directions to levels of glycaemia.

INTRODUCTION

Decreased peripheral glucose clearance is a characteristic feature of type I and type II diabetes mellitus, as well as of many animal models of diabetes, including the high-dose streptozotocin-induced hyperglycaemic/hypoinsulinaemic rat model. In this model, because hyperglycaemia and hypoinsulinaemia are present concomitantly, it is not possible to differentiate which of these variables is responsible for the changes in glucose uptake. In contrast with this most frequently employed model, we have previously demonstrated that when streptozotocin is injected at a low dose (65 mg/kg dissolved in saline), a fraction of the pancreatic insulin production capacity can be spared at least for 10 days [1]. Under these conditions, the pancreas is unable to produce a normal postprandial insulin response and hyperglycaemia develops; however, basal (fasting-state) insulin production is unaffected. This amount is subnormal relative to the prevailing hyperglycaemia, and hence the rats are relatively insulin-deficient, yet they exhibit absolute fasting insulin levels comparable with those of control rats. This hyperglycaemic/normoinsulinaemic model allows one to dissect out the effects of hyperglycaemia from those of combined hyperglycaemia and hypoinsulinaemia. In a previous study we used this model to show a decrease in the amount of cytochalasin B binding sites in muscle from such diabetic animals [1], suggesting, but not proving, that glycaemia, irrespective of insulin levels, could dictate the number of glucose transporters present in the muscle plasma membrane in this animal model.

Manipulation of glycaemia in these hyperglycaemic/normoinsulinaemic rats can be achieved by altering urinary glucose excretion. Gauthier *et al.* [2] first showed that acute administration of phlorizin, which inhibits the Na^+–glucose cotransporter without affecting the passive glucose transport systems of non-epithelial cells, can decrease blood glucose levels in dogs by inhibiting renal-tubular glucose reabsorption. Subsequently, Lussier *et al.* [3], Starke *et al.* [4] and Hetenyi *et al.* [5] used this method to correct glycaemia in diabetic dogs. More recently, Rossetti *et al.* [6] showed that correction of glycaemia by 3 weeks of continuous administration of phlorizin to partially pancreatectomized or alloxan-treated rats improved peripheral glucose utilization, suggesting that chronic changes in glycaemia could control glucose uptake *in vivo*. The cellular basis for this control remained unknown.

Two glucose transporter isoforms are expressed in rat skeletal muscle, GLUT1 and GLUT4 [7–11]. In this tissue, GLUT1 transporters are almost exclusively located in plasma membranes, whereas GLUT4 transporters are present in plasma membranes and in a higher yield in an intracellular membrane fraction that is responsive to insulin [12,13]. Our membrane-preparation procedure yields isolated plasma membranes and intracellular membranes, neither of which is contaminated with endothelial-cell membranes, suggesting that the GLUT1 and GLUT4 glucose transporter isoforms present are of muscle origin [14].

The objectives of the present study were, firstly, to determine whether the decrease in number of glucose transporters at the plasma membrane of muscle from post-absorptive hyperglycaemic/normoinsulinaemic rats can be reversed by correction of glycaemia independently of changes in plasma insulin concentration, and secondly, to characterize the regulation by glucose of the individual glucose transporter isoforms. To this effect, blood glucose levels in diabetic rats were restored by decreasing the rate of renal glucose reabsorption with phlorizin. The number

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of glucose transporters was measured by cytochalasin B binding, and specific isoforms were detected immunologically in isolated membrane fractions from skeletal muscle. The results point to a relatively rapid and selective regulation by circulating glucose of the glucose transporter number and isoform present at the muscle cell surface.

EXPERIMENTAL

Materials

Cytochalasin B and E, L-glucose, gradient-grade sucrose, phlorizin, propylene glycol and streptozotocin were purchased from Sigma (St. Louis, MO, U.S.A.). [^{3}H]Cytochalasin B was purchased from Amersham (Arlington Heights, IL, U.S.A.). GF/B filters were obtained from Whatman (Clifton, NJ, U.S.A.). Pig insulin (regular iletin) was obtained from Eli Lilly Co. R820 antibody (a rabbit antiserum containing antibodies to a synthetic peptide of the 12-amino-acid *C*-terminal sequence of GLUT4) and a full-length rat GLUT4 cDNA probe were kindly given by Dr. D. E. James, Washington University, St. Louis, MO, U.S.A. The anti-GLUT1 rabbit antibody was raised in our department to a synthetic peptide of the 15-amino-acid *C*-terminal sequence of GLUT1 and affinity-purified on to the immobilized synthetic polypeptide.

