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Functional involvement of protein kinase C- β II and its substrate, myristoylated alanine-rich C-kinase substrate (MARCKS), in insulin-stimulated glucose transport in L6 rat skeletal muscle cells

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Abstract

Aims/hypothesis—Insulin stimulates phosphorylation cascades, including phosphatidylinositol-3-kinase (PI3K), phosphatidylinositol-dependent kinase (PDK1), Akt, and protein kinase C (PKC). Myristoylated alanine-rich C-kinase substrate (MARCKS), a PKC β II substrate, could link the effects of insulin to insulin-stimulated glucose transport (ISGT) via phosphorylation of its effector domain since MARCKS has a role in cytoskeletal rearrangements.

Methods—We examined phosphoPKC β II after insulin treatment of L6 myocytes, and cytosolic and membrane phosphoMARCKS, MARCKS and phospholipase D1 in cells pretreated with LY294002 (PI3K inhibitor), CG53353 (PKC β II inhibitor) or W13 (calmodulin inhibitor), PI3K, PKC β II and calmodulin inhibitors, respectively, before insulin treatment, using western blots. ISGT was examined after cells had been treated with inhibitors, small inhibitory RNA (siRNA) for

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MARCKS, or transfection with MARCKS mutated at a PKC site. MARCKS, PKC β II, GLUT4 and insulin receptor were immunoblotted in subcellular fractions with F-actin antibody immunoprecipitates to demonstrate changes following insulin treatment. GLUT4 membrane insertion was followed after insulin with or without CG53353.

Results—Insulin increased phosphoPKC β II(Ser660 and Thr641); LY294002 blocked this, indicating its activation by PI3K. Insulin treatment increased cytosolic phosphoMARCKS, decreased membrane MARCKS and increased membrane phospholipase D1 (PLD1), a protein regulating glucose transporter vesicle fusion resulted. PhosphoMARCKS was attenuated by CG53353 or MARCKS siRNA. MARCKS siRNA blocked ISGT. Association of PKC β II and GLUT4 with membrane F-actin was enhanced by insulin, as was that of cytosolic and membrane MARCKS. ISGT was attenuated in myocytes transfected with mutated MARCKS (Ser152Ala), whereas overproduction of wild-type MARCKS enhanced ISGT. CG53353 blocked insertion of GLUT4 into membranes of insulin treated cells.

Conclusions/interpretation—The results suggest that PKC β II is involved in mediating downstream steps of ISGT through MARCKS phosphorylation and cytoskeletal remodelling.

Keywords

F-actin; Glucose transporter 4; Insulin-stimulated glucose uptake; L6 myocytes; MARCKS; Phospholipase D1; PKC β

Introduction

Insulin is responsible for translocating glucose transporter 4 (GLUT4), a transmembrane protein, to the membrane of muscle and fat cells. Involvement of upstream kinases, such as Akt (also called protein kinases B), has been studied extensively. The molecular scenario nearer the membrane is less understood. One kinase known to support insulin's role in skeletal muscle is protein kinase C (PKC) β II. Physiologically, there are reports of polymorphisms in the PKC β promoter that reduce promoter activity in humans. One polymorphism is associated with the decreased expression of PKC β II and results in decreased peripheral insulin-dependent glucose uptake [1]. Other polymorphisms in the PKC β promoter are associated with diabetic vascular complications and diabetic neuropathy in people with type 1 diabetes [2,3]. Despite the finding that PKC β II modulates insulin action in human skeletal muscle [4] and rat skeletal muscle and cell lines (for review see [5]), little is known about its molecular mechanisms.

Isozymes of PKC β are implicated as both signal transducers and modulators of insulin signalling [6,7]. PKC comprises a family of 12 serine/threonine kinase isozymes and their splice variants, which exhibit differential cellular distributions and substrate specificities [8]. To be active kinases, the conventional PKC isozymes PKC α , PKC β I, PKC β II and PKC γ require a series of post-translational modifications. These include phosphorylation by phosphatidylinositol-dependent kinase (PDK) 1/2 and autophosphorylation, the binding of two or three Ca²⁺ ions, followed by interaction with membrane lipids: acidic phosphatidylserine and non-polar diacylglycerol [8]. The β isoforms, PKC β I and PKC β II, are encoded by the same gene and differ in the last exon as a product of alternative splicing of PKC β pre-mRNA, producing proteins that differ by their carboxyl-terminal 50–52 amino acids [9].

Previous studies show that insulin stimulates alternative splicing of PKC β pre-mRNA in favour of the PKC β II isoform in BC3H-1 myocytes, L6 skeletal muscle cells, rat hepatocytes, HepG2 cells, 3T3-L1 preadipocytes, vascular smooth muscle cells and embryonic fibroblasts [10–12]. This occurs through enhanced exon inclusion [11]. Using a dominant-negative-acting PKC β II construct expressed in L6 myotubes, PKC β II rather than PKC β I was shown to be the splice variant promoting insulin-stimulated glucose transport [13]. Insulin also stimulates the

phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS), a high-affinity conventional PKC substrate, in cells, consistent with the activation of calcium-dependent conventional PKCs [14]. MARCKS is an acidic protein with a central 25 amino acid basic region, termed the effector domain (ED), which is a site of interaction with Ca^{2+} -calmodulin, F-actin and membrane phospholipids [15]. The ED contains three PKC phosphorylation sites (Ser-152, Ser-156 and Ser-163), and ED phosphorylation by PKC on Ser-152 or Ser-156 disrupts interactions with Ca^{2+} -calmodulin and aids in releasing MARCKS from membranes as well as altering its interactions with F-actin [16]. Phosphorylation of MARCKS by PKC unmasks membrane phosphatidylinositol 4,5-bisphosphate (PIP_2), thus initiating cytoskeletal reorganisation through PIP_2 -mediated recruitment of actin polymerisation and treadmilling or tethering of actin filament cross-linking proteins, and proteins required to attach F-actin to membranes [17,18]. $\text{PKC}\beta$ phosphorylation of MARCKS plays a role in vesicular trafficking in neurons [19]. Thus, the potential molecular role for $\text{PKC}\beta\text{II}$ in insulin-stimulated glucose transport is hypothesised to occur via phosphorylation of MARCKS. MARCKS movement may also be reciprocal to phospholipase D binding to PIP_2 in the membrane. Phospholipase D1 (PLD1), an enzyme regulated by agonist stimulation, has been proposed to function at many steps in vesicle trafficking and fusion [20,21], and is associated with GLUT4 trafficking to exocytic sites [22].

Here, we studied the phosphorylation states of MARCKS and $\text{PKC}\beta\text{II}$ and the distribution of PLD1 during insulin action and mutated a crucial PKC phosphorylation site in the MARCKS ED to determine whether it blocked glucose transport in cells expressing the mutant.

Methods

Cell culture

L6 rat skeletal myoblasts, obtained from Dr Amira Klip (The Hospital for Sick Children, Toronto, ON, Canada), were grown in α -minimal essential medium (α -MEM) containing 10% FBS at 37°C in a humidified 5% CO_2 , 95% air atmosphere in 24-well, six-well, 100-mm or 245-mm plates. Cells were grown to 80% confluence and induced to differentiate into multinucleated myotubes by reducing the FBS concentration to 2% for 2–4 days prior to the experiment. Cells were then washed with PBS and incubated in serum-free α -MEM for 4–6 h prior to each experiment. Insulin (Sigma-Aldrich, St Louis, MO, USA) was used at a final concentration of 10 $\mu\text{IU}/\text{ml}$ and was added to the cells 30 min prior to cell lysis.

