Inhibition of insulin-stimulated phosphorylation of the intracellular domain of phospholemman decreases insulin-dependent GLUT4 translocation in streptolysin-O-permeabilized adipocytes

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A variety of studies indicate that protein kinase C might be involved in the insulin signalling cascade leading to translocation of the insulin-regulated glucose transporter GLUT4 from intracellular pools to the plasma membrane. Phospholemman is a plasma-membrane protein kinase C substrate whose phosphorylation is increased by insulin in intact muscle [Walaas, Czernik, Olstad, Sletten and Walaas (1994) *Biochem. J.* **304**, 635–640]. The present study examined whether the inhibition of phospholemman phosphorylation modulates the effects of insulin on GLUT4 translocation. For this purpose, a synthetic peptide derived from the intracellular domain of phospholemman with the phosphorylatable serine residues replaced with alanine residues was prepared. This peptide was found to decrease the protein kinase C-catalysed phosphorylation of a synthetic phospholemman peptide *in vitro*. When introduced into

streptolysin-O-permeabilized adipocytes, the peptide decreased the effects of insulin on both the phosphorylation of phospholemman and the recruitment of GLUT4 to the plasma membrane. Similarly, the internalization of phospholemman antibodies, which also decreased the protein kinase C-mediated phosphorylation of the synthetic phospholemman peptide *in vitro*, decreased the effect of insulin on GLUT4 translocation in the adipocytes. The results suggest that phosphorylation of the intracellular domain of phospholemman might be involved in modulating the insulin-induced translocation of GLUT4 to the plasma membrane.

Key words: exocytosis, fat cells, glucose transport, protein kinase C.

INTRODUCTION

Translocation of the insulin-regulated glucose transporter GLUT4 from an intracellular pool to the plasma membrane is an essential step in the stimulation of glucose transport by insulin [1]. The mechanisms involved in this regulation remain incompletely understood. The involvement of phosphoinositide 3-kinase is well documented [1–4] but other signalling systems might also be involved [5]. Evidence suggesting an involvement of protein kinase C (PKC) includes the ability of insulin to induce the translocation of both conventional and atypical PKC isoforms to the plasma membrane [6–8]. Moreover, the insulin-induced stimulation of GLUT4 translocation can be partly mimicked by activators of PKC [9–12] and prevented by non-specific protein kinase inhibitors [13,14]. Using adipocytes permeabilized with streptolysin-O (SLO), we have recently demonstrated [15] that insulin-induced increases in PKC activity [16] and GLUT4 translocation are both blocked by internalization of the PKC(19–36) pseudosubstrate peptide, a highly specific inhibitor of PKC. Moreover, an atypical PKC isoform is required for the stimulation of glucose uptake by insulin in 3T3-L1 adipocytes [17]. These observations indicate that the PKC-dependent phosphorylation of plasma membrane substrate(s) might be involved in insulin-dependent GLUT4 vesicle translocation. The present study examines whether the

inhibition of the insulin-stimulated phosphorylation of phospholemman (PLM), a plasma membrane PKC substrate, leads to the modulation of insulin-dependent GLUT4 translocation.

PLM, originally observed in skeletal muscle sarcolemma as a substrate for an endogenous insulin- and GTP-sensitive protein kinase [18], is a 72-residue transmembrane plasmalemma protein with an intracellular, positively charged C-terminal phosphorylation domain (G⁵⁸TFRSSIRRLSTRRR⁷²) [19]. Studies *in vitro* indicate that PKC phosphorylates both Ser-63 and Ser-68, and cAMP-dependent protein kinase (PKA) phosphorylates Ser-68, whereas studies on intact muscle indicate that insulin or active phorbol esters increase the ³²P labelling of both Ser-63 and Ser-68 *in situ* [20,21]. The function of PLM, which seems to be enriched in striated muscle [19] and the gene for which is localized to human chromosome 19q13.1 [22], is not well defined. Because the expression of PLM in *Xenopus* oocytes induces anion-permeable conductances [23], and the reconstitution of PLM in lipid bilayers forms taurine-selective channels [24], the transmembrane PLM domain seems to behave like a channel. Here we demonstrate that the internalization into permeabilized adipocytes of either a synthetic peptide encompassing the intracellular PLM(58–72) phosphorylation domain but with alanine residues replacing Ser-63 and Ser-68, or of antibodies directed towards this PLM domain, decreases the insulin effects on both PLM phosphorylation and GLUT4 translocation in the

