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Changes in glucose transport and protein kinase Cβ_2 in rat skeletal muscle induced by hyperglycaemia

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Abstract

Aims/hypothesis. We have previously reported that hyperglycaemia activates glucose transport in skeletal muscle by a Ca^{2+}-dependent pathway, which is distinct from the insulin-signalling pathway. The aim of this study was to explain the signalling mechanism by which hyperglycaemia autoregulates glucose transport in skeletal muscle.
Methods. Isolated rat soleus muscle was incubated in the presence of various concentrations of glucose or 3-*O*-methylglucose and protein kinase C and phospholipase C inhibitors. Glucose transport activity, cell surface glucose transporter 1 and glucose transporter 4 content and protein kinase C translocation was determined.
Results. High concentrations of 3-*O*-methylglucose led to a concentration-dependent increase in [^{3}H]-3-*O*-methylglucose transport in soleus muscle. Dantrolene, an inhibitor of Ca^{2+} released from the sarcoplasmic reticulum, decreased the V_{max} and the K_m of the concentration-response curve. Protein kinase C inhibitors (H-7 and GF109203X) inhibited the stimulatory effect of high glucose concentrations on hexose transport, whereas glucose transport stimulated by insulin was unchanged. Incubation of muscle with glucose (25 mmol/l) and 3-*O*-methylglucose (25 mmol/l) led to a three fold gain in protein kinase Cβ_2 in the total membrane fraction, whereas membrane content of protein kinase Cα, β_1, δ, ε and ϑ were unchanged. A short-term increase in the extracellular glucose concentration did not change cell surface recruitment of glucose transporter 1 or glucose transporter 4, as assessed by exofacial photolabelling with [^{3}H]-ATB-BMPA bis-mannose.
Conclusion/interpretation. Protein kinase Cβ_2 is involved in a glucose-sensitive, Ca^{2+}-dependent signalling pathway, which is possibly involved in the regulation of glucose transport in skeletal muscle. This glucose-dependent increase in 3-*0*-methylglucose transport is independent of glucose transporter 4 and glucose transporter 1 translocation to the plasma membrane and may involve modifications of cell surface glucose transporter activity. [Diabetologia (1999) 42: 1071–1079]

Keywords Protein kinase C, Phosphatidylinositol 3 kinase, Phospholipase C, GLUT 4, skeletal muscle, hyperglycaemia.

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Abbreviations: PKC, Protein kinase C; PI, phosphatidylinositol; DAG, 1,2-diacylglycerol; PLC, Phospholipase C; $InsP_3$, inositol 1,4,5-triphosphate; KHB, Krebs-Henseleit Bicarbonate; DMSO, dimethyl sulphoxide; PMSF, Phenylmethanesulphonyl fluoride; ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(d-mannose-4-yloxy)-2-propylamine; SR, sarcoplasmic reticulum.

Skeletal muscle is quantitatively the most important tissue involved in maintaining glucose homeostasis, accounting for about 80 % of glucose disposal after a glucose infusion or ingestion [1]. Glucose transport is the rate-limiting step for glucose metabolism in insulin sensitive tissues [2, 3]. In skeletal muscle, glucose transport is mediated by a process involving the translocation of glucose transporters (GLUT), namely GLUT4, from an intracellular site to the plasma membrane [4, 5]. Glucose transporter 4 translocation

can be achieved in response to the activation of several separate pathways in skeletal muscle. One pathway is known to be stimulated by insulin, insulin mimicking agents and insulin-like growth factors [4–10]. Another is activated by muscle contraction/exercise, hypoxia or by pharmacological agents which increase cytoplasmic Ca^{2+} [5, 11–16].

Acute hyperglycaemia has also been shown to directly increase plasma membrane GLUT4 content [17], providing evidence for an insulin-independent autoregulation of the glucose transport system in skeletal muscle. We have shown that glucose transport activity which is induced by hyperglycaemia is blocked by dantrolene [18], an inhibitor of Ca^{2+} release from the sarcoplasmic reticrum. In contrast, inhibition of phosphatidylinositol (PI) 3 kinase by wortmannin had no effect on such glucose transport [18]. Thus, hyperglycaemia may activate glucose transport by a Ca^{2+}-dependent mechanism.

The protein kinase C (PKC) family consists of at least 12 serine/threonine kinase isoforms that phosphorylate and activate multiple proteins. The conventional PKC isoforms, include α, β, and γ and are activated by Ca^{2+}, phosphatidylserine and diacylglycerol (DAG); the novel isoforms include δ, ε, η, and θ and are Ca^{2+}-independent and activated by phosphatidylserine and DAG. The atypical PKC isoforms include ζ and λ and are insensitive to Ca^{2+} and DAG [19]. Protein Kinase Cs are important regulators of cell growth, differentiation, and metabolism [20,21]. Protein Kinase C isoforms differ substantially in structure, regulation, tissue distribution, expression throughout development and substrate specificity. The basic mechanism of action for most PKC isoforms involves translocation from cytosol to cellular membranes and a subsequent response to changes in intracellular Ca^{2+} or 1,2-DAG concentration [20, 21]. The exact mechanism regulating isoform-specific PKC activation in skeletal muscle is poorly defined. Protein Kinase C has been reported to stimulate glucose transport in skeletal muscle by a mechanism distinct from the insulin and hypoxia pathways [22]. Since hyperglycaemia induces translocation of PKC isoforms in rat-1 fibroblasts [23] and rat glomerular mesangial cells [24], we hypothesised that a short-term increase in the extracellular concentration of glucose can increase 3-*O*-methylglucose transport by a PKC-dependent pathway.