PI3 kinase and $\text{PKC}\beta\text{II}$ inhibitors

LY294002 (Sigma-Aldrich) is a phosphatidylinositol 3-kinase (PI3K) inhibitor. LY294002 inhibits purified PI3K with an IC_{50} of 1.4 $\mu\text{mol}/\text{l}$ [23]. Inhibition of PI3K is commonly associated with the inhibition of Akt phosphorylation on Thr308 by PDK1 [24,25].

$\text{PKC}\beta\text{II}$ was inhibited pharmacologically by preincubating cells for 30 min with 1 $\mu\text{mol}/\text{l}$ CG53353 (also known as DAPH 2), a gift from Dr Dorianno Fabbro, Novartis, Basel, Switzerland. CG53353 is concentration-selective for $\text{PKC}\beta\text{II}$ inhibition (IC_{50} for $\text{PKC}\alpha$ 1.9 $\mu\text{mol}/\text{l}$, $\text{PKC}\beta\text{I}$ 3.8 $\mu\text{mol}/\text{l}$, $\text{PKC}\beta\text{II}$ 0.41 $\mu\text{mol}/\text{l}$, $\text{PKC}\gamma$ 22 $\mu\text{mol}/\text{l}$, $\text{PKC}\zeta$ > 500 $\mu\text{mol}/\text{l}$ [26]). CG53353 was used at a concentration shown not to affect the activation of the insulin receptor, the EGF receptor (HER-1), c-erbB2 (HER-2), the platelet-derived growth factor receptor or IGF-1 receptor [26]. W13, a calmodulin inhibitor, was from Sigma-Aldrich.

Western blot analysis

Protein extracts from cells grown on 100 mm plates were prepared by adding 500 μl lysis buffer per plate (20 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 1% Triton X-100, 50 mmol/l NaF, 0.2 mmol/l Na_3VO_4 and complete protease inhibitor cocktail (Roche, Basel, Switzerland),

scraping cells and transferring them to a 1.5 ml tube. Between 10 and 20 μg of total protein from each sample was separated by SDS-PAGE (10% acrylamide) followed by electroblotting onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were probed in TBST buffer (20 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 0.05% Tween-20) using anti-PKC β II (Ser641) (Abcam, Cambridge, MA, USA). Anti-phosphoMARCKS antibody was from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Phospholipase D1 and anti-phosphoAkt (Thr308 and Ser473) antibodies were from Cell Signaling Technology, Danvers, MA, USA. Anti-phosphoPKC β II (Ser660) to NH₂-EGF(pS) FVNSEFLKPEVKSCOOH (657–673) was raised in rabbits by Bio-Synthesis, Lewisville, TX, USA. This peptide epitope is unique to PKC β II and the antibody does not cross-react with other PKCs. MARCKS antibody has been described earlier [26]. Non-specific binding was blocked by the presence of 5% dried skimmed milk or porcine gelatine.

Membrane fractionation

Protein extracts from cells grown on 100 mm plates were prepared by adding 5 ml ice-cold PBS per plate, scraping the cells and transferring them to 15 ml tubes. Cells were pelleted (centrifugation at 700 \times g for 5 min), resuspended in 500 μl swelling buffer (20 mmol/l Tris, pH 7.5, 10 mmol/l NaCl, 50 mmol/l NaF, 0.2 mmol/l Na₃VO₄ and Complete Protease Inhibitor Cocktail) for 10 min before addition of Triton X-100 (to 1%), and disrupted by 20 passes in a Dounce homogeniser. The homogenate was then centrifuged (3000 \times g, 10 min) to remove nuclei and debris. The supernatant fraction was then centrifuged at 25,000 \times g for 30 min. The cytosolic fraction was present in the supernatant fraction and the pellet was resuspended in 40 μl lysis buffer to produce a plasma membrane fraction.

Immunoprecipitation with anti-actin antibody

L6 myotubes were treated for 30 min with or without 10 $\mu\text{IU/ml}$ insulin (0.41 mg/l), washed with cold PBS then incubated with 2 mmol/l DSP (a cleavable cross-linker; Pierce, Rockford, IL, USA) at room temperature for 30 min, followed by 15 min with stop buffer (20 mmol/l Tris, 20 mmol/l glycine). Cells were scraped, homogenised in 500 μl lysis buffer and incubated at 4°C with agitation overnight with 20 μl (200 μg) protein-A magnetic beads prebound to anti-actin antibody from Santa Cruz Biotechnology (sc-10731) according to NEB protocol S1425S. The relevant proteins were then extracted by magnetic separation (magnetic separator from New England Biolabs, Ipswich, MA, USA), washed three times in lysis buffer and resuspended in 50 μl Laemmli buffer containing 5% β -mercaptoethanol. Lysates were subjected to western analysis as described above.

Measurement of insulin-stimulated glucose transport by 2-deoxyglucose uptake

L6 myoblasts were cultured as described above but in 24-well plates. Cells were rinsed with PBS and incubated in serum-free α -MEM for 4 h prior to experiments. Inhibitors were added 2 h prior to experiments. Cells were then washed and incubated in PBS with 1% BSA at 37°C with inhibitors and/or 10 $\mu\text{IU/ml}$ insulin 30 min prior to addition of 10 nmol 2-deoxy[³H] glucose (50–150 $\mu\text{Ci}/\mu\text{mol}$; Perkin Elmer, Boston, MA, USA) and incubation (6 min, 37°C). Cells were washed three times with cold PBS and lysed in 1% SDS. Radioactivity was determined by liquid scintillation counting.

Inhibition of PKC β II and MARCKS production with siRNA

Small inhibitory RNA (siRNA) was performed using the Silencer siRNA Cocktail Kit (Ambion/Applied Biosystems, Foster City, CA, USA). Two approaches were used for PKC β II siRNA to avoid ‘off-target’ involvement. For the first siRNA, the PKC β II-specific exon (exon 17–156 nucleotides) was amplified by PCR using the primers (forward) 5'-TAATACGACTCACTATAGGGTACTTG TGGCGAAACGCTG-3' and (reverse) 5'-

TAATACGA CTCACT ATAGGGTACTTTAGCTCTTGACTTC-3'. These were purified, digested by RNase III and repurified. The digested exon (100 nmol/l) was then transfected into cells with Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) using the standard protocol. Next, siRNA to PKC β II (no. 103309), MARCKS (no. 59351) or Silencer negative control (no. 4611; Ambion) was transfected into cells. Silencer and scrambled siRNA were the controls for the two methods. Briefly, RNA and Lipofectamine were mixed before adding to 80% confluent myotubes in serum-free medium. Serum was added after 5 h and cells were incubated for 36–48 h at 37°C. Western analysis and ISGT assays were performed as described above.

Overproduction of MARCKS and the MARCKS effector domain mutant S152A cDNA

Rat MARCKS cDNA with the serine→alanine point mutation in Ser-152 was constructed as described [27]. Myotubes in 24-well plates were transiently transfected with 0.1 μ g pcDNA3 plasmid containing wild-type or mutated MARCKS using Lipofectamine as described above. After transfection (48 h), cells were serum-starved for 6 h prior to determining 2-deoxy-[³H] glucose uptake. S152A MARCKS and wild type MARCKS overproduction and phosphorylation were verified by western blot analysis [27].