Animals and tissue preparation

Male specific-pathogen-free Sprague–Dawley rats from Charles River, Canada, were received at 250 g body wt. and maintained on Prolab animal diet for rats (Agway). Animals were separated into three experimental groups for subsequent study. Group 1 received a single tail-vein injection of streptozotocin (65 mg/kg body wt., freshly reconstituted with 0.9% NaCl) on day 0; these rats were given 10% (w/v) sucrose in filtered tap water for 24 h after injection, and thereafter they received food *ad libitum* for 7 days. Group 2 received a single injection of streptozotocin as above, and 10% sucrose in filtered tap water for the next 24 h. This was followed by food *ad libitum*, and on day 5 by twice-daily subcutaneous injections of phlorizin [0.4 g/kg as a 40% (w/v) suspension in propylene glycol]. Group 3 consisted of control rats without any treatment (previous studies in our laboratory have shown that sham injections of 0.9% NaCl have no observable effects on metabolism or on the number of glucose transporters). All animals were monitored each day for body weight, glycosuria and urine excretion. Rats from all groups fed until night 6; on night 7 they were fasted, and then killed on day 8 by cervical dislocation after rapid ether-inhalant anaesthesia. Because the rats feed at night only, the post-absorptive state of day 7, followed by an imposed overnight fast, leads to a 24 h post-absorptive period before death. Trunk blood was collected into heparinized tubes for glucose and hormone assays (see below). Immediately after death, rats were placed on ice, and muscles from both hindlimbs were dissected out, trimmed of fat and connective tissues, weighed, and frozen at −120 °C until used for membrane preparation.

Radioimmunoassays

Plasma glucose was determined with a Beckman autoanalyser (Palo Alto, CA, U.S.A.). Plasma insulin was determined by a radioimmunoassay using anti-(bovine insulin) antiserum (kindly supplied by Dr. P. Wright, Indianapolis, IN, U.S.A.), a purified rat insulin standard and pig insulin labelled with ^{125}I (Novo Research Institute, Copenhagen, Denmark). Dextran-coated charcoal was used to separate free from bound hormone. Plasma immunoreactive glucagon levels were determined with a radioimmunoassay as described in [15]. Plasma non-esterified fatty acids were determined by a radiochemical assay as described in [16].

Membrane preparation and characterization

Plasma and intracellular membranes from rat skeletal muscle were prepared by the protocol described previously [17,18]. Briefly, 20–25 g of tissue was minced in buffer 1 [20 mM-$NaHCO_3$ (pH 7.0), 5 mM-NaN_3, 250 mM-sucrose and 100 μM-phenylmethanesulphonyl fluoride], homogenized in a Polytron (Brinkman Instruments, Westbury, NJ, U.S.A.) by using a Kinematica Speed Control set at 65000 rev./min for 5 s. This homogenate was centrifuged at 1200 *g* for 10 min, and the pellet was re-homogenized and re-centrifuged to remove debris. The combined supernatants were centrifuged at 9000 *g* for 10 min to sediment mitochondria and nuclei, and the resulting supernatant was centrifuged at 190000 *g* for 60 min to obtain the crude membrane (CM) pellet. This pellet was resuspended in buffer 1, layered on a discontinuous sucrose gradient [25%, 30% and 35% (w/w) sucrose], and centrifuged at 50000 *g* for 16 h. Membranes were collected at each sucrose interface (i.e. on top of 25% sucrose, 30% sucrose, 35% sucrose and at the bottom of the gradient), diluted 10-fold with buffer 2 [20 mM-$NaHCO_3$ (pH 7.0) and 5 mM-NaN_3], recovered by centrifugation at 190000 *g*, and membrane pellets were resuspended in buffer 1. Cytochalasin B binding assays were carried out immediately, and membranes were stored at −20 °C for further analysis. Enzyme assays were completed within 48 h. The plasma-membrane marker 5′-nucleotidase was measured by the method of Song & Bodansky [19]. Protein was assayed by the method of Lowry *et al.* [20]. We have previously shown [1,12,17,18] that membranes banding on top of 25% and 30% sucrose are enriched in plasma-membrane markers, and are here termed PM-I and PM-II respectively; membranes banding on top of 35% sucrose are devoid of plasma-membrane markers, and are hence termed internal membranes (IM). Importantly, all three fractions contain glucose transporters, and are free or contain only small levels of sarcoplasmic-reticulum markers, which are found in the pellet of the gradient (SR fraction). The PM-I membranes have been further characterized by an insulin-dependent increment, and the IM by an insulin-dependent decrement (in IM), in glucose transporter number [13,17].

Cytochalasin B binding

D-Glucose-protectable binding of cytochalasin B was used to measure the number of glucose transporters, as validated previously [18]. The binding of 0.2 μM-[^{3}H]cytochalasin B (2.3 μCi/ml) was measured at equilibrium in the presence of 2.5 μM-cytochalasin E and 20 mM of either D- or L-glucose, each in quintuple determinations. Binding in the presence of D-glucose was subtracted from that in the presence of L-glucose to yield the specific [^{3}H]cytochalasin B binding.