Phospholipase C (PLC), is possibly one early signal which potentially mediates PKC regulation of glucose transport. Phospholipase C is activated by a G-protein-mediated pathway or by receptor tyrosine kinase. It is involved in the break down of phosphatidylinositol and leads to the production of inositol 1,4,5-triphosphate ($InsP_3$) and DAG [25]. Inositol 1,4,5-triphosphate is a second messenger known to stimulate Ca^{2+} release from sarcoplasmic reticulum by binding to $InsP_3$-gated Ca^{2+} channels [26, 27]. The increased cytosolic DAG and Ca^{2+} concentrations can induce translocation and activation of conventional (Ca^{2+}-dependent) PKC isoforms [21, 28]. Thus, we determined whether high extracellular concentrations of glucose increase 3-*O*-methylglucose transport in skeletal muscle by a mechanism involving PLC or PKC or both.

Here we show that increased concentrations of glucose increase the rate of 3-*O*-methylglucose transport by a PKC-mediated mechanism, which is independent of the PLC pathway. Furthermore, incubation of skeletal muscle with a high concentration of glucose or 3-*O*-methylglucose promotes a redistribution of PKCβ_2 to the total muscle membranes. Finally, this increase in glucose transport activity does not involve recruitment of GLUT4 or GLUT1 to the cell surface in skeletal muscle as assessed by exofacial photolabelling with ATB bis-mannose. Thus, "mass-action" cannot fully account for increased glucose uptake under hyperglycaemic conditions.

Materials and methods

Materials. We obtained U-73122 (1-[6-(17b-3-methoxyestra-1,3,5(10)-trien-17-yl-amino)-hexyl]-1H-pyrole-2,5-dione) and GF109203X (2-[1-(3-dimetylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) from Calbiochem-Novabiochem (La Jolla, Calif., USA). The insulin (Actrapid) was a product of Novo-Nordisk (Copenhagen, Denmark). Radioactive products were from American Radiolabelled Chemicals (St. Louis, Mo., USA). Materials for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, Calif., USA). All other chemicals were from Sigma Chemical Company (St. Louis, Mo., USA). Protein kinase Cβ antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). All other antibodies were from Transduction Laboratories (Lexington, Ky., USA).

Animals. Male Wistar rats (95–115 g) were purchased from B&K Universal (Sollentuna, Sweden). Rats were housed in a climate-controlled environment with 12-h alternating light-dark cycle for 1 week before the experiments. Rats received standard rodent chow and water ad libitum and were fasted overnight before the study. The principles of laboratory animal care (NIH publication n° 85–23, revised 1985) were followed.

Muscle dissection and incubation. Rats were anaesthetised by an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). Thereafter, soleus muscles were dissected and divided into two equal portions. The muscles were initially incubated for 15 min in sealed glass flasks containing Krebs-Henseleit bicarbonate (KHB) buffer, supplemented with 0.1% bovine serum albumin (radioimmunoassay grade), 5 mmol/l HEPES and 40 mmol/l mannitol. Thereafter, the muscles were incubated for 15–60 min in KHB containing 5 to 35 mmol/l glucose, with or without insulin or inhibitors or both in the concentrations indicated in the tables and figures. When inhibitors or cytochalasin B were used, muscles were pre-exposed to these compounds (20 min) before stimulation with insulin or a high concentration of glucose. Dimethyl sulphoxide (DMSO) was added (0.2%) to the media for experiments done with U-73122, GF109203X or dantrolene. Ethanol was used (0.5%) for experiments using cytochalasin B. Insulin

was used at a concentration of 1000 μU/ml. We have previously shown that this concentration of insulin elicits the maximum effect on glucose transport in rodent skeletal muscle [29]. Each vial was continuously gassed with 95% O_2/5% CO_2 and maintained in a shaking water incubator (30 °C).

3-O-methylglucose transport measurements. Before glucose transport was measured, muscles were incubated in glucose-free media with or without inhibitors as indicated above. Mannitol was adjusted to 40 mmol/l to maintain constant osmolarity. Muscles were rinsed with fresh glucose-free media three times for 3 min. Thereafter, glucose transport was assessed using [^{3}H] 3-*O*-methylglucose as described [29]. The extracellular space was estimated using [^{14}C] mannitol. The concentration of 3-*O*-methylglucose was matched to the concentration of glucose used in the earlier incubations and mannitol was added to maintain constant osmolarity (40 mmol/l). Muscles were incubated for 10 or 20 min (30 °C) in KHB with concentrations of 3-*O*-methylglucose and mannitol as indicated in the figures. Muscles were processed as described [29]. Glucose transport activity is expressed as μmol per ml of intracellular water per hour. When indicated, results are presented as per cent of basal glucose transport.