Assay for membrane-associated GLUT4

Membrane recycling of glucose transporters occurs in metabolically active cells. To inhibit this, cells were incubated with 2 mmol/l potassium cyanide [28], then L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich) was added to the cells at a final concentration of 1 mg/ml to cleave the exofacial loops of membrane-inserted GLUT4, and digestion proceeded for 30 min at 37°C. At the end of the digestion period, soybean trypsin inhibitor (Sigma-Aldrich) was added to a final concentration of 2 mg/ml and the cell monolayer was quickly washed twice with PBS containing trypsin inhibitor and 2% albumin. Cells were pelleted (3 \times g, 5 min) and homogenisation buffer was added prior to disrupting cells. Cells were centrifuged (100,000 \times g, 45 min) and the membrane pellet was resuspended in lysis buffer; proteins were resolved by SDS-PAGE. GLUT4 transporters inserted into the plasma membrane are detected as fragments at a lower, approximately 33 kDa, band on immunoblot probed with an anti-GLUT4 antibody (sc-7938; Santa Cruz Biotechnology) generated to the central domain of the protein (amino acids 230–290).

Results

Insulin activation of PKC β II by PI3K pathway

PI3K activates numerous kinases, including Akt and PKC isozymes, by its subsequent activation of PDK1 and its phosphorylation of serine/threonine residues in their activation loops [29]. However, since insulin activation of PKC β II by PI3K had not been demonstrated in L6 myocytes, we examined phosphoPKC β II (Thr641 and Ser660) in cells treated with serum, insulin and LY294002, a PI3K inhibitor, or the Src inhibitor PP1 (Fig. 1). When cells were serum-starved for 6 h and treated with insulin for 30 min, phosphoPKC β II (Thr641 and Ser660) levels more than doubled, and with Thr641 there was >40-fold stimulation over control (lane 3). Serum re-addition was also an activator of both phosphorylation sites (lane 2). PKC β II Ser660 phosphorylation was blocked by LY294002 at both concentrations, but Thr641 phosphorylation was blocked better by 20 μ mol/l LY294002. Ser660, a residue in the alternatively spliced region, is autophosphorylated and thought to require the prior phosphorylation of activation loop residues. Ser660 phosphorylation was more sensitive to LY294002 inhibition. 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*] pyrimidine (PP1), a tyrosine kinase inhibitor, had a small inhibitory effect on both sites. Akt Thr308 and Ser473 phosphorylation was also stimulated by insulin, but serum was not a good activator in L6 myotubes. LY294002 blocked insulin-induced phosphorylation of both sites. PP1 inhibited insulin-induced Akt Ser473 phosphorylation, indicating the potential upstream regulation of

this site by tyrosine kinases. The extracellular signal-regulated kinase or ERK1/2 pathway, activated equally by serum and insulin and not regulated by PI3K or Src kinases, is shown as a control. Thus, PKC β II Ser660 and Thr641 phosphorylation was activated in a PI3K-dependent manner by insulin.

Insulin induces PKC β II and Ca²⁺-calmodulin-dependent phosphorylation of MARCKS

We hypothesised that MARCKS, a conventional PKC substrate, was the potential mediator of PKC β II action related to GLUT4-storage vesicle movement. We previously identified MARCKS as a target of insulin-stimulated PKC activity in L6 myotubes [14]; however, a molecular role for MARCKS was not hypothesised. To further investigate the effects of MARCKS in the context of PKC-mediated stimulation of insulin action, we examined phosphorylated MARCKS in cytosol and membrane fractions of L6 cells. Previous studies reported that the calmodulin antagonist W13 inhibits the effect of insulin on glucose transport in 3T3L1 adipocytes [30]. Both activated PKC and calcium-bound calmodulin (CaCaM) act on MARCKS [31]. When MARCKS is phosphorylated by PKC β II or bound by CaCaM, the ED is masked and MARCKS, released from the membrane, is detected as phosphoMARCKS in the cytosol [15,32].

To determine whether this occurred in L6 myotubes, cells were pretreated with CG53353 (a PKC β II-specific inhibitor) or W13 before insulin treatment and fractionated, and extracts were examined by western blotting. In this protocol, nuclei and other heavy organelles are removed first, and the remaining extract is divided into soluble (cytosol) and non-soluble (membrane-containing) fractions as described in the Methods. After 30 min of insulin treatment, cytosolic phosphoMARCKS was twofold more intense on the blot. The cytosolic increase was blocked when cells were treated with CG53353 or W-13 (Fig. 2b, lanes 4–9). Membrane phosphoMARCKS decreased somewhat with insulin treatment but was not significantly altered by inhibitor treatment (Fig. 2f). Cytosolic MARCKS pools were increased with insulin treatment but decreased with inhibitors compared with their controls in lanes 4 and 7 (Fig. 2c). Membrane MARCKS pools decreased following insulin treatment but not with inhibitors (Fig. 2g, lanes 4–9). Since PLD1 is thought to be targeted to MARCKS sites in the membrane following the release of phosphoMARCKS to the cytosol, PLD1 was immunoblotted; membrane PLD1 increased in the membrane following insulin treatment (Fig. 2h, lanes 1–3). Both inhibitors blocked this increase (lanes 5–9). A decrease in cytosolic PLD1 was demonstrated in insulin-treated control cells, and the trend was muted by the inhibitors (Fig. 2d). Actin levels did not significantly change, although a modest decrease in the presence of insulin alone was noted in the membrane (Fig. 2i). Prior studies indicate that the cytosolic translocation of MARCKS is increased both by PKC-mediated phosphorylation and by the binding of CaCaM (for review see [33]). PLD1 activity was also associated with GLUT4 vesicle trafficking to exocytic sites [22]. Here, insulin stimulated MARCKS phosphorylation and its release from the membrane was countered by an increase in cytosolic phosphoMARCKS. In addition, PKC β II appeared to phosphorylate MARCKS in response to insulin, as indicated by the inhibition of phosphoMARCKS by CG53353 (Fig. 2b).

These inhibitors also blocked (>60%) ISGT (Fig. 3a). In order to mimic the activation of calmodulin, we increased intracellular calcium levels by addition of the ionophore A23187. This addition, in the absence of insulin, produced a moderate but significant stimulation of glucose uptake (Fig. 3a). To determine whether the inhibition of MARCKS phosphorylation by CG53353 noted in Fig. 2b was due to PKC β II inhibition, two different PKC β II siRNAs were transfected into L6 myotubes and phosphoMARCKS was examined (Fig. 3b–d). The siRNA reduced PKC β II levels by 50–60%, and MARCKS phosphorylation was reduced by more than 80–90% in insulin-treated cells.

Next, a commercial siRNA (Ambion) to reduce MARCKS levels was transfected into the cells (Fig. 4a). Decreasing MARCKS production fully blocked the insulin-induced increase in 2-deoxy[³H]glucose uptake ($p < 0.05$) (Fig. 4b).