Abbreviations used: ICM, intracellular membrane fraction; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PLM, phospholemman; PM, plasma membrane fraction; SLO, streptolysin-O; SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor.

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cells. Our results indicate that phosphorylation of the intracellular domain of PLM might modulate insulin-induced trafficking of GLUT4 vesicles.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\text{P}^{32}]\text{P}_i$ were from Amersham (Little Chalfont, Bucks., U.K.); ^{125}I -Protein A was from ICN Pharmaceuticals (Irvine, CA, U.S.A.). PMA and SLO were from Research Biochemicals International (Natick, MA, U.S.A.). Collagenase and ATP were from Sigma Chemicals (St. Louis, MO, U.S.A.). PVDF membranes were from Bio-Rad (Richmond, CA, U.S.A.). Highly purified insulin was a gift from Novo Nordisk (Copenhagen, Denmark). Male Wistar rats (150–200 g body weight) were from Møllegaard (Copenhagen, Denmark). A synthetic peptide [PLM(58–72), GTFRSSIRRLSTRRR in single-letter code], containing the 15 C-terminal amino acid residues of PLM, and a synthetic alanine analogue of the PLM peptide [Ala-PLM(58–72), GTFRSA⁶³IRRLA⁶⁸TRRR] were prepared at the Peptide Synthesis Facility, Forskningsparken, University of Oslo, Norway. Rabbit anti-[glucose transporter (insulin-regulated, GLUT4)] was from East Acres Biologicals (Southbridge, MA, U.S.A.). PKC β_{11} (recombinant) was obtained from PanVera Corp. (Madison, WI, U.S.A.); the catalytic subunit of PKA was a gift from Dr. S. O. Døskeland (University of Bergen, Bergen, Norway).

Antibody preparation

Polyclonal antibody against whole PLM was raised by isolating rat muscle PLM [20], immunizing rabbits as described [25] and purifying the antiserum by the method of Olmsted [26]. Antiserum against the PLM(58–72) peptide was prepared (MedProbe AS, Oslo, Norway) by conjugating the synthetic peptide to keyhole limpet haemocyanin and immunizing rabbits with it. Both

antibodies recognized essentially only one band of approx. molecular mass 15 kDa when tested by immunoblotting on a total membrane preparation (prepared by centrifugation at 100 000 *g*; see below) from rat adipocytes (Figure 1) and skeletal muscle (results not shown). Immunoblotting was performed by separating proteins by SDS/PAGE, transferring the sample to PVDF membranes [27] and incubating the membranes with primary antibodies followed by ^{125}I -Protein A as described (see [15]).

Cell preparation

Adipocytes were isolated from epididymal fat pads from 8–12 rats by the method of Rodbell [28]. After dissection, the fat pads were minced and incubated in 'extracellular' buffer [129 mM NaCl/5 mM KCl/1.2 mM MgCl_2 /1.3 mM CaCl_2 /1.2 mM KH_2PO_4 /20 mM Hepes (pH 7.4)] containing 2 mM sodium pyruvate and 2% (w/v) BSA. Collagenase was added and the incubation was continued for 90 min at 37 °C. The cells were filtered through a nylon screen and washed four times by flotation and resuspension in extracellular buffer. In permeabilization experiments, the cells were subsequently washed once and then resuspended in 'intracellular' buffer [20 mM Hepes (pH 7.4)/120 mM KCl/4 mM NaCl/10 mM MgCl_2 /1.2 mM KH_2PO_4 /1.3 mM CaCl_2 /1 mM EGTA/10 $\mu\text{g}/\text{ml}$ leupeptin/2 mM sodium pyruvate/2% (w/v) BSA].