Preparation of total muscle membrane fractionation. Soleus muscle was incubated as described in the figures and tables. The muscles were homogenised (4 °C) in buffer containing 20 mmol/l TRIS (pH 7.5) 1 mmol/l EDTA, 1 mmol/l EGTA, 0.5 mmol/l phenylmethanesulphonyl fluoride (PMSF), 0.1 μg/ml leupeptin, 0.1 μg/ml aprotinin and 10 mmol/l 2-Mercaptoethanol. The homogenate was centrifuged (100 000 × *g* for 35 min at 4 °C). The supernatant was collected as the cytosolic fraction. The pellet was re-suspended in TRIS buffer with a further addition of 0.5% Triton X-100. Muscle membranes were made soluble and centrifuged (15 000 × *g* for 15 min at 4 °C). The supernatant was collected as a total membrane fraction and stored at –80 °C until analysis.

Western blot analysis. Protein concentration was determined in cytosolic and total membrane fractions using a kit from Bio-Rad (Richmond, Calif., USA). Aliquots (30 μg) were made soluble in Laemmli buffer, separated by SDS-PAGE (7.5% resolving gel), and transferred to PVDF membranes. Membranes were blocked overnight (4 °C) in TBST (5% non-fat milk in 10 mmol/l TRIS-HCl, 100 mmol/l NaCl and 0.1% Tween-20, pH 7.6). Membranes were incubated with anti-PKC isozyme-specific antibodies (as specified in Fig. 4 and Table 2) washed (3 × 15 min with TBST), incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody, then washed with TBST. Protein kinase C protein expression was made visible by enhanced chemiluminescence and quantified by densitometry.

Photolabelling of cell surface glucose transporters. Soleus muscle was incubated in pre-incubation media described above (40 min), in the presence of glucose or insulin in concentrations as specified in the figures. Muscles were incubated in glucose-free media (3 × 7 min at 18 °C) to wash glucose from the extracellular space. Muscles were transferred to a dark room and incubated for 8 min (18 °C) in rinse buffer containing 37 MBq (1 mCi/ml) ATB[2-^{3}H]BMPA. The use of the photolabelling reagent for assessing the cell surface recruitment of glucose transporters has been described [30]. Muscles were irradiated with ultraviolet light as described [31] for 2 × 3 min in a photochemical reactor. Thereafter, muscles were blotted, trimmed of visible tendons and frozen in liquid nitrogen. Muscles were weighed and further processed as described [31, 32] to determine cell surface GLUT4 content. Results are expressed as cpm per 100 mg frozen muscle.