Western-blot analysis

Membranes (40 μg of protein) were subjected to SDS/PAGE on 10%-polyacrylamide gels as described by Laemmli [20a] and electrophoretically transferred to nitrocellulose filter membranes for 2 h as described previously [13]. The nitrocellulose filters were incubated for 1 h at room temperature with Tris/saline/Tween (50 mM-Tris/HCl, pH 7.4, 150 mM-NaCl, 0.04% Tween 20) containing 3% BSA (buffer 3), followed by overnight incubation at 4 °C with either anti-GLUT4 polyclonal antibody R820 (1:500 dilution) or anti-GLUT1 polyclonal antibody (1:25 dilution) in buffer 3. The nitrocellulose papers were washed for 3 × 15 min at room temperature in buffer 3 and then incubated for 90 min with ^{125}I-labelled Protein A (1 μCi/10 ml) in buffer 3, washed three ties (15 min each) with buffer 3, air-dried, and exposed to XAR-

Table 1. Characteristics of diabetic animals and effect of phlorizin

Rats were injected on day 1 with saline (Controls) or with 45 mg of streptozotocin/kg (Diabetic) as indicated in the Experimental section. On day 4, diabetic animals were separated into two groups, one of which (Diabetic/phlorizin) was treated twice daily with phlorizin as indicated in the Experimental section. On day 7, all animals were deprived of food for 24 h, and then killed on day 8. Results are of parameters at death (i.e. day 8, fasted or post-absorptive state) unless otherwise stated. The day-7 measurements refer to values in the early morning after the nightly feeding. The numbers of animals in each group were 10 Controls, 6 Diabetic, 6 Diabetic/phlorizin. None of the animals investigated was ketotic at any time of the study. Values in all Tables are means ± S.E.M.

			Controls	Diabetic	Diabetic/phlorizin
Weight	(day 8 fasted)	(g)	309 ± 11	293 ± 5	287 ± 6
Glycaemia	(day 7)	(mM)	5.4 ± 0.3	> 22	14.2 ± 0.7
Glycaemia	(day 8 fasted)	(mM)	5.5 ± 0.5	19.6 ± 1.6	5.3 ± 0.7
Glycosuria	(day 8 fasted)	(g/l)	0.21 ± 0.2	85 ± 10	105 ± 17
Urine volume	(day 8 fasted)	(ml/day)	26 ± 5	160 ± 15	170 ± 15
Circulating insulin	(day 8 fasted)	(μ-units/ml)	10.1 ± 1.0	9.1 ± 1.1	7.5 ± 0.6
Non-esterified fatty acids	(day 8 fasted)	(μ-equiv./l)	661 ± 55	625 ± 71	674 ± 42
Glucagon	(day 8 fasted)	(pg/ml)	250 ± 41	314 ± 72	493 ± 44

5 Kodak film for 24 h. Autoradiographs were quantified by laser scanning densitometry, using X-ray films showing bands within the linear range of optical density.

RNA isolation and Northern-blot analysis

Total cellular RNA was extracted from excised muscle of rats from the various experimental groups by the acid guanidinium isothiocyanate/phenol/chloroform method of Chomzynski & Sacchi [21]. The RNA isolated was assessed for purity by the A_{260} and A_{280}, and subjected to denaturing gel electrophoresis in 0.9% agarose gels containing phenol and Mops, with 1 × Mops running buffer [20 mM-Mops/5 mM-sodium acetate/0.5 mM-EDTA (pH 8.0), pH adjusted to 7.0 with acetic acid]. At the end of the run, the gels were photographed under u.v. trans-illumination to record the 28 S and 18 S marker positions and to determine the integrity of the RNA preparation. After electrophoresis, gels were rinsed free of formaldehyde, and RNA was transferred on to a nitrocellulose membrane and vacuum-dried at 80 °C for 2 h. The nitrocellulose membranes were photographed under u.v. trans-illumination to produce a 10 cm × 10 cm (4 in × 4 in) negative, and the relative RNA content of each lane was confirmed by laser scanning densitometry. Prehybridization and hybridization were carried out as described by Sambrook *et al.* [22] by using [α-^{32}P]dCTP-labelled probes (random-priming method) at 5×10^9 c.p.m./μg of cDNA. After hybridization, blots were stringently washed free of non-specific radioactivity in one change of cold wash buffer and two changes of hot wash buffer, and subjected to autoradiography at −120 °C in Kodak X-OMAT casettes. The bands were then quantified by laser scanning densitometry.

Statistics

Unless otherwise stated, results were analysed by Student's *t* test for unpaired data, and statistical significance was assigned to results where $P < 0.05$.