Immunoprecipitation demonstrated that PKC β II, GLUT4 and MARCKS were associated with actin in an insulin-dependent manner

Another indication of the interaction of PKC β II, MARCKS and GLUT4 following insulin treatment would be changes in their subcellular localisation and interactions. To approach this, cortical F-actin was used as a common cytoskeletal component and its antibody was used to immunoprecipitate cytosolic and membrane fractions of insulin-treated (30 min) and control cells. Proteins were separated by electrophoresis, and candidate proteins were probed by immunoblot analysis. In order to preserve the possible fragile nature of the interactions, cells were treated with the cleavable cross-linking agent dithiobis-succinimidyl propionate (DSP) before fractionation. After addition of insulin, PKC β II and GLUT4 decreased in the cytosolic fraction and increased in the particulate fraction (Fig. 5a–c). MARCKS increased in both cytosolic and particulate fractions after insulin treatment (Fig. 5d), indicating that insulin mobilised a pool of MARCKS that was previously unassociated with actin. Interestingly, the insulin receptor associated with actin, but only in the particulate fraction after insulin treatment (Fig. 5e). Levels of cytosolic and membrane actin changed only slightly (Fig. 5f). Thus, insulin stimulation resulted in a coordinated shift in the association of actin with PKC β II, GLUT4, MARCKS and the insulin receptor.

Overproduction of MARCKS mutated in the effector domain inhibited 2-deoxyglucose uptake

If MARCKS is necessary for GLUT4 transporter translocation to plasma membranes, it was hypothesised that phosphorylation of MARCKS ED by PKC β II was the putative site of action. Serine 152 and 156 are key phosphorylation sites for PKC β II in MARCKS; mutation of either site to alanine blocks the activation by MARCKS of phospholipase D [27], which has recently been implicated in GLUT4 docking [22]. The MARCKS S152A mutant was used here to investigate the potential involvement of MARCKS phosphorylation. Increasing MARCKS S152A to a twofold greater level than endogenous MARCKS blocked insulin-stimulated glucose transport by 50% (Fig. 6a). This was expected since blocking PKC β II sites would reduce the number of sites for GLUT4 transporters to insert. Overproduction of native MARCKS increased the effect of insulin on 2-deoxyglucose uptake by 40%. This suggested that by increasing available PKC β II phosphorylation sites in overproducing MARCKS cells, more transporters could potentially be inserted into the membrane. Figure 6b verifies the stimulation by insulin of MARCKS phosphorylation in pcDNA3 and wild-type MARCKS-transfected cells. Cells transfected with mutant S152A confirmed the increased amount of MARCKS, but increased phosphorylation was not detected (Fig. 6b [IB: pMARCKS]).

GLUT4 translocation is reduced by inhibiting PKC β II activity

Our previous studies and others indicate that specific inhibition of PKC β II activity blocks ISGT in myotubes [34]. The transporters GLUT4 and GLUT1 are both translocated to cell membranes following insulin stimulation; however, since the insulin regulation of GLUT4 has been more widely studied, we focused on it. Evidence of the involvement of PKC β II activity in GLUT4 trafficking was demonstrated when GLUT4 membrane insertion was examined using insulin and CG53353. A trypsin digestion assay that cleaves GLUT4 protein at specific exofacial portions was used. The result is a 33 kDa tryptic fragment that can be detected by western analysis using an antibody recognising the internal domain of GLUT4 [28]. Insulin treatment increased membrane-fused GLUT4 (33 kDa) and this was markedly inhibited by 1 μ mol/l CG53353, a specific PKC β inhibitor used at a concentration approximately tenfold more selective for PKC β II than for PKC β I (Fig. 7). Another GLUT4 fragment resulting from

incomplete trypsin cleavage of exofacial loops was noted at a slightly higher molecular weight, and it was also blocked by CG53353.

Discussion

Whereas a number of proteins are identified as being associated with the insulin receptor and others as co-localising to the GLUT4 compartment, the linkage between the insulin signalling cascade and the GLUT4 compartment is not well defined. This study addressed the role of PKC β II as a transducer between these systems. As part of the transducer pathway, the roles of PI3K and Akt are established (reviewed in [35]). We found that PKC β II was activated in a PI3K-dependent manner, since Ser660 and Thr641 phosphorylation was blocked by the PI3K inhibitor LY294002. PKC β II is a known substrate of PDK1 [36] and PI3K is a known activator of PDK1 [25]; however, placement of phosphoPKC β II in the insulin signalling cascade had not previously been demonstrated in skeletal muscle cells.

The involvement of various PKCs in insulin signalling is widely reported; however, there appears to be conflicting evidence regarding which isoforms are involved and whether they transduce or modulate insulin action [5]. Wright et al. [37] presented evidence that PKCs may have opposing effects in different muscle fibre types. They suggested that PKC β II may act as a transducer of insulin signalling preferentially in fast-twitch muscle fibres, which coincides with the production of other established insulin-signalling molecules [38].

The observation by Blobe et al. [39] that activated PKC β II (but not PKC β I) associates with F-actin and the finding of others that F-actin is involved in the movement of glucose transporters [35] led us to hypothesise that PKC β II may facilitate GLUT4 translocation into the plasma membrane via MARCKS and F-actin tethering. Here we observed that insulin stimulated the phosphorylation of MARCKS in a PKC β II-dependent manner. MARCKS proteins are substrates for most isoforms of PKC (with the exception of atypical PKCs [40]). There is a possibility that phosphorylation of MARCKS by PKC β II altered the cortical F-actin mesh mediating GLUT4 translocation and allowed GLUT4 movement to the plasma membrane.

GLUT4 translocation in insulin-responsive cells has been compared to that of neurotransmitter vesicles in neuronal dendrites [41]. In neuronal cells, phorbol esters induce vesicle movement [42], a redistribution of the actin cytoskeleton [43] and phosphorylation of MARCKS (for review see [15]). Results of antisense treatment indicated that vesicular trafficking mediated by MARCKS was related to PKC β in neurons [19]. Our studies using siRNA and CG53353 showed inhibition of MARCKS phosphorylation and GLUT4 membrane insertion. Insulin treatment of L6 cells altered the subcellular distribution of PKC β II, GLUT4, insulin receptor and MARCKS with F-actin. Moreover, mutation of a serine PKC phosphorylation site to alanine within the MARCKS ED caused it to act as a dominant negative and attenuate ISGT. This mutation blocks PKC-mediated activation of PLD1 [27]. In the plasma membrane, MARCKS associates with acidic phospholipids (mainly PIP₂), which are also associated with the actin cytoskeleton [35].

One model proposed for MARCKS action in the unstimulated state is that it binds to and masks membrane PIP₂ groups [44]. When phosphorylated by PKC β II, MARCKS is released, allowing PIP₂ to interact with actin scaffold proteins [15] and/or to activate PLD1 [27]. We found that insulin treatment increased membrane PLD1 levels and that CG53353 blocked this increase. Thus, insulin-stimulated MARCKS phosphorylation could provide a crucial link to the observation that PLD1 regulates the fusion of GLUT4 vesicles with plasma membranes [22]. Phosphorylation of MARCKS by PKC β II would allow it to become cytosolic, as we observed here, and promote the interaction of PLD1 with PIP₂, as reported for GLUT4 translocation [22].

The stimulatory effects of A23187 on glucose uptake in adipocytes suggest a role for free Ca^{2+} in glucose uptake [45], while chelation of intracellular Ca^{2+} inhibited the effects of insulin on glucose uptake [46]. In skeletal muscle insulin increases near-membrane but not global Ca^{2+} levels [47]. It is likely that the Ca^{2+} -dependent proteins PKC β II and calmodulin would operate downstream of this event. The inhibition of ISGT by W13 here is similar to that reported in 3T3-L1 cells [46] and consistent with CaCaM being involved in vesicular movement.