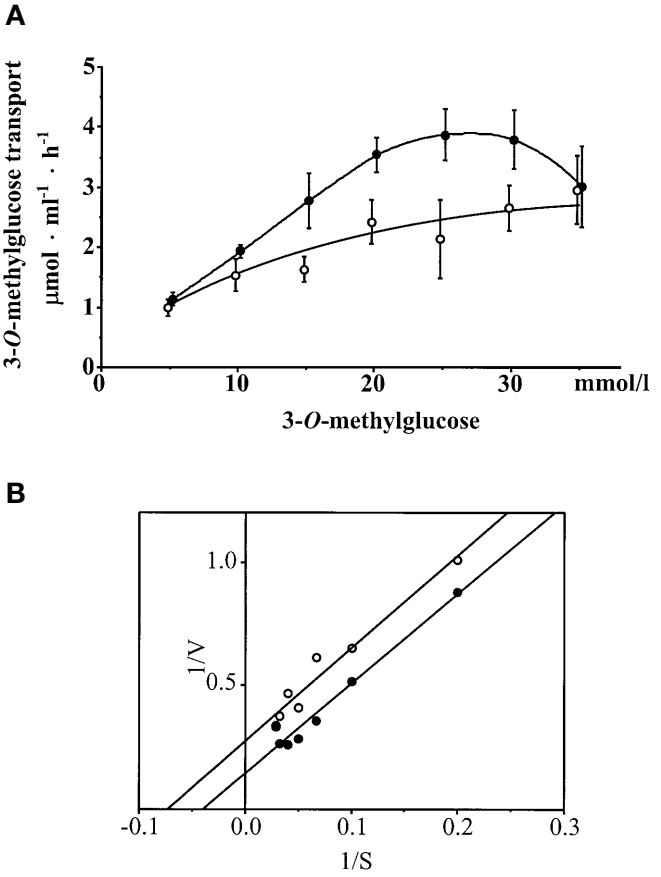

Fig. 1A,B. Effect of dantrolene on the rate of 3-*O*-methylglucose glucose transport in rat soleus muscle. **A** Soleus muscles were incubated for 15 min in KHB containing 40 mmol/l mannitol + /– 25 μmol/l dantrolene, followed by incubation for 20 min with 3-*O*-methylglucose and mannitol in KHB + /– dantrolene. Mannitol was included in the media to keep the osmolarity at 40 mmol/l in the presence of different concentrations of 3-*O*-methylglucose. Data were calculated from two independent experiments (n = 7–10 muscles for each condition). Significant difference was observed between concentration dependencies of 3-*O*-methylglucose transport in the absence (●) or presence (○) of dantrolene. **B** Reciprocal of the 3-*O*-methylglucose transport rate with or without dantrolene (Lineweaver-Burke analysis). Without dantrolene the V_{max} is 6.91 μmol · ml^{-1} · h^{-1} and the K_m is 25.0 mmol/l. With 25 μmol/l dantrolene the V_{max} is 3.65 μmol · ml^{-1} · h^{-1} and the K_m is 13.7 mmol/l

Statistical analysis. All data are expressed as means ± SEM. Data were analysed using a one-way ANOVA and differences determined by Tukey-Kramer post hoc analysis for multiple comparison. For the experiments using dantrolene, a two-way ANOVA was used and differences determined by Dunnet 2-tail analysis. Significance was set at $p < 0.05$.

Results

Kinetic analysis of 3-O-methylglucose transport. An increase in the extracellular concentration of 3-*O*-

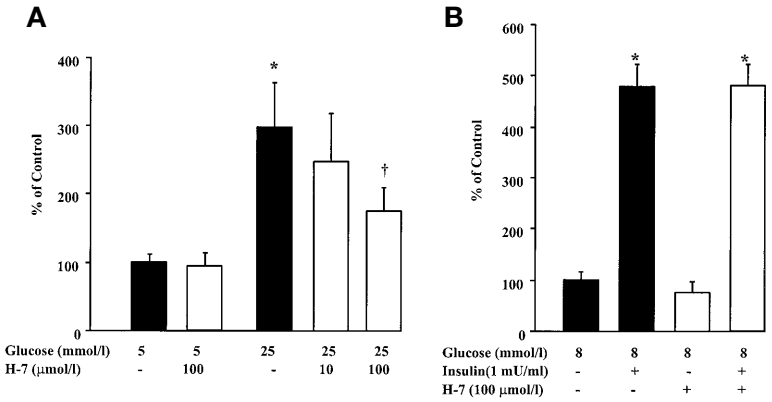

Fig. 2A,B. Effect of H-7 on 3-*O*-methylglucose transport. **A** Soleus muscles were pre-exposed to 5 or 25 mmol/l glucose as described in "Materials and methods". Thereafter, glucose was rinsed from the extracellular space of the muscle and glucose transport was assessed in the presence of increasing concentrations of H-7. Soleus muscle were incubated in the presence of 8 mmol/l 3-*O*-methylglucose with or without insulin (1 mU/ml) or H-7 (100 μmol/l) or both. Results are means ± SEM for $n = 5$–7 muscles per group. *$p < 0.05$ vs muscles exposed to 5 mmol/l glucose without H-7 (**A**) or vs muscles exposed to 8 mmol/l glucose and insulin with or without H-7 (**B**). $p < 0.05$ vs muscles exposed to 25 mmol/l glucose without H-7 (**A**)

Table 1. Effect of U-73122 on 3-*O*-methylglucose transport

	8 mmol/l glucose	20 mmol/l glucose
U-73122		
–	100.0 ± 7.6	199.6 ± 17.0[a]
10 mmol/l	130.2 ± 20.6	195.5 ± 18.2[a]
20 mmol/l	108.0 ± 9.4	182.7 ± 24.0[a]

Muscles were incubated as described in "Materials and methods". Data was calculated from two independent experiments. Values are expressed as % of the response observed at 8 mmol/l glucose for each corresponding condition. Results are means ± SEM for 6–13 muscles. [a] $p < 0.05$ vs 8 mmol/l glucose without inhibitor

methylglucose was associated with a corresponding concentration-dependent increase in the rate of 3-*O*-methylglucose transport (Fig. 1). The increase in 3-*O*-methylglucose transport was linear to 20 mmol/l 3-*O*-methylglucose, saturated at 25–30 mmol/l and decreased at 30–35 mmol/l. Rates of 3-*O*-methylglucose were greater ($p < 0.05$)at all concentrations tested between 15 mmol/l and 30 mmol/l, compared with 5 mmol/l. A concentration-response curve for 3-*O*-methylglucose transport was assessed in the presence of 25 μmol/l dantrolene (Fig. 1). Although 3-*O*-methylglucose activity was increased in a concentration-dependent manner, the concentration-response curve was suppressed ($p < 0.005$) by addition of 25 μmol/l dantrolene. Linweawer Burke analysis of the concentration-response curves showed that dantrolene decreased both V_{max} and K_m for 3–*0*-methylglucose.