Determination of protein kinase activity

Determination of PKC activity *in vitro* with the PLM(58–72) peptide as substrate was done as reported previously [21]. In brief, 250 μM PLM(58–72) peptide was incubated at 30 °C in 50 mM Hepes, pH 7.4, with 1.25 mM CaCl_2 , 1 mM EGTA, 10 mM MgCl_2 , 200 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 50 $\mu\text{g}/\text{ml}$ phosphatidylserine and 2.5 nM PKC β_{11} . PKA activity was determined as described [21]. Reactions were terminated after 5 min by the addition of 7% (w/v, final concn.) trichloroacetic acid; aliquots were spotted on P81 phosphocellulose paper squares (Whatman). After washing, radioactivity was determined by Cerenkov counting.

Determination of PKC activity *in situ* in SLO-permeabilized adipocytes was done essentially as described [15,29]. Washed cell suspension in 'intracellular' buffer was incubated with SLO (2 i.u./ml) at 37 °C in plastic vials containing (final concns.) 25 mM Hepes, pH 7.4, 600 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 10 mM MgCl_2 , with 250 μM PLM(58–72) as substrate, with or without 5–40 μM Ala-PLM(58–72) and in the absence or presence of 100 nM insulin. Reactions were terminated after 5 min as described above. Samples were centrifuged at 100 000 *g* for 8 min, aliquots of the supernatants were spotted on P81 phosphocellulose paper and radioactivity was determined as described.

Analysis of PLM phosphorylation in adipocytes

For the determination of PLM phosphorylation *in vitro* in adipocyte membranes, untreated adipocytes were homogenized in 250 mM sucrose/25 mM NaF/1 mM EDTA/10 $\mu\text{g}/\text{ml}$ leupeptin/25 mM Hepes (pH 7.4). Membranes were prepared by centrifugation at 100 000 *g* for 5 h, then suspended in 50 mM Hepes (pH 7.4)/1 mM EDTA/2.5 mM EGTA/10 $\mu\text{g}/\text{ml}$ leupeptin/0.5% CHAPS and phosphorylated as described [20]. After SDS/PAGE, the ^{32}P -labelled PLM band was identified by autoradiography.

For the determination of PLM phosphorylation *in situ* in permeabilized adipocytes, cells were incubated with SLO and 600 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, in the absence or presence of 100 nM insulin and with or without 40 μM Ala-PLM(58–72), as described above.

Figure 1 Immunoblot of PLM in rat adipocyte membranes

A 100 000 *g* membrane pellet from rat adipocytes, together with prestained molecular mass markers (Rainbow; Amersham) included in the outer lanes, was subjected to SDS/PAGE on gels containing 15% (w/v) polyacrylamide; proteins were electrophoretically transferred to PVDF membranes. After blocking, the membrane was incubated with preimmune rabbit IgG (lane A) or anti-peptide PLM(58–72) antibody (lane B) (both at 1:100 dilution) for 1 h, followed by washing. The immune complex was detected by incubation with ^{125}I -protein A and autoradiography. The 15 kDa immunoreactive PLM band is indicated.

Reactions were terminated by the addition of 250 mM sucrose/25 mM NaF/10 μ g/ml leupeptin/25 mM Hepes (pH 7.4) containing 0.1 M EDTA; membranes were prepared by centrifugation at 100 000 *g* for 5 h. Membrane phosphoproteins were solubilized in SDS sample buffer, identified by SDS/PAGE and autoradiography, and quantified by Cerenkov counting of the excised gel bands.

For the determination of PLM phosphorylation *in situ* in intact adipocytes, the cells were preincubated with [32 P]P_i (0.5 mCi/ml) for 2 h. Cell aliquots were then incubated with insulin (final concn. 100 nM) or PMA, a specific activator of PKC (final concn. 500 nM) for 10 min. After homogenization and centrifugation as described above, the membrane fractions were subjected to SDS/PAGE and the 32 P-labelled PLM band was detected by autoradiography. Thermolytic digestion of PLM and the two-dimensional separation of phosphopeptides on silica-containing thin-layer plates were done as previously described [20].