RESULTS

Metabolic characteristics of control, diabetic and phlorizin-treated animals

Metabolic characteristics of experimental rats on mornings of days 7 and 8 (after overnight feeding and 24 h after feeding respectively) are listed in Table 1. Both the diabetic (group 1) and phlorizin-treated diabetic (group 2) rats experienced a negligible decrease in body weight over the experimental period (about 5% on average). Streptozotocin-treated rats became markedly hyperglycaemic in the fasting (post-absorptive) state (> 22 mM) within 1 day of injection, and remained hyperglycaemic until either killing (group 1) or the beginning of the phlorizin treatment (group 2). In the fed state (morning of day 7), phlorizin treatment decreased blood glucose from > 22 mM to 14.2 mM. After the overnight fast, the diabetic (group 1) rats had an average glycaemia of 19.6 mM, whereas the phlorizin-treated diabetic rats (group 2) showed normal glucose levels (5.3 mM). Phlorizin-treated diabetic rats exhibited an increase in glycosuria (105 g/l) compared with diabetic rats (85 g/l), accounted for by the ability of phlorizin to inhibit renal glucose reabsorption. There was no significant difference in the fasting insulin levels of diabetic rats compared with controls (group 3), or of diabetic compared with phlorizin-treated diabetic rats. The insulin levels immediately after feeding are expected to have been lower in the diabetic groups relative to the controls; however, these values are not expected to have fallen below basal levels at any point in time. Hence, neither the diabetic nor the phlorizin-treated diabetic rats were ever hypoinsulinaemic (that is, they did not have insulin levels lower than those in control fasted rats). Moreover, the muscles in all three groups were exposed to equal levels of insulin during the diurnal post-absorptive state, and during the 24 h elapsed between the last feeding period and killing. The statistical equivalence of circulating non-esterified fatty acid levels in the three groups also reflects their similarity in insulin levels. The equivalence in fasting insulin and non-esterified fatty acid concentrations in the three groups therefore suggests that glycaemia, rather than insulinaemia, was the main distinguishing metabolic parameter. In addition, glucagon levels were elevated in the diabetic rats (group 1) and consequently increased further upon normalization of glycaemia with phlorizin treatment (group 2). Owing to the absence of glucagon receptors in muscle, these changes in glucagon were not further controlled. In a separate set of experiments, phlorizin treatment was found not to change the fed-state insulin levels (results not shown).

Characteristics of subcellular membrane fractions of rat skeletal muscle

Table 2 summarizes the biochemical characteristics of skeletal-muscle membranes isolated from control, diabetic and phlorizin-treated diabetic rats. Protein yield is the average amount of protein recovered in each membrane fraction, expressed per g of starting tissue. There was no significant difference between the three groups in the amount of protein recovered in the CM, PM-I, PM-II or SR fractions. However, the protein yield was lower in the IM fraction of the phlorizin-treated group relative to the

Table 2. Characterization of membranes isolated from control, diabetic and diabetic/phlorizin-treated-rat skeletal muscle

Membranes were isolated from Control, Diabetic and Diabetic/phlorizin rat skeletal muscles as described in the Experimental section. The isolated fractions were assayed for protein and 5′-nucleotidase activity. Results are for 7 independent preparations from Control rats, 7 from Diabetic rats and 5 from Diabetic/phlorizin rats, each utilizing about 20 g of muscle. Each assay was carried out in triplicate.

	Protein yield (μg/g of tissue)			5′-Nucleotidase (nmol/min per mg of protein)		
Fraction	Control	Diabetic	Diabetic/phlorizin	Control	Diabetic	Diabetic/phlorizin
Crude membranes (CM)	1487±134	1307±94	1307±84	80±10	76±9	64±5
25% sucrose (plasma membranes I) (PM-I)	52±5	51±3	52±6	358±33	320±28	296±32
30% sucrose (plasma membranes II) (PM-II)	80±7	82±5	80±1	326±29	319±25	293±21
35% sucrose (internal membranes) (IM)	411±59	374±46	250±30	79±9	76±8	79±4
Gradient pellet (sarcoplasmic reticulum) (SR)	304±74	216±41	319±33	45±5	51±2	43±4

Table 3. Effect of hyperglycaemia and its restoration on glucose transporter number and isoforms in membranes of rat skeletal muscle

The values for GLUT4 and GLUT1 are in densitometric units relative to the PM-I fraction of control rats. Note that the densitometric units of GLUT4 transporters cannot be compared numerically with those of GLUT1 transporters, given differences in antibody reactivity. Results are of 3–6 independent membrane preparations (ND, not detectable): $*P < 0.05$ versus control.

Fraction	Parameter	Control	Diabetic	Diabetic/phlorizin
CM	CB binding (pmol/mg of protein)	0.89±0.10	0.55±0.08*	0.72±0.05
PM-I	CB binding (pmol/mg of protein)	2.88±0.29	2.16±0.30*	3.06±0.30
	GLUT4 (relative units)	1.0	0.70±0.18*	1.32±0.37
	GLUT1 (relative units)	1.0	1.76±0.47*	1.30±0.78
PM-II	CB binding (pmol/mg of protein)	5.75±0.84	3.72±0.86*	4.98±0.53
	GLUT4 (relative units)	5.30	3.06±0.64*	3.83±1.70
	GLUT1 (relative units)	0.52	2.63±0.99*	1.38±0.57
IM	CB binding (pmol/mg of protein)	1.71±0.20	0.99±0.19*	1.46±0.11
	GLUT4 (relative units)	0.71±0.07	0.43±0.10*	0.45±0.01*
	GLUT1 (relative units)	ND	ND	ND
SR	CB binding (pmol/mg of protein)	0.98±0.21	0.96±0.36	0.71±0.13

other two groups. Table 2 also summarizes the activity of the plasma-membrane marker 5′-nucleotidase in each membrane fraction from control, diabetic and phlorizin-treated diabetic rats. The data show the abundance of this known plasma-membrane marker in each of the isolated fractions, and hence their relative resemblance to plasma membranes. Characterization of these membranes has been reported previously by our laboratory [1,12,13,17,18]; we have recently also shown that PM-I, PM-II and IM are not enriched in sarcoplasmic-reticulum or transverse-tubule markers [14]. Only PM-I and PM-II, but not IM, show positive immunodetectable α1 subunit of the plasma-membrane marker Na^+/K^+-ATPase (H. Hundal, A. Marette, Y. Mitsumoto & A. Klip, unpublished work). To date, there is no unique chemical marker for the intracellular pool of glucose transporters other than the functional response to insulin (given intravenously in acute-insulin-injection experiments), namely a decrease in glucose transporter number [13,14,17,18]. However, contamination of intracellular membranes with plasma membranes is ruled out by the absence of GLUT1 transporters and of the α1 subunit of the Na^+/K^+-ATPase. In addition, since the three rat groups yielded essentially the same level of enzymic activity in each of the isolated subcellular membrane fractions (expressed per mg of protein), it is suggested that membranes of similar degree of purity were obtained regardless of the metabolic condition of each rat group.