Effect of cytochalasin B on 3-O-methylglucose transport. Cytochalasin B was used to evaluate whether the increase in 3-*O*-methylglucose transport activity which is induced by glucose is mediated by facilitated glucose transporters (GLUT1 or GLUT4 or both), or by non-specific diffusion. Incubation of soleus muscle with 50 μmol/l cytochalasin B reduced 3-*O*-methylglucose transport by 93% and 85%, for 5 mmol/l and 25 mmol/l extracellular 3-*O*-methylglucose, respectively ($p < 0.01$). Thus, hyperglycaemia appears to increase glucose entry into the muscle cell by a regulated transport process, rather than by diffusion.

Effect of PLC inhibition on 3-O-methylglucose transport. We used the PLC inhibitor, U-73122, to test the hypothesis that high concentrations of hexose increase glucose transport by a PLC -inositol triphosphate-mediated pathway. The twofold increase in 3-*O*-methylglucose transport activity induced by a pre-exposure of muscle to high concentrations of glucose was not changed by 10 or 20 mmol/l U-73122 (Table 1). Thus, PLC does not appear to mediate the increase in glucose transport that is induced by hyperglycaemia.

Effect of PKC inhibitors on 3-O-methylglucose transport. Since Ca^{2+} release from the sarcoplasmic reticulum (SR) is known to lead to PKC activation [21], we next tested whether PKC is involved in the signalling pathway that is induced by glucose to increase 3-*O*-methylglucose transport in skeletal muscle. We pre-exposed soleus muscle to media containing low or high concentrations of glucose, with or without H-7, a widely used PKC inhibitor. After incubation with 25 mmol/l glucose, H-7 (100 mmol/l) inhibited 3-*O*-methylglucose transport by 41% ($p < 0.05$; Fig. 2). Basal 3-*O*-methylglucose transport and that stimulated by insulin were not changed by H-7 (Fig. 2). We did additional experiments using a PKC-specific inhibitor (GF109203X). The increased in 3-*O*-methylglucose transport induced by glucose was significantly reduced by 41% and 28%, for muscle incubated in the presence of 1 and 10 μmol/l GF109203X, respectively (Fig. 3).

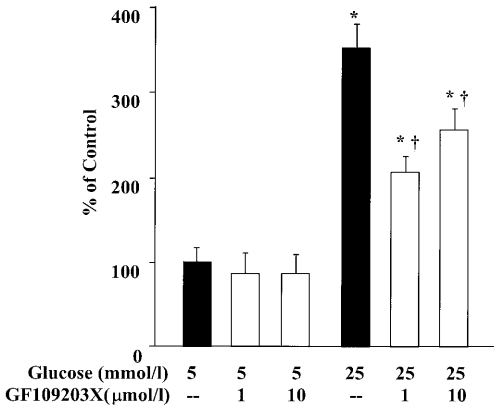

Fig. 3. Effect of GF109203X on 3-*O* -methylglucose transport. Soleus muscles were pre-exposed to glucose as described in "Materials and methods". Thereafter, glucose was rinsed from the extracellular space of the muscle and 3-*O*-methylglucose transport was assessed. Data was calculated from two independent experiments. Results are means ± SEM for 3–12 muscles per group. *$p < 0.05$ vs muscles exposed to 5 mmol/l 3-*O*-methylglucose without GF109203X, $p < 0.05$ vs muscles exposed to 25 mmol/l 3-*O*-methylglucose without GF109203X

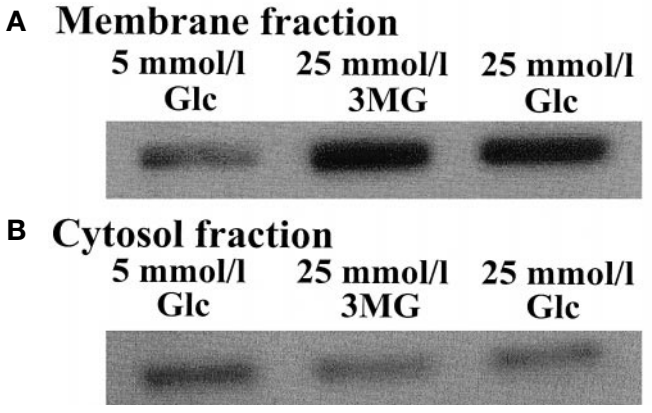

Fig. 4A,B. Representative immunoblot of PKCβ2 expression in muscle membrane fractions (**A**) or cytosol fractions (**B**). Soleus muscle was incubated for 30 min in KHB containing 5 mmol/l glucose (Glc), 25 mmol/l 3-*O*-methylglucose (3MG), or 25 mmol/l glucose. Total membrane or cytosolic fractions were prepared as described in "Materials and methods". Data expressed in arbitrary units using paired muscle comparisons for 5–6 rats is presented in Table 2