Immunoprecipitation of PLM

Immunoprecipitation of PLM from 32 P-labelled fat cells was done by solubilizing the 100 000 *g* membrane pellet in 1% (w/v) SDS, followed by the addition of a 5-fold excess of Triton X-100. After the addition of anti-peptide antiserum (final dilution 1:100) or purified anti-PLM antibody (0.46 μ g) in the presence of 1% (w/v) BSA, the antigen-antibody complex was isolated as described [30], dissolved in SDS sample buffer and subjected to SDS/PAGE. 32 P-labelled PLM was demonstrated by autoradiography.

Translocation of GLUT4

Washed cell suspensions were suspended in 'intracellular' buffer containing SLO (2 i.u./ml), 200 nM adenosine and 5 mM ATP. Incubation was done at 37 °C for 10 min in plastic vials in the absence or presence of 100 nM insulin, and with the addition of various concentrations of the Ala-PLM(58–72) peptide or PLM antibodies as reported in the text. Control samples were incubated in preimmune serum or rabbit IgG (Sigma), none of which had any effect on the distribution of GLUT4 in the absence or presence of insulin (results not shown). The cells were floated and washed in homogenization buffer [250 mM sucrose/10 mM NaF/10 mM sodium pyrophosphate/10 μ g/ml leupeptin/25 mM Tris/HCl buffer (pH 7.4)]. The plasma membrane (PM) and total intracellular membrane (ICM) fractions were prepared as reported previously [15] by differential centrifugation by a modification of the method of Karnieli et al. [31]. PM was isolated by centrifugation at 10600 *g* for 20 min; ICM was isolated by centrifugation of the supernatant at 100 000 *g* for 5 h. The pelleted fractions were suspended in SDS sample buffer, boiled for 2 min and subjected to SDS/PAGE. Quantitative immunoblotting analysis of GLUT4 was done as described [15].

RESULTS

Effects of Ala-PLM(58–72) peptide on PLM phosphorylation *in vitro*

When the phosphorylation of PLM(58–72) catalysed by PKC β_{II} was examined *in vitro* [21], addition of the Ala-PLM(58–72) peptide in the low micromolar range gave a marked but partial inhibition of enzyme activity (Figure 2). In contrast, Ala-PLM(58–72) had no inhibitory effect on the PKA-catalysed phosphorylation of PLM(58–72). A kinetic analysis with double-reciprocal plots indicated a non-competitive type of PKC in-

Figure 2 Effects of Ala-PLM(58–72) on PKC activity *in vitro*

(A) Dose-dependent effects of Ala-PLM(58–72), added in the concentrations indicated, on PKC and PKA activity *in vitro*. Enzymes were assayed with the synthetic PLM(58–72) peptide (final concn. 250 μ M) as substrate. Results are means $\pm$ S.E.M. for four to six experiments and are plotted as percentages of enzyme activity observed in the absence of Ala-PLM(58–72). Symbols: $\bullet$, PKC; $\triangle$, PKA. The significance of Ala-PLM-peptide effects was examined by ANOVA and *t* test. **P* < 0.05; ***P* < 0.01. (B) Time course of PKC activity without ($\circ$) or with ($\bullet$) the addition of 40 μ M Ala-PLM(58–72), examined *in vitro* with the synthetic PLM(58–72) peptide (final concn. 250 μ M) as substrate as described in the Materials and methods section. Results are means $\pm$ S.E.M. for three experiments and show the incorporation of 32 P into substrate under standard conditions. The significance of Ala-PLM-peptide effects was determined by ANOVA and *t* test. **P* < 0.05.

hibition (results not shown). These results suggest that PKC is subject to a mixed type of inhibition by Ala-PLM(58–72), similar to the effects found with other Ala-substituted PKC substrate peptides, which might be caused by multiple binding sites with distinct affinities [32].