Stimulation of L6 myotubes by insulin correlated with changes in the distribution of PKC β II, GLUT4, insulin receptor and MARCKS bound to membrane actin. Moreover, the reduction in the amount of MARCKS by siRNA resulted in decreased ISGT, and overproduction of the MARCKS ED mutant Ser-152-Ala blocked ISGT. This suggests that PKC β II acts on MARCKS to modulate glucose transporter movement since inhibition of PKC β II reduced membrane insertion of GLUT4. This scenario provides a molecular mechanism for PKC β II via MARCKS involvement in insulin action.

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Abbreviations

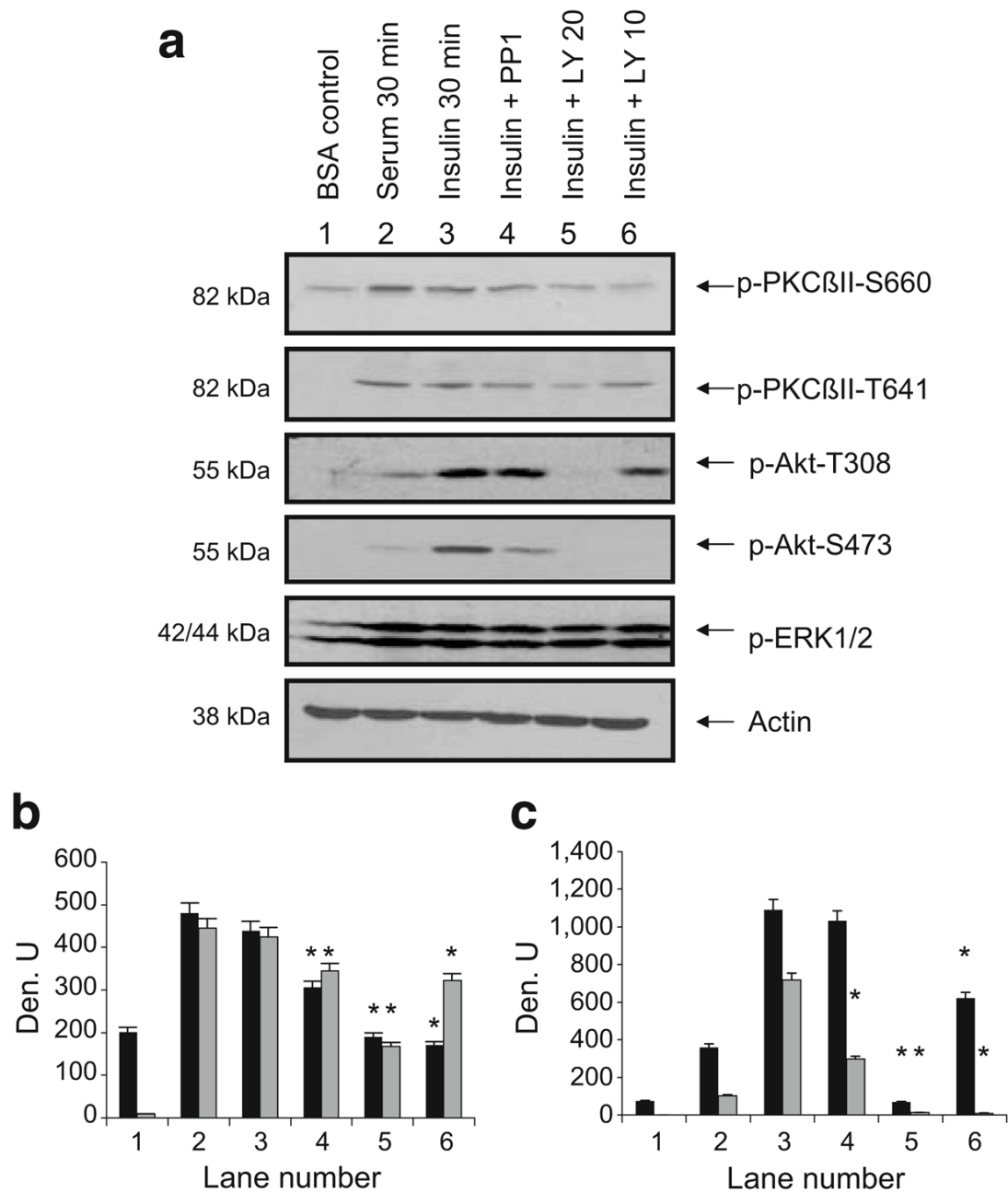
CaCaM	Calcium-bound calmodulin
ED	Effector domain
ISGT	Insulin-stimulated glucose transport
MARCKS	Myristoylated alanine-rich C kinase substrate
MEM	Minimal essential medium
PDK1	Phosphatidylinositol-dependent kinase
PI3K	Phosphatidylinositol-3-kinase
PIP₂	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLD1	Phospholipase D1

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**Fig. 1.**

a Insulin increased phosphoPKCβII. L6 myotubes were serum-starved for 6 h prior to pretreatment with PP1 (10 μmol/l), LY294002 (20 or 10 μmol/l) for 30 min followed by serum (10%) or insulin (10 μIU/ml) for 30 min. Whole-cell lysates were separated by SDS-PAGE (10%) and transferred to membranes for western blot analysis as indicated with anti-phosphoPKCβII, phosphoAkt, phosphoERK1/2 or actin. Blots were scanned and quantitated using UnScanIt gel digitising software (Silk Scientific, Orem, UT, USA). **b, c** Relative densitometric units (Den. U) for phosphoPKCβII S660 and T641 and for phosphoAkt T308 and S473. Black bars show units for pPKCβII-S660 (**b**) and pAkt-T308 (**c**) and grey bars show units for p-PKCβII-T641 (**b**) and pAkt-S473 (**c**). The experiment was repeated three times.

Significant inhibition ($*p < 0.05$) of insulin effects by inhibitors on pPKC β II and pAkt sites, as determined using Prism4 (GraphPad Software, La Jolla, CA, USA) by one-way ANOVA. p, phospho