Effect of high concentrations of glucose or 3-O-methylglucose on the distribution of PKC isoforms in skeletal muscle. To investigate whether glucose or 3-*O*-methylglucose activate specific PKC isoforms, immunoblot analysis of total muscle membrane or cytosol fractions was done using isoform-specific PKC antibodies (Fig. 4). Protein kinase Cβ_2 increased by three fold in total membranes prepared from soleus muscle after incubation with either high concentrations of glucose or 3-*O*-methylglucose (Table 2). Protein kinase Cβ_2 expression in the cytosol fraction tended to be lower after exposure to high concentrations of glucose or 3-*O*-methylglucose. Since high concentrations of glucose and 3-*O*-methylglucose independently led to a gain of PKCβ_2 in total membranes, glucose rather than glucose metabolites appear to activate PKCβ_2. Protein kinase Cζ increased by 47 % ($p < 0.05$) in the membrane fraction of muscles that were incubated with 25 mmol/l glucose (Table 2). In contrast, membrane expression of PKC α, β_1, δ, ε and ϑ (Table 2) were not changed after exposure to high glucose concentrations. Furthermore, cytosolic expression of all other PKC isoforms tested was not changed by prior exposure of soleus muscle to high concentrations of glucose or 3-*O*-methylglucose in vitro.

Effect of prior exposure to high concentrations of glucose on cell surface glucose transporter content. We next tested whether exposure of isolated skeletal muscle to high concentrations of glucose changed the cell surface content of GLUT1 or GLUT4 using the exofacial photolabelling technique with ATB-[^{3}H]BMPA bis-mannose (Fig. 5). Cell surface GLUT1 content was similar under basal conditions with those stimulated by insulin in isolated soleus skeletal muscle. Insulin induced a 3.5-fold increase in cell surface GLUT4 content ($p < 0.01$). In contrast, cell surface GLUT1 and GLUT4 content were not changed in soleus muscle after exposure to 25 mmol/l glucose in vitro.

Discussion

The increase in the rate of glucose uptake in response to high concentrations of extracellular glucose has mainly been attributed to the mass-action effect of glucose on glucose uptake, rather than to activation of glucose transport by specific glucose transport proteins [33,34]. Here we provide evidence that prior exposure of isolated soleus muscle to high concentrations of glucose leads to an increase in glucose transport activity by a regulated process involving Ca^{2+} release from the SR, possibly by a PKCβ_2-mediated signalling pathway. Thus, the mass-action of glucose cannot fully account for the increase in glucose uptake in skeletal muscle, since glucose transport activity in the presence of 20 mmol/l 3-*O*-methylglucose was reduced in soleus muscle incubated with PKC inhibitors. Furthermore, we show that prior exposure of isolated skeletal muscle to high concentrations of glucose does not lead to recruitment of GLUT1 or GLUT4 to the cell surface. Thus, our results provide evidence that hyperglycaemia could lead to the modification of GLUT activity at the cell surface.

Short-term stimulation of skeletal muscle with phorbol esters increases glucose transport and GLUT4 translocation in skeletal muscle and adipocytes by a PKC-mediated mechanism [35–39]. Here we provide evidence for an auto regulation of the glucose transport system by a calcium-sensitive pathway,