Phosphorylation of PLM in adipocytes

Incubation of intact adipocytes in the presence of [32 P]P_i, followed by the isolation of the membrane fraction and analysis by SDS/PAGE and autoradiography, showed a broad 32 P-labelled band with an molecular mass of approx. 15 kDa (Figure 3A). This electrophoretic mobility, which is similar to that of PLM from skeletal muscle [19,20], was shared by a 32 P-labelled 15 kDa band present in a 100 000 *g* adipocyte membrane fraction that had been phosphorylated *in vitro* in the presence of [γ - 32 P]ATP and purified PKC or PKA (results not shown). Moreover, the phosphorylation of this band seemed to be increased when the cells had been incubated in the presence of 500 nM PMA (Figure 3A). The 15 kDa 32 P-labelled protein was also recognized

Figure 3 Effects of PMA and insulin on PLM phosphorylation in intact adipocytes

Adipocytes were prelabelled with 0.5 mCi/ml [32 P]P_i for 2 h followed by incubation for 10 min in the absence (lanes 1 and 3) or presence of either 500 nM PMA (lane 2) or 100 nM insulin (lane 4). After isolation of the membranes by 100 000 *g* centrifugation, the membrane pellets from samples shown in (A) (lanes 1 and 2) were subjected to protein separation by SDS/PAGE. The membrane pellets from samples shown in (B) (lanes 3 and 4) were solubilized in 1% (w/v) SDS; 32 P-labelled PLM was immunoprecipitated with polyclonal antibodies raised against the PLM(58–72) peptide as described in the Materials and methods section. The 32 P-labelled 15 kDa PLM bands (arrowed) were detected by autoradiography. Results are taken from one representative experiment, which was repeated twice with similar results.

Figure 4 Effect of PMA on 32 P-labelling of Ser-63 and Ser-68 in adipocyte PLM

(A) Peptide map of PLM(58–72) phosphorylated by PKC *in vitro* (see Figure 2). (B, C) Peptide maps of the 15 kDa bands phosphorylated in intact adipocytes in the absence (B) or presence (C) of 500 nM PMA. The 32 P-labelled 15 kDa bands (see Figure 3) were excised from the gel and subjected to phosphopeptide mapping with thermolysin as described in the text. The spots labelled a and b are peptides comprising phosphoserine-63 and phosphoserine-68 respectively, the phosphorylation sites in PLM.

by antibodies raised against the C-terminal PLM(58–72) peptide (Figure 3B) and by polyclonal antibodies raised against skeletal muscle PLM (results not shown); the incorporation of 32 P into this immunoreactive band was markedly increased in adipocytes that had been incubated with 100 nM insulin (Figure 3B).

The phosphorylation sites in the 15 kDa protein labelled by PKC in intact adipocytes were characterized further. Extensive two-dimensional thermolytic peptide mapping of the 32 P-labelled 15 kDa bands isolated from adipocytes incubated in the absence or presence of 500 nM PMA showed two major 32 P-labelled peptides, designated a and b (Figures 4B and 4C). These peptides co-migrated with thermolytic peptides derived from the PLM(58–72) peptide phosphorylated with PKC *in vitro* (Figure 4A), and also with thermolytic peptides derived from skeletal muscle PLM phosphorylated in the presence of insulin or PMA (results not shown). The latter have been identified as PLM residues Ser-63 and Ser-68 respectively [21]. A third, apparently unrelated, 32 P-labelled thermolytic peptide (designated c), although present in control samples, was absent from the PLM(58–72) peptide and was not investigated further.

Taken together, these results indicate that PLM is expressed in fat cells, where it is subject to the same enzymic and hormonal regulation of phosphorylation as in skeletal muscle [20,21].