Effect of glycaemia on glucose transporters (cytochalasin B binding sites)

Table 3 illustrates the cytochalasin B binding content per mg of protein in the CM, PM-I, PM-II, IM and SR fractions derived from control (group 3), diabetic (group 1) and phlorizin-treated diabetic (group 2) animals. In the CM, there was a significant decrease in cytochalasin B binding with diabetes, as seen previously [8]. This diabetes-induced decrease in glucose transporter number in CM was partly reversed upon normalizing the fasting glucose concentration with phlorizin. In the CM-derived fractions PM-I, PM-II and IM of diabetic animals there was also a decrease in cytochalasin B binding activity (in pmol/mg of protein). Importantly, these decreases were largely reversed upon normalizing blood glucose with phlorizin. It must be remembered that the IM contain the insulin-responsive pool of glucose transporters. In the SR fraction, the amount of cytochalasin B binding sites was the lowest of all purified fractions, and did not seem to be affected by either diabetes or phlorizin treatment.

Effect of glycaemia on GLUT4 and GLUT1 glucose transporter isoforms

Fig. 1 shows a representative Western blot of immunoreactive GLUT4 protein in PM-I, PM-II and IM derived from muscle of control, diabetic and phlorizin-treated diabetic rats. Identical amounts of protein of each fraction were applied to the gels. In each panel of Fig. 1, the autoradiographic film was exposed to the gel for a different length of time to allow for better observation of all panels. GLUT4 transporters in the PM-I and PM-II fractions decreased in the hyperglycaemic state. This decrease varied quantitatively among the independent membrane preparations, being more marked for the PM-I fraction than for the PM-II fraction in two experiments, and more marked for

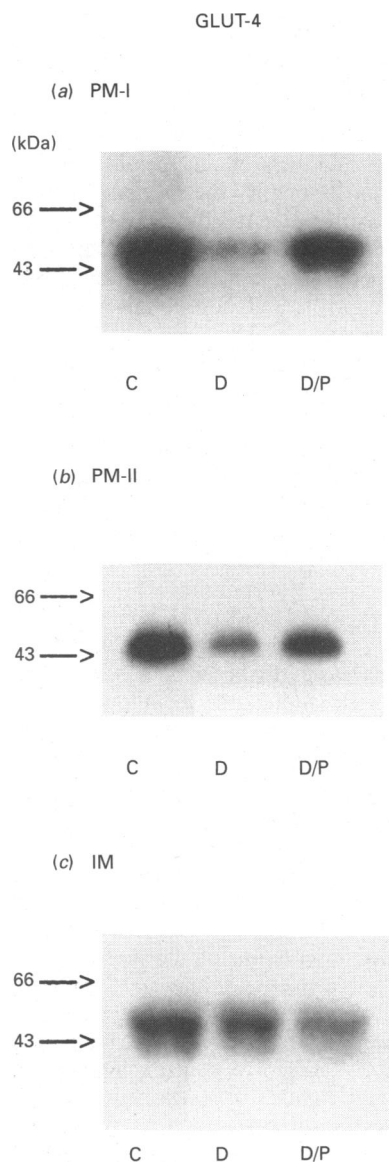

Fig. 1. Effect of glycaemia on GLUT4 protein content in plasma membranes and internal membranes from rat hindlimb muscles

Isolated membrane fractions (40 μg of protein) were resolved by SDS/PAGE and analysed by Western blotting using an anti-GLUT4 antibody, as described in the Experimental section. (*a*) Plasma-membrane fraction I (PM-I); (*b*) plasma-membrane fraction II (PM-II); (*c*) intracellular membranes (IM). The time of exposure to the X-ray film differed for each panel, in order to give a comparable range of intensities, for purposes of clarity. An estimate of the relative amount of GLUT4 protein in each fraction is given in Table 3, for results of 3–6 similar experiments. Abbreviations: C, control rats; D, diabetic rats; D/P, phlorizin-treated diabetic rats.

the PM-II fraction in a third experiment. Quantitative results of three independent experiments are further shown in Table 3 (see above). Importantly, phlorizin treatment caused normalization of GLUT4 protein levels in the PM-I and PM-II fractions, in parallel with the changes observed in cytochalasin B binding described above. In the IM, however, GLUT4 protein levels were not restored (even though the cytochalasin B binding per mg of protein was restored upon re-establishing normoglycaemia; see the Discussion section).