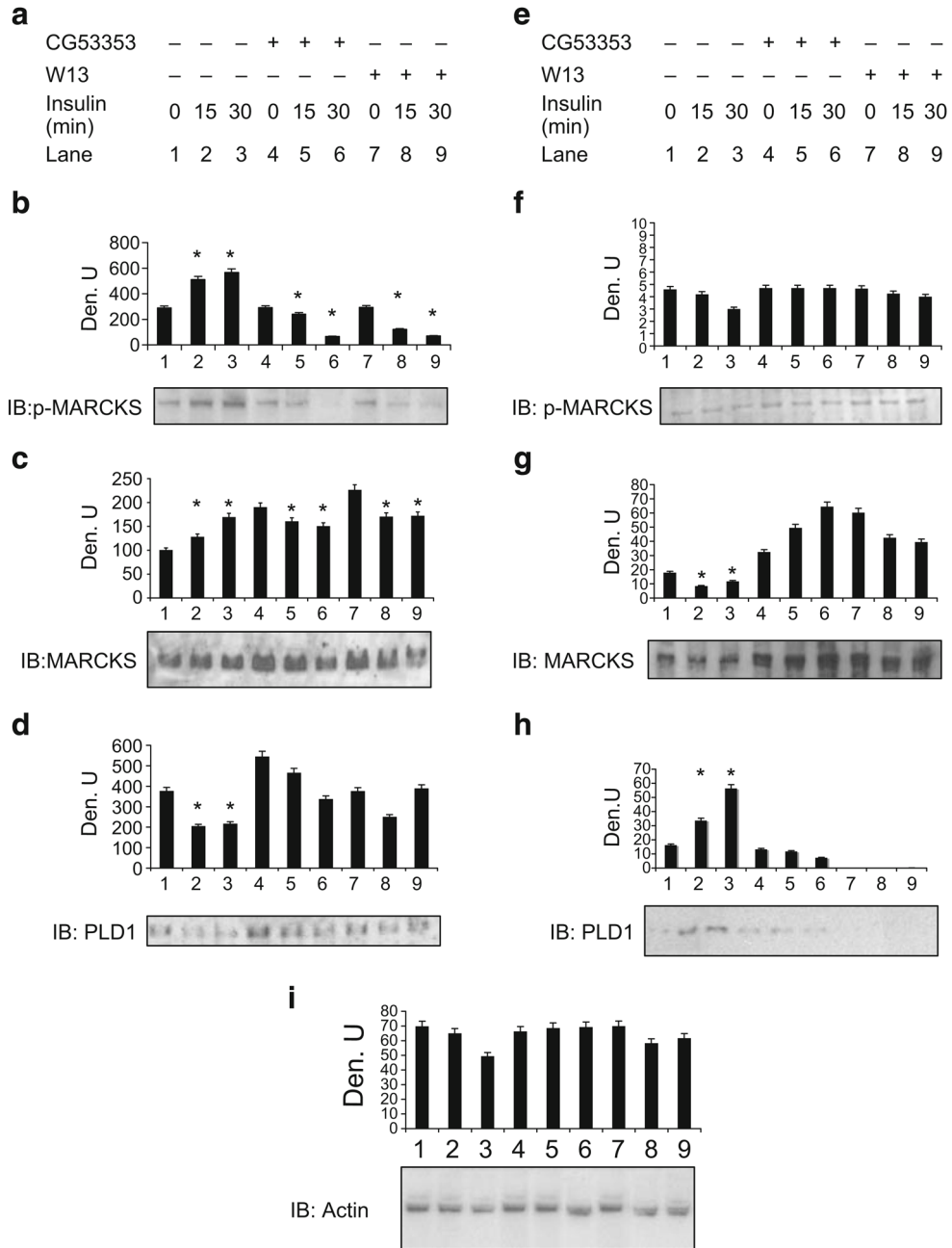
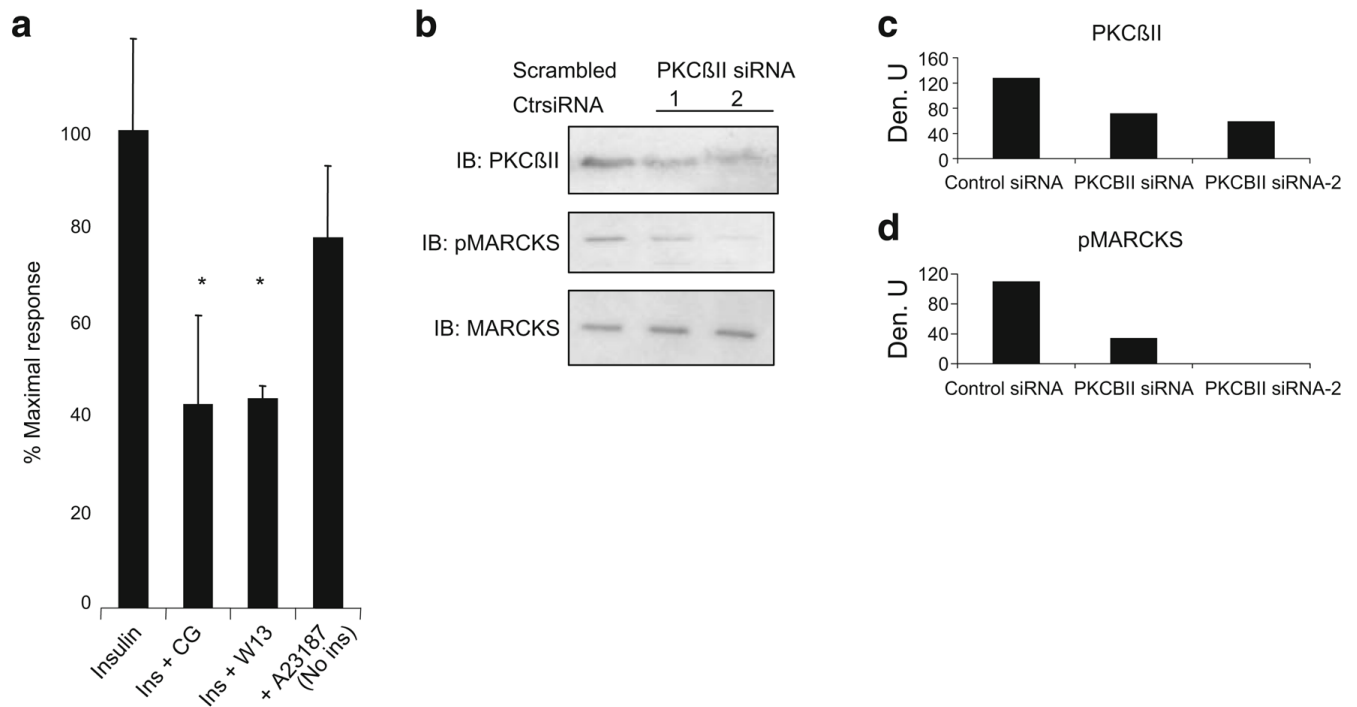


Fig. 2. Cytosolic MARCKS phosphorylation was blocked by PKC β II inhibition. L6 cells were grown as described in Methods. **a–d** Cytosol; **e–i** membrane. Subcellular fractions were separated by SDS-PAGE, transferred to nitrocellulose and probed using phosphoMARCKS (p-MARCKS), MARCKS, PLD1 or actin antibodies as indicated. The experiment is representative of three that showed similar results on immunoblots (IB). Densitometric scans (Den. U), determined as described in Fig. 1, are shown above each blot/panel. * $p < 0.05$, unpaired t test

**Fig. 3.**

Insulin-stimulated glucose uptake was reduced by inhibiting either PKC β II (CG, CG53353) or CaCaM (W13) and mimicked by addition of a calcium ionophore (A23187). **a** L6 myotubes were grown as described and 2-deoxy-D-[1,2- 3 H]glucose uptake was then determined as outlined in Methods. The experiment was performed in duplicate and repeated on two occasions with similar results. Data are expressed as percentage of maximal insulin response. * $p < 0.05$ for inhibition of ISGT in the presence of inhibitors, Student's t test. **b** Cells were transfected with two different PKC β II siRNAs or a scrambled control siRNA as described in Methods. Forty-eight hours later, cells were exposed to insulin (10 μ IU/ml) for 30 min. Cell lysates were analysed by immunoblotting (IB) for PKC β II, pMARCKS and MARCKS as described in the Methods. The siRNA lanes represent two separate experiments with different siRNA preparations. Lanes were scanned as described in the legend of Fig. 1 and results are shown in the graphs (**c**, **d**). Ctr, control; Den. U, densitometric units; Ins, insulin