Figure 5 Effect of Ala-PLM(58–72) on insulin-stimulated PLM phosphorylation in SLO-permeabilized adipocytes

(A) SLO-permeabilized adipocytes were phosphorylated with exogenous PLM(58–72) internalized as substrate in the absence or presence of insulin (final concn. 100 nM) and Ala-PLM(58–72) (final concn. 20 μ M) as indicated; the reactions were terminated after 5 min as described in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 3$). (B) Concentration-dependent inhibition of the insulin-stimulated PLM(58–72) peptide phosphorylation by Ala-PLM(58–72) in SLO-permeabilized adipocytes. Results are means $\pm$ S.E.M. ($n = 3$). The significance of differences from control was determined by ANOVA and *t* test. *** $P < 0.01$.

Effects of Ala-PLM(58–72) peptide on insulin-stimulated PLM phosphorylation in SLO-permeabilized adipocytes

The efficacy of insulin-induced protein phosphorylation in SLO-permeabilized adipocytes was first examined with exogenous PLM(58–72) added as substrate [15]. This phosphorylation of PLM(58–72), which seems to be catalysed by PKC [15], reached 218% of control activity in the presence of 100 nM insulin (Figure 5A). This insulin effect was inhibited in a dose-dependent manner by the addition of Ala-PLM(58–72) to the permeabilization medium, with an apparent IC_{50} of 6–8 μ M and a maximal effect of 75% inhibition at 20 μ M peptide (Figure 5B).

In some experiments (results not shown), we examined the phosphorylation of native PLM *in situ*; it was also found to be sensitive to internalization of Ala-PLM(58–72). In these experiments, SLO-permeabilized adipocytes were incubated with 300 μ M [γ - 32 P]ATP for 5 min and PLM phosphorylation was analysed by SDS/PAGE of membrane proteins as described

Figure 6 Effect of Ala-PLM(58–72) on insulin-dependent translocation of GLUT4 in SLO-permeabilized adipocytes

(A) Inhibition of insulin-induced increase in GLUT4 in PM and decrease in the ICM fraction by Ala-PLM(58–72) (final concn. 20 μM). Adipocytes were incubated for 10 min with SLO in the absence or presence of insulin (Ins) (100 nM final concn.) and Ala-PLM(58–72) (final concn. 20 μM). The GLUT4 transporters in the PM and ICM were isolated and identified by immunoblotting and quantified as described in the Materials and methods section. Results are presented as relative values (control = 1), and are means ± S.E.M. ($n = 4$ to 6). The significance of insulin- and Ala-PLM-peptide-induced effects on GLUT4 translocation, compared with control values, was examined by ANOVA and t test. * $P < 0.05$; ** $P < 0.01$. (B) Concentration-dependent inhibition of insulin-dependent translocation of GLUT4 to PM by Ala-PLM(58–72). Adipocytes were incubated as described above in the absence or presence of insulin (100 nM final concn.) and Ala-PLM(58–72). Results are means ± S.E.M. for four to six experiments and are plotted as percentages of insulin-induced GLUT4 translocation observed in the absence of Ala-PLM(58–72). The significance of the Ala-PLM-peptide effects on insulin-induced GLUT4 translocation was examined by ANOVA and t test. * $P < 0.05$; ** $P < 0.01$.

above. A considerable increase in 32 P-labelling of the 15 kDa PLM band was found in the insulin-treated samples; this effect was markedly decreased when 20 μM Ala-PLM(58–72) was present during incubation (results not shown).

Effects of Ala-PLM(58–72) peptide on insulin-induced GLUT4 translocation in SLO-permeabilized adipocytes

To examine the effect on GLUT4 trafficking, the relative concentrations of GLUT4 in fractions enriched in PM and ICM from the SLO-permeabilized adipocytes were quantified by immunoblotting. We observed recently [15] that incubation of SLO-

Figure 7 Effects of PLM antibodies on PKC activity and insulin-dependent translocation of GLUT4