Fig. 2 shows a representative Western blot of immunoreactive

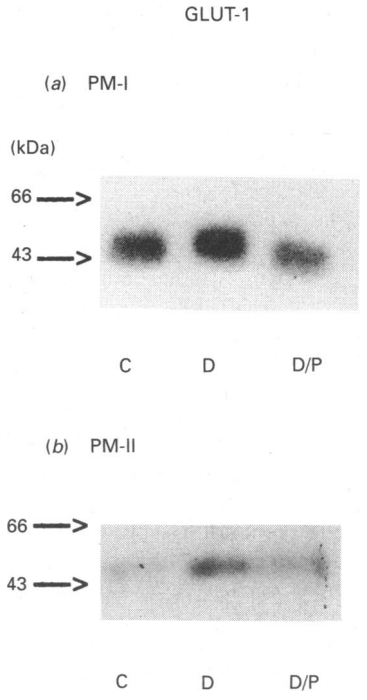

Fig. 2. Effect of glycaemia on GLUT1 protein content in plasma membranes from rat hindlimb muscles

All details are as described in Fig. 1, except that only the PM-I and PM-II fractions are illustrated (since these were the only ones with detectable amounts of GLUT1 immunoreactive protein). For an estimate of relative amounts of GLUT1 in several similar experiments, see Table 3. Abbreviations: C, control rats; D, diabetic rats; D/P, phlorizin-treated diabetic rats.

GLUT1 protein in membranes derived from muscle of control, diabetic and phlorizin-treated diabetic rats. Identical amounts of protein of each fraction were applied. GLUT1 was virtually undetectable in IM from all three animal groups. In contrast with GLUT4 protein, the GLUT1 protein levels increased in the PM-I and PM-II of diabetic rats and then decreased back towards control levels upon phlorizin treatment.

The results in three to six independent experiments are shown in Table 3. Values of each transporter isoform in the different fractions are normalized to their respective values in the PM-I of control rats, taken from autoradiographic films, with each film containing the three fractions within one preparation (control, diabetic and diabetic/phlorizin). Hence, within each isoform, the relative units are a true reflection of the relative content of immunoreactive bands in the different membrane fractions. GLUT4 transporters in both PM-I and PM-II fractions were decreased by hyperglycaemia, and recovered towards control levels in the phlorizin-treated diabetic rats. GLUT1 transporters showed the opposite pattern, augmenting with diabetes in the PM-I and PM-II, and diminishing upon correction of glycaemia. In the IM, hyperglycaemia resulted in a decrease in GLUT4 protein, which did not recover upon phlorizin treatment. To ascertain that the effect of phlorizin was related to correction of glycaemia and not to direct effects of the drug on skeletal muscle, two types of control experiments were performed (results not shown): (i) non-diabetic phlorizin-treated rats did not show any changes in glucose transporter number or isoform content, (ii) in L6 muscle cells in culture, hexose transport did not change upon acute or chronic exposure to phlorizin (A. Klip, L. Lam, D. Dimitrakoudis, T. Ramlal & M. Vranic, unpublished work).

In order to investigate whether the changes in glucose trans-

porter number and isoform seen in the isolated membranes of rat muscle of the three rat groups were a reflection of overall changes in the total amount of muscle glucose transporters, GLUT4 and GLUT1 proteins were measured in the corresponding muscle homogenates (before subcellular fractionation). The results of three independent experiments indicated that GLUT4 protein in homogenates was on average lower in the diabetic than in non-diabetic animals: The average radioactivity in the immunoreactive bands of Western blots was 71.5 ± 3.6 c.p.m. for the control and 57.5 ± 5.9 c.p.m. for the diabetic rats. This is in agreement with previous observations by Garvey *et al.* [23]. In phlorizin-treated animals, the GLUT4 transporters in homogenates, although recovering somewhat, remained lower than the levels observed in control muscles (65.5 ± 8.9 c.p.m.). Although the experimental errors in these homogenates suggest overlap between the control and phlorizin-treated diabetic group, in the three individual experiments analysed the latter group remained lower than the control. The GLUT1 transporter was not detectable in these crude preparations, presumably owing to its much lower abundance compared with the GLUT4 transporter, and hence its variations could not be studied at the level of unfractionated homogenates.

Effect of glycaemia on GLUT4 mRNA

Since GLUT4 protein levels were restored in PM-I and PM-II but not in IM and only partly in total membranes, it was considered that synthesis *de novo* of this protein may not explain in full the response to restoration of normoglycaemia. Therefore, the effects of diabetes and of the phlorizin treatment in the diabetic animals were also investigated on the levels of GLUT4 mRNA of skeletal muscle. Northern-blot analysis showed a single band of 2.8 kb for the GLUT4 transcript, in accordance with other results in the literature analysing skeletal muscle [9–11,23,24]. Five independent experiments with each of the three rat groups were performed, and two gels were run with all five sets of RNA. The gels were scanned by laser densitometry. The results showed that after 1 week of diabetes, GLUT4 mRNA decreased markedly (to 0.40 ± 0.07 of the controls). Importantly, the 2-day treatment with phlorizin, which restored glycaemia back to control levels for at least an overnight period, was able to restore only in part the GLUT4 mRNA levels (to 0.68 ± 0.06 of the controls). The phlorizin treatment did not alter GLUT4 mRNA levels in non-diabetic rat muscles (results not shown), confirming that the increase in GLUT4 mRNA in the phlorizin-treated diabetic rats was not the result of a non-specific effect of phlorizin.