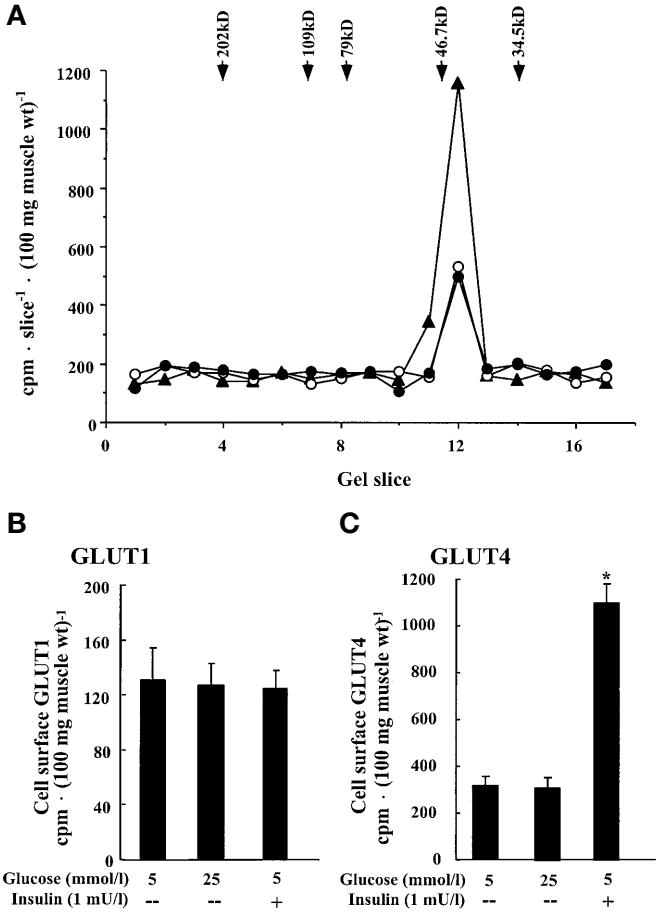

Fig. 5A–C. Cell surface GLUT1 and GLUT4 content in skeletal muscle. **A** Representative gel profile of cell surface GLUT4 content in soleus muscle incubated with 5 mmol/l glucose (○), 25 mmol/l glucose (●) or 5 mmol/l glucose + insulin (▲). Photolabelled cell surface GLUT4 was immunoprecipitated from total membranes and separated by SDS-polyacrylamide gel electrophoresis. The gel was cut into slices and the amount of ATB-[2-^{3}H]BMPA associated with each solubilised gel slice was determined by scintillation counting. Results are expressed as cpm per slice per 100 mg of muscle. **C** Skeletal muscle cell surface GLUT1 (**B**) and GLUT4 (**C**) content. Results are means ± SEM for four to five independent observations. *$p < 0.01$ vs muscles exposed to 5 mmol/l glucose or 25 mmol/l glucose

since dantrolene, an inhibitor of Ca^{2+} release from the SR, partly inhibits 3-*O*-methylglucose transport. The dantrolene-resistant component of 3-*O*-methylglucose transport appears to represent the mass-action effect of glucose on glucose transport. Since inhibition of Ca^{2+} release from the SR led to inhibition of 3-*O*-methylglucose transport, we next determined whether PLC or PKC pathways or both were involved. Exposure of rat epitrochlearis muscles to exogenous PLC in vitro is associated with increased PKC activity associated with the membrane [36], providing evidence for activation of this pathway in skeletal muscle. We used pharmacological inhibitors to determine whether glucose transport was mediated by a PLC or PKC pathway or both. Using U-73122, a phosphatidylinositol specific PLC (PI-PLC) inhibitor, we found no evidence for a role of PI-PLC in glucose transport that is induced by either insulin or hexose. This compound was used in concentrations that have been previously used to inhibit PLC activity in isolated vascular smooth muscle cells [40,41], intact aorta [42] and airway smooth muscle [43]. Thus, pathways that are independent of PLC could stimulate Ca^{2+} release from SR in response to hyperglycaemia.

To determine whether PKC mediates glucose transport, we pre-incubated soleus muscle in the presence of high concentrations of glucose and either H-7, a broad base cell permeable serine-threonine kinase inhibitor known to inhibit PKC [44,45] or GF109203X, a highly selective cell-permeable PKC inhibitor [46,47]. Thereafter, glucose was rinsed from the muscle and glucose transport was assessed using 3-*O*-methylglucose. Both compounds inhibited 3-*O*-methylglucose transport activity, providing evidence that PKC plays an important part in a Ca^{2+}-dependent pathway by which high concentrations of glucose activate glucose transport. Furthermore, since H-7 failed to suppress glucose transport that is stimulated by insulin, the inhibitory effect of this compound is not due to non-specific effects on either GLUT4 vesicle recycling or direct inhibition of glucose transport. Rather, these effects are likely to be due to specific inhibition of a signalling pathway in skeletal muscle that

Table 2. PKC isoform expression in membrane and cytosolic fractions

	PKCα	PKCβ1	PKCβ2	PKCδ	PKCε	PKCθ	PKCζ
Membrane							
5 mmol/l glucose	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
25 mmol/l 3MG[a]	0.78 ± 0.09	1.02 ± 0.13	2.96 ± 0.54[b]	1.07 ± 0.17	1.00 ± 0.10	1.12 ± 0.16	1.31 ± 0.14
25 mmol/l glucose	0.88 ± 0.14	1.68 ± 0.39	2.99 ± 0.60[b]	0.90 ± 0.05	0.86 ± 0.07	1.33 ± 0.46	1.47 ± 0.16[b]
Cytosol							
5 mmol/l glucose	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	n.d.	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
25 mmol/l 3MG[a]	0.99 ± 0.16	1.11 ± 0.12	0.77 ± 0.15	n.d.	1.26 ± 0.20	1.40 ± 0.47	0.89 ± 0.19
25 mmol/l glucose	1.07 ± 0.18	1.12 ± 0.22	0.79 ± 0.29	n.d.	1.04 ± 0.12	1.91 ± 0.17	1.19 ± 0.27

Muscles were incubated and processed as described in the legend to Figure 5. Data is expressed in arbitrary units using paired muscle comparisons within rats. Values are reported as % of effect for each animal at 5 mmol/l glucose. Results are means ± SEM for 5–6 rats. [a] 3MG, 3-*O*-methylglucose. [b] $P < 0.05$ vs 5 mmol/l glucose. n.d., PKCδ in cytosol fraction was not detectable

is induced by glucose. Western-blot analysis showed that high concentrations of either glucose or 3-*O*-methylglucose promoted an approximate threefold increase in PKCβ_2 content in the total muscle membrane fraction. Conversely, high glucose or 3-*O*-methylglucose did not lead to a gain in either α, $\beta 1$, δ, ε or ϑ PKC isoforms in the total membrane fraction. Thus, activation of PKCβ_2 could be an important component of the signalling cascade that is induced by glucose to increase glucose transport in skeletal muscle.