(A) Antibody-induced inhibition of PLM(58–72) phosphorylation *in vitro*. PLM(58–72) was phosphorylated with PKC and PKA as described in the legend to Figure 2, in the presence of preimmune serum (–) or anti-PLM antiserum (+) (both at a final dilution of 1:100). Results are means ± S.E.M. ($n = 3$). (B) Inhibition of insulin-dependent translocation of GLUT4 to PM in SLO-permeabilized adipocytes by anti-PLM antibodies. Adipocytes were incubated for 10 min with SLO in the absence or presence of 100 nM insulin (100 nM final concn.) and preimmune serum or anti-PLM(58–72) (AB-PLM, final dilution 1:100) as indicated. The GLUT4 transporter in the plasma membrane fraction was isolated, identified by immunoblotting (autoradiography of a representative experiment is shown; GLUT4 is indicated by arrows) and quantified as described in the Materials and methods section. Results are presented as relative values (control = 1) and are means ± S.E.M. ($n = 3$). The effects of anti-PLM antibody on PKC activity and GLUT4 translocation were both significant ($P < 0.01$, t test).

permeabilized adipocytes with insulin gives an approx. 4-fold increase in immunoreactive GLUT4 levels in PM, with a simultaneous decrease of GLUT4 in the ICM fraction (Figure 6A). These observations are similar to previous results obtained in intact adipocytes and SLO-permeabilized 3T3-L1 cells [33]. In this study, the addition of Ala-PLM(58–72) to the permeabilization medium inhibited the insulin effect in a dose-dependent manner, with an apparent IC_{50} of 6–8 μM and a maximal effect of 70% inhibition at 20 μM peptide (Figure 6B).

Effects of PLM antibodies on insulin-induced GLUT4 translocation in SLO-permeabilized adipocytes

Both anti-[PLM(58–72)] antiserum and antibodies raised against the muscle PLM protein inhibited the phosphorylation *in vitro* of the (58–72)PLM peptide catalysed by PKC, but not that by PKA (Figure 7A, and results not shown). Internalization of these antibodies into SLO-treated adipocytes gave an approx. 65% inhibition of insulin-dependent GLUT4 translocation to PM in comparison with incubation with preimmune serum or rabbit IgG (Figure 7B). The preimmune sera or IgG preparations had no effect by themselves (results not shown). The antibodies thus

mimicked the inhibition obtained by the Ala-PLM(58–72) peptide under similar experimental conditions.

DISCUSSION

PLM is one of the major insulin-regulated PKC substrates in skeletal muscle [20]. Here we demonstrate that this protein is also present in fat cells and that it might be involved in the modulation of insulin-induced GLUT4 translocation. The experimental approach that we used to examine this question employed the introduction of distinct peptide and antibody inhibitors of PLM phosphorylation into permeabilized adipocytes, followed by an analysis of insulin responses in these cells.

We have previously shown that the C-terminal PLM(58–72) peptide is a good substrate for PKC, with insulin-induced phosphorylation in skeletal muscle *in situ* occurring at Ser-63 and Ser-68 [21]. The present results show that a synthetic PLM(58–72) peptide, where the two phosphorylation sites were replaced by alanine residues, can serve as an inhibitor of PLM phosphorylation. Employing the cognate PLM(58–72) peptide as substrate, this Ala-PLM(58–72) peptide behaved as a non-competitive inhibitor of PKC activity *in vitro*, inducing an incomplete inhibition with an apparent IC_{50} of 6–8 μ M. When this Ala-PLM(58–72) peptide was introduced into SLO-permeabilized adipocytes, the insulin-induced increases of phosphorylation of both internalized exogenous PLM(58–72) and of the native PLM protein present in the SLO-permeabilized adipocytes were decreased. Moreover, our results indicate that this peptide also antagonizes effects of insulin on GLUT4 translocation in the SLO-permeabilized adipocytes, again with an apparent IC_{50} comparable to that found when PKC was assayed *in vitro*.