DISCUSSION

It is well established that skeletal muscle is the tissue responsible for most ($> 90\%$) of the glucose uptake in the post-prandial state *in vivo* [25]. Muscle and adipose tissues are the major targets for stimulation of glucose uptake by insulin, and transmembrane transport of glucose is a primary regulated step in these tissues [26]. However, muscle and fat often do not respond in parallel to the same metabolic alterations. For instance, in hyperglycaemic/hypoinsulinaemic streptozotocin-diabetic rats, basal glucose uptake is decreased in skeletal muscle [12,27,28] but not in adipose cells [23,24,29]; furthermore, fasting decreases the number of glucose transporters in fat, but not in muscle [24,30]. These differences and others [29,31] are likely to result from fundamental divergence in the regulation of the number and type of glucose transporter molecules in the two tissues. Since conclusions based on work done in adipose tissue cannot always be extrapolated to skeletal muscle, the tissue responsible for most of the insulin-stimulated glucose uptake, studies designed to investigate mechanisms of insulin resistance in human or animal diabetes should systematically be performed on skeletal muscle. The objectives of the present study were to establish the effects of glycaemia on the regulation of glucose transporter number in plasma membranes and intracellular membranes of diabetic-rat skeletal muscle, and to identify the specific glucose transporter isoform(s) affected. In order to study the contribution of glycaemia, two experimental strategies were used in concert, namely the use of the hyperglycaemic model with fasting insulin levels similar to those in controls, and the phlorizin model of restoration of normoglycaemia.

Since the amount of glucose transporters in total muscle homogenates does not necessarily reflect the number of transporters in the plasma membrane under all metabolic conditions, to understand the molecular basis and dynamics of transport regulation it is important to measure transporter number in subcellular membrane fractions. Recent independently developed procedures for subcellular fractionation of rat muscle have allowed us and others to isolate PM and IM from this tissue, endowed with glucose transporters that respond to insulin [13,17,32,33] and exercise [13,18,32,34] challenges.

The precedent for the hyperglycaemia/corrected-hyperglycaemia experiments was partly based on previous studies in dogs. Importantly, in those studies, the metabolic clearance rate of glucose (conceptually equivalent to the transport rate in muscle tissue, independent of the prevailing glycaemia) was decreased in hyperglycaemia and increased towards normal levels upon correction of glycaemia with phlorizin [35]. Since this clearance rate parameter is likely to be determined by transporter number, we looked for possible changes in transporter number in skeletal-muscle membrane fractions under the experimental conditions described above.

The first notable observation of the present study was that normalization of glycaemia by administration of phlorizin restored the content of cytochalasin B binding per mg of protein in the PM-I, PM-II and IM fractions from the depressed levels seen in diabetes to levels similar to control. This restoration was specific, since similar changes in marker enzyme activities were not detected. These results suggest that the down-regulating influence on the glucose transport system caused by the investigated diabetic state is reversible. Moreover, since the only parameter differing in the diabetic rats and the phlorizin-treated diabetic rats was their glycaemia (in the post-absorptive state), we surmise that glycaemic level is by itself able to dictate the overall glucose transporter number independently of changes in insulin. It must be emphasized that changes in other hormones or metabolites cannot be ruled out definitively as contributing factors to the restoration of transporter number in the phlorizin-treated rats. The potential contribution of catecholamines or glucocorticoids was not examined in the present study. However, it is important to note that fasting insulin, glucagon or fatty acid levels did not correlate, directly or inversely, with transporter levels. In contrast, a relationship between glycaemia and transporter number was established across the three animal groups studied.

When the contribution of the individual transporters was assessed by Western blotting, two other striking observations were made.

(*a*) The GLUT4 protein level decreased in parallel with the cytochalasin B binding in PM-I and PM-II, whereas the GLUT1 protein level reacted oppositely, actually increasing with diabetes in both PM-I and PM-II. Since the overall glucose transporter number (i.e. cytochalasin B binding) was lower in each PM-I and PM-II of diabetic animals, in spite of the increase in GLUT1 protein, it can be suggested that GLUT1 proteins are present in

lower absolute amount in the PMs of these samples than is GLUT4. Hence, GLUT4 proteins seem to be a major transporter species in the PM in the basal state. However, because it is not known at present whether GLUT1 and GLUT4 possess the same intrinsic transport activity per molecule in muscle, the relative contribution of the increase in GLUT1 protein to glucose uptake remains to be determined. Importantly, the content of GLUT4 protein per mg of protein of the PM-I and PM-II was restored by normalization of glycaemia with phlorizin, following the pattern of total glucose transporter number and possibly accounting for much of it. The GLUT1 protein content was also corrected, decreasing back to control levels with normalization of glycaemia. This evidence strongly suggests that the effects of glycaemia on glucose transporter number are distinct and specific for each transporter isoform. Interestingly, it was recently reported that GLUT1 levels also rise in livers of streptozotocin-diabetic rats [36], and in erythrocytes of type II diabetic patients, in the latter correlating with patients' glycaemia and not insulinaemia [37].