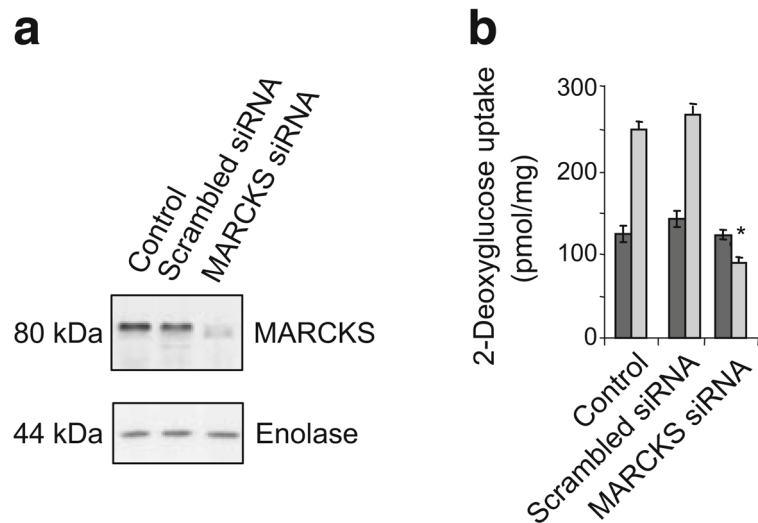


Fig. 4. Insulin-stimulated increase in 2-deoxyglucose uptake in L6 cells was blocked by MARCKS siRNA. **a** L6 cells were grown to 80% confluence and allowed to fuse into myotubes for 4 days. Cells were transfected with siRNA for MARCKS purchased from Ambion as described. Forty-eight hours later, cells were treated with insulin for 30 min. Half of the cells were lysed to perform western blot analysis (**a**), in which enolase was used to access loading of the gel and half were assayed for 2-deoxy-D-[1,2-³H]glucose uptake (**b**). The uptake assay was performed in triplicate on two occasions with similar results. Dark grey bars show basal uptake and light grey bars show insulin-stimulated uptake. Results are mean±SEM. * $p < 0.05$ compared with scrambled siRNA for insulin response, Student's *t* test

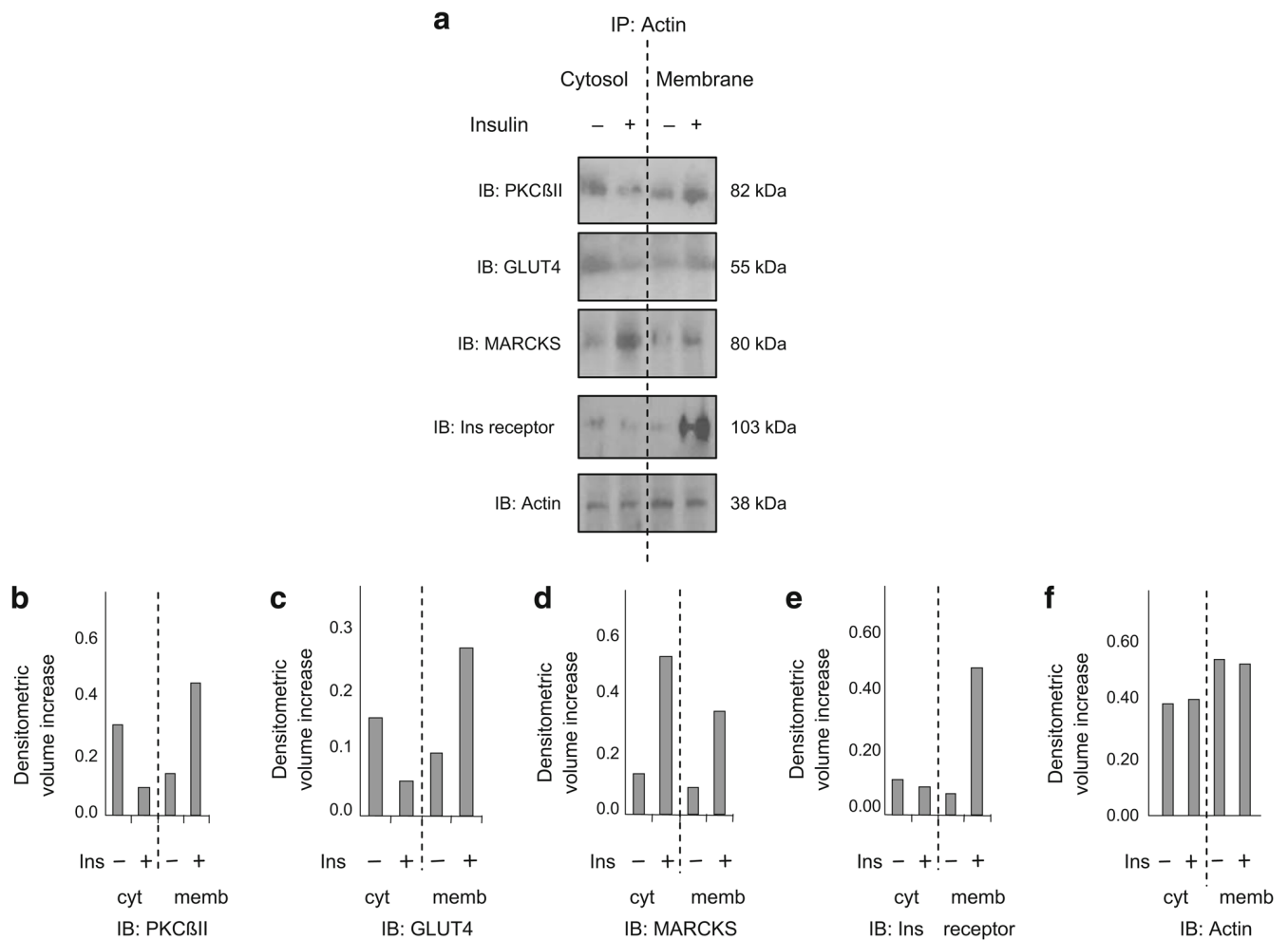


Fig. 5. PKC β II, GLUT4 and MARCKS bound actin in an insulin-dependent manner. L6 myotubes were treated for 30 min with and without 10 μ U/ml insulin, treated with cross-linking agent and then immunoprecipitated (IP) with anti-actin antibody. The bound proteins were then extracted by magnetic separation and treated with dithiothreitol to break cross-links. **a** Proteins were separated by SDS-PAGE and subjected to immunoblotting (IB) for actin, PKC β II, GLUT4, MARCKS and the insulin receptor. **b–f** Densitometric analysis (as described in the legend of Fig. 1) for the relevant proteins in (a) are shown beneath the blots. The experiments were repeated on two occasions in separate experiments with similar results. Ins, insulin; cyt, cytosol fraction; memb, membrane fraction