We noted a 47% increase in PKCζ in the muscle membrane fraction after glucose exposure. Thus, PKCζ could play an important part in the signalling cascade that is induced by glucose. Insulin activates PKCζ through a PI 3-kinase-mediated mechanism and PKCζ has been suggested to be a down-stream effector of PI 3-kinase that contributes to glucose transport that is stimulated by insulin [48, 49]. Our earlier study [18] shows, however, inhibition of PI 3 kinase by wortmannin has no effect on glucose transport induced by hyperglycaemia, thus transport activity induced by glucose appears to be mediated by a PI 3-kinase-independent pathway. Thus, PKCζ is not likely to be a candidate for the transport activity induced by glucose.

Release of Ca^{2+} from SR could be sufficient to activate PKCβ_2, a calcium sensitive PKC isoform. Evidence has accumulated to show that direct PKC activation by phorbol esters or DAG leads to the stimulation of glucose transport in adipocytes [30, 50, 51] and skeletal muscle [35–37], and GLUT4 translocation to the cell surface in adipocytes [30, 51, 52]. Insulin [38, 53] and muscle contraction [54, 55] have been reported to activate the PKC pathway. Furthermore, PKC activation through stimulation with phorbol esters increases glucose transport [35–37]. Using the exofacial photolabelling technique with [^{3}H]-ATB-BMPA bismannose, we found no evidence, however, to support the hypothesis that a high concentration of glucose leads to a gain of GLUT4 or GLUT1 at the cell surface. Our results are in contrast with a previous report [17] whereby hyperglycaemia was shown to directly increase plasma membrane GLUT4 content in rat skeletal muscle. Whether high concentrations of glucose directly modulate the activity of GLUT4 or GLUT1 or both at the plasma membrane, rather than changing the number of glucose transporters at the skeletal muscle plasma membrane, is still not known.

Glucose directly stimulates PKC translocation/activation [17, 23]. The $\beta 2$ isoform of PKC, can be translocated/activated in response to prior exposure to high concentrations of glucose in rat aortic smooth muscle cells in vitro [56] and in human platelets in vivo [57]. The mechanism behind PKC activation by high concentrations of glucose involves de novo synthesis of DAG from glucose. Diacylglyceride is a physiological activator of conventional and novel PKC isoforms. High concentrations of DAG have been observed in vascular tissue in diabetic animals [58–60]. Furthermore, in cultured vascular tissues [56] and adipocytes, exposure to high concentrations of glucose leads to an accumulation of DAG [61]. We show that a high concentration of extracellular 3-*O*-methylglucose (a glucose analogue transported into cells by the glucose transporters but not further metabolised) led to a gain in PKCβ_2 in the total membrane fraction. The magnitude of this increase was similar to that after incubation of soleus muscle in the presence of a high concentration of glucose. This indicates that the effect of high glucose on PKCβ_2 translocation is independent of glucose metabolism. Exposure of soleus muscle to a high concentration of glucose was also associated with a 47% increase in PKCζ content in the membrane fraction. Protein kinase Cζ is an atypical PKC isoform that is activated independent of DAG, although it can be activated by phosphatidic acid, a precursor of DAG that can be formed from glucose. Exposure of rat-1 fibroblast [23] or rat glomerular mesangial cells [24] to high concentrations of glucose leads, however, to translocation of PKCζ. Although our results suggest there could be a mechanism for the stimulation of PKC which is independent of DAG in response to prior exposure to high concentrations of glucose, we cannot rule out the possibility that glucose metabolism is required for PKC activation.

Several studies provide evidence that changes in the distribution of PKC isoforms occur in skeletal muscle from animal models of diabetes [62]. Some of these changes can be long-therm and occur as a consequence of increased concentrations of glucose, insulin and lipids. Changes in PKCθ and PKCε are evident in skeletal muscle from rats made insulin resistant by diet (high-fat-fed) and these changes appear to be related to muscle triglyceride and diglyceride concentrations [63]. In the Goto-Kakizaki (GK) diabetic rat [64], PKC enzyme activity and expression of PKCα, PKCβ, PKCε, and PKCδ are increased in membrane fractions and decreased cytosolic fractions of soleus muscles, relative to control Wistar rats. In addition, PKCθ expression in GK soleus muscles is decreased in both membrane and cytosol fractions, whereas PKC-ζ expression is not changed in either fraction. Thus, more extreme changes in the distribution or expression or both of the PKC isoforms could occur in response to increased lipid availability and this might not be evident in a short-term in vitro situation.

In conclusion, we provide evidence that glucose can directly modify glucose transport activity by a mechanism that possibly involves PKCβ_2. The increase in PKCβ_2 content in the membrane fraction is independent of glucose metabolism, since similar effects were observed using 3-*O*-methylglucose. Moreover, we show that exposure of isolated skeletal muscle to high concentrations of glucose does not lead to changes in cell surface content of GLUT1 or

GLUT4, despite increased glucose transport activity. This short-term effect of hyperglycaemia on glucose transport provides a novel mechanism for the regulation of glucose transport in skeletal muscle.

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