(*b*) In the IM, the decrease in cytochalasin B binding was again mirrored by a decrease in GLUT4 protein content. Interestingly, however, GLUT4 protein levels in this fraction did not recover with this treatment. Since the GLUT1 protein was not measurable in the IM samples, it could not account for the changes in cytochalasin B binding to a significant degree. It could be, therefore, that other transporter isoform(s) may be present in muscle cells, which bind cytochalasin B but are not recognized by the anti-GLUT1 or anti-GLUT4 antibodies.

It is important to note that GLUT4 transporter number decreased in muscle total membranes of post-absorptive hyperglycaemic/normoinsulinaemic rats relative to controls, suggesting that the net amount of this transporter is decreased in the diabetic state, presumably reflecting decreased transporter synthesis. This was confirmed by the lower amount of GLUT4 mRNA detected in diabetic-rat muscles relative to control. Normalization of glycaemia did not lead to a full recovery of GLUT4 transporters in muscle total membranes, nor to a full recovery of GLUT4 mRNA. These results strongly suggest that redistribution of GLUT4 transporters to the PM fraction is restored more effectively, and that perhaps a later adaptive response to the restoration of normoglycaemia involves synthesis and replenishment of the intracellular GLUT4 pool. These results further emphasize that total muscle extracts may not reflect accurately the changes occurring in the plasma membrane, and call for caution in relating levels of total glucose transporter content to regulation of glucose uptake.

It is an inevitable and insurmountable problem in studies *in vivo* that a hyperglycaemic/normoinsulinaemic state cannot be continuously maintained throughout the considerable period which may be necessary to achieve a steady state of glucose transporter expression. Hence, the conclusions of the present study cannot be unambiguously extended to the long-term situation of diabetes without reservation. Hence it is possible that recurrence of relative hypoinsulinaemia in otherwise normoinsulinaemic streptozotocin-treated rats may contribute to their overall transport insufficiency. However, at least for a period of about 24 h, correction of glycaemia reverses the abnormalities in glucose transporter content in the plasma membrane. Biosynthetic processes taking a longer time are harder to assess by our model.

Our observations in muscle of post-absorptive normoinsulinaemic rats indicate that a 24 h restoration of glycaemia leads to a partial recovery of GLUT4 mRNA levels. In contrast, a more prolonged phlorizin treatment did not restore GLUT4 mRNA levels or GLUT4 transporter number in adipose tissue of hyperglycaemic/hypoinsulinaemic depancreatized rats [38]. It is possible that GLUT4 mRNA and protein levels in fat and muscle are not regulated by the same factors, that the prevailing hypoinsulinaemia of the rats in the studies using adipose tissue prevented the regulatory effect of glucose on GLUT4 mRNA and protein, and/or that the opposing results are due to differences in the animal models and treatment protocols used.

In summary, our results indicate that in post-absorptive hyperglycaemic/normoinsulinaemic rats the number of GLUT4 transporters in the plasma membranes is decreased, whereas that of GLUT1 transporters is increased. Further, a short-term normalization of fasting glycaemia leads to normalization of GLUT4 and GLUT1 levels in these membranes. In contrast, in intracellular membranes the overall number of glucose transporters is decreased by the diabetic state and recovers with normoglycaemia, but the recovery is not attributed to an increase in GLUT4 (or GLUT1) transporters. It is possible that, at this early stage of normalization of glycaemia, GLUT4 transporters migrate from the IM to the PM, restoring the PM levels and hence masking any biosynthetically induced increase in GLUT4 in the IM fraction. Longer maintenance of normoglycaemia with phlorizin may reveal if the levels of GLUT4 in this fraction also recover to normal values. In the short term investigated, restoration of normoglycaemia sufficed to cause a partial recovery of the diminished GLUT4 mRNA levels towards control levels. We speculate that a longer maintenance of normoglycaemia may suffice to restore muscle GLUT4 mRNA back to normal values. *In vivo*, a 3-week exposure of partly depancreatized or alloxan-treated rats to phlorizin has been shown to lead to restoration of insulin-stimulated glucose uptake [6].

The relevance of the present study lies in the understanding that the rate of glucose uptake into muscle *in vivo* depends on the number of glucose transporter molecules in the plasma membrane of the cells as much as on the mass action of glucose. This latter parameter alone could cause a doubling in glucose uptake in diabetic animals when the circulating glucose is doubled. It is possible that the decrease in glucose transporters in the PM of hyperglycaemic rats observed in this study represents a compensatory effort of the muscle to prevent the elevation of glucose influx caused by the glucose mass action. Furthermore, this study may have implication for our understanding of the pathogenesis of type II diabetes in humans. We speculate that in type II diabetes the hyperglycaemia resulting from increased hepatic glucose production or inadequate glucose disposal in the fed state, could by itself down-regulate the glucose transporters in the muscle plasma membrane. In keeping with this hypothesis, hindquarter-muscle perfusion with high glucose concentrations has been demonstrated to generate a state of muscle insulin resistance [39].

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