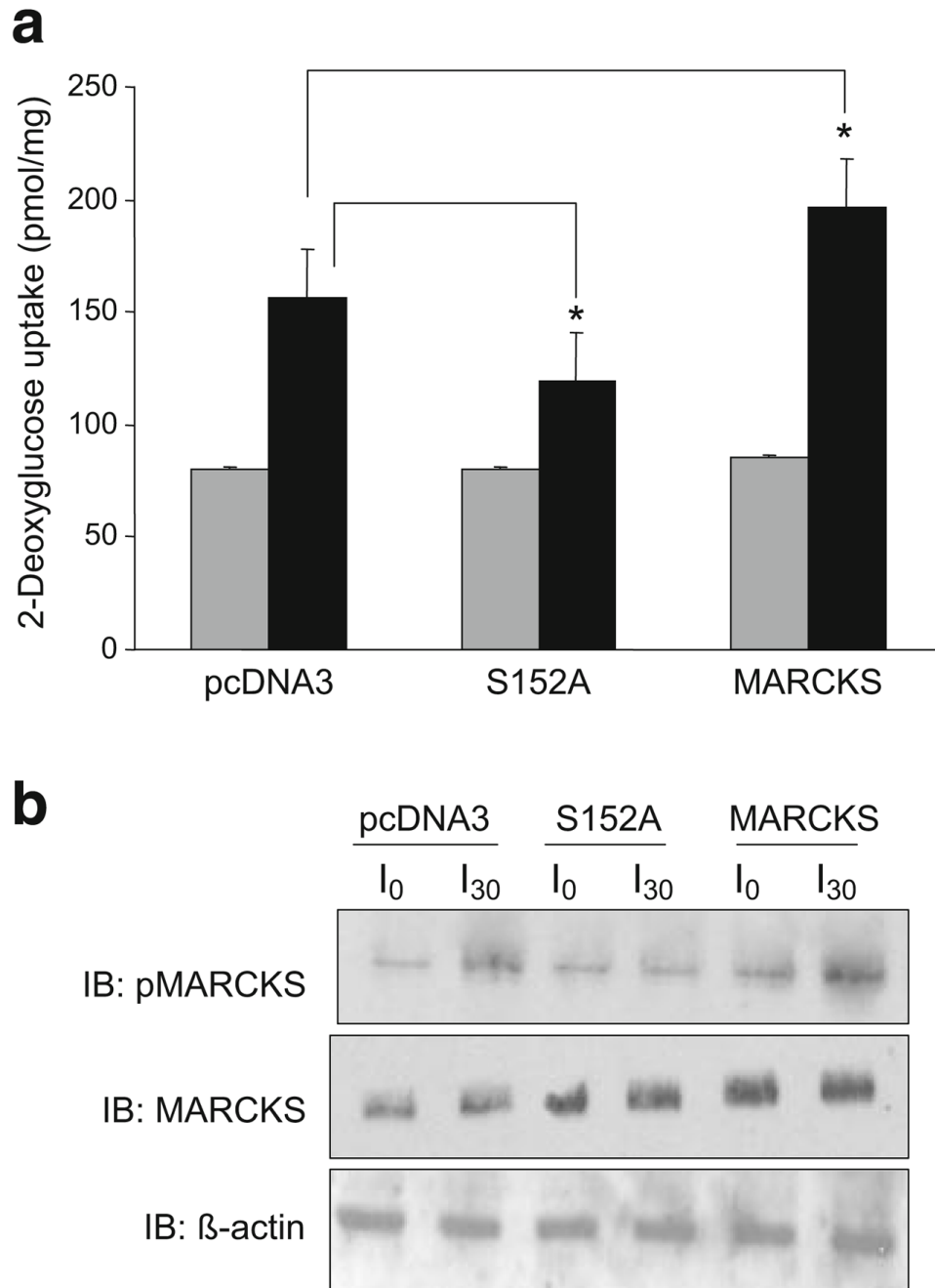
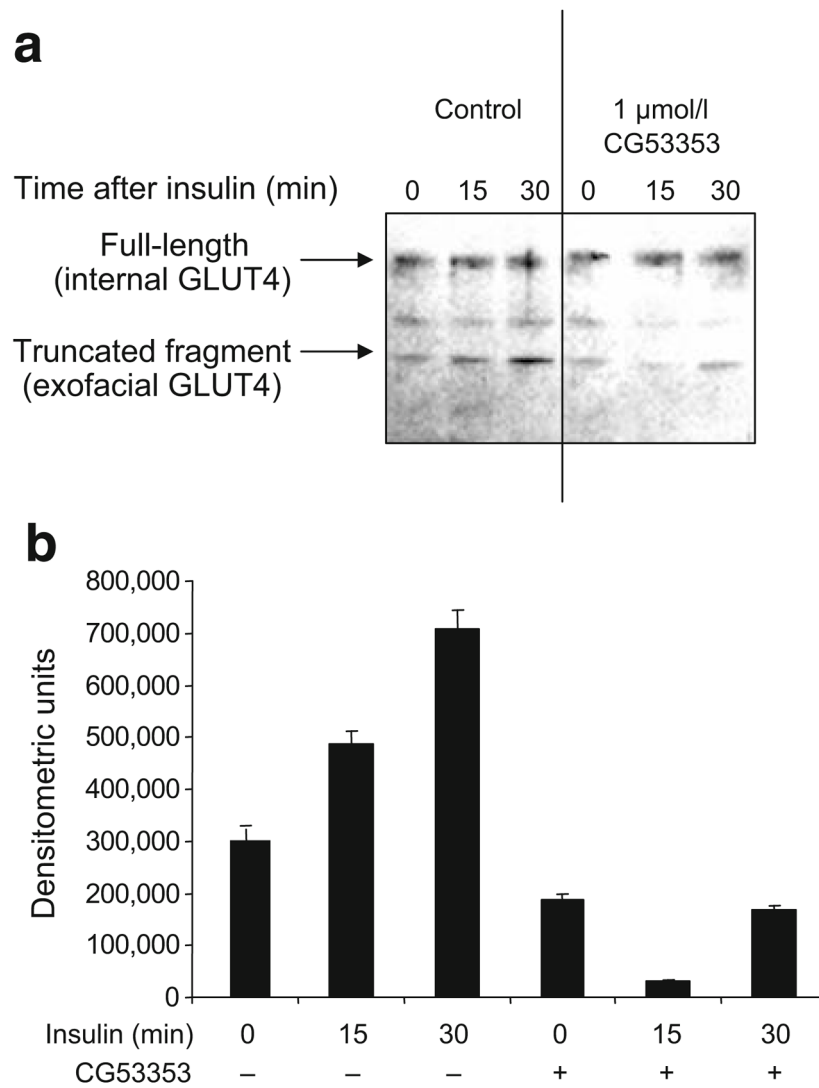


Fig. 6. Transient transfection of MARCKS serine-152-alanine mutant blocked insulin-stimulated 2-deoxyglucose uptake. L6 myotubes at 80% confluence were transiently transfected with pcDNA3, S152A MARCKS or wild-type MARCKS as described in the Methods for 48 h. 2-Deoxyglucose uptake was measured after 10 μ IU/ml insulin treatment for 30 min. Duplicate wells were harvested for western blot analysis of phosphoMARCKS, MARCKS and actin (**b**). Transfection was performed in three separate experiments run in duplicate for 2-deoxyglucose uptake. Light grey shaded bars show basal uptake and dark grey bars show insulin-stimulated uptake. Results are mean \pm SEM. * p < 0.05 compared with pcDNA3 for insulin response, Student's t test

**Fig. 7.**

a GLUT4 in the plasma membrane was reduced by inhibiting PKC β II activity. L6 cells were grown as described in the Methods. Prior to insulin treatment (10 $\mu\text{U/ml}$ for 30 min), cells were treated for 30 min with or without 1 $\mu\text{mol/l}$ CG53353 or DMSO. Cells were then treated with KCN to halt metabolic activity and trypsin to cleave exofacial proteins. GLUT4 transporters inserted into the plasma membrane would be susceptible to proteolysis of the exofacial loops by trypsin. Cells were then lysed, membranes isolated and proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed using anti-GLUT4 antibodies recognising the intracellular region of the protein. This detects the full-length (near membrane) and the cleaved (inserted in plasma membrane) protein. The experiment was performed on three occasions with similar results. **b** The change in the lower molecular mass, fully cleaved fragment determined by scanning as described in the legend of Fig. 1