To complement the studies with the PLM peptide analogue, which presumably interferes with PLM functions, PLM antibodies were generated and tested as insulin antagonists. Polyclonal antibodies raised either against the PLM(58–72) peptide or the whole PLM protein each behaved as monospecific reagents, recognizing only the PLM band on immunoblots of adipocyte membrane proteins. Employing these antibodies during phosphorylation assays *in vitro* showed that they specifically decreased the phosphorylation of PLM(58–72) catalysed by PKC but not that by PKA. This suggests that these antibodies specifically interfered with interactions between PKC and the intracellular PLM domain. When these antibodies were added to the permeabilized adipocytes, they mimicked the ability of the Ala-PLM(58–72) peptide to inhibit the effects of insulin on GLUT4 translocation.

Therefore two distinct inhibitors of PLM phosphorylation both decreased the effects of insulin on GLUT4 translocation. The mechanisms involved in GLUT4 translocation remain incompletely understood [1,5,34]. GLUT4 vesicle trafficking shares many characteristics with the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) model for the exocytosis of synaptic vesicles in nerve terminals [35–37]. Insulin has been reported to translocate v-SNAREs of the synaptobrevin family, present in GLUT4 vesicles, to the plasma membrane; syntaxin 4, a member of the syntaxin t-SNARE family, has been identified in a complex with GLUT4, the *N*-ethylmaleimide-sensitive fusion protein and α -soluble *N*-ethylmaleimide-sensitive fusion ('NSF') protein attachment protein (' α -SNAP') in adipocyte plasma membranes [38–41].

A role for PKC in neurotransmitter release (see [42,43]), possibly modulating SNARE interactions [44], is well documented. Similarly, strong support for a role of PKC in insulin-dependent GLUT4 translocation was recently obtained

[15,17]. PKC substrate(s) might therefore be involved in exocytotic reactions. The present results indicate that the inhibition of insulin-stimulated, PKC-mediated phosphorylation of PLM leads to a significant inhibition of insulin-dependent translocation of GLUT4 to PM. These results are consistent with the hypothesis that a causal relationship between insulin-stimulated phosphorylation of the C-terminal intracellular PLM domain and insulin-induced GLUT4 translocation might exist.

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REFERENCES

- Holman, G. D. and Kasuga, M. (1997) *Diabetologia* **40**, 991–1003
- Ruderman, N. B., Kapeller, R., White, M. F. and Cantley, L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1411–1415
- Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J. and Kahn, C. R. (1994) *Mol. Cell. Biol.* **14**, 4902–4911
- Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) *J. Biol. Chem.* **269**, 3568–3573
- Guilherme, A. and Czech, M. P. (1998) *J. Biol. Chem.* **273**, 33119–33122
- Egan, J. J., Saltis, J., Wek, S. A., Simpson, I. A. and Londos, C. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1052–1056
- Mosthaf, L., Kellerer, M., Mühlhölzer, A., Mushack, J., Seffer, E. and Häring, H. U. (1996) *Exp. Clin. Endocrinol.* **104**, 19–24
- Bandyopadhyay, G., Standaert, M. L., Zhao, L., Yu, B., Avignon, A., Galloway, L., Karnam, P., Moscat, J. and Farese, R. V. (1997) *J. Biol. Chem.* **272**, 2551–2558
- Mühlbacher, C., Karnieli, E., Schaff, P., Obermaier, B., Mushack, J., Rattenhuber, E. and Häring, H. U. (1988) *Biochem. J.* **249**, 865–870
- Vogt, B., Mushack, J., Seffer, E. and Häring, H. U. (1991) *Biochem. J.* **275**, 597–600
- Gibbs, E. M., Calderhead, D. M., Holman, G. D. and Gould, G. W. (1991) *Biochem. J.* **275**, 145–150
- Saltis, J., Habberfield, A. D., Egan, J. J., Londos, C., Simpson, I. A. and Cushman, S. W. (1991) *J. Biol. Chem.* **266**, 261–267
- Tanti, J. F., Rochet, N., Gremeaux, T., Van Obberghen, E. and Le Marchand-Brustel, Y. (1989) *Biochem. J.* **258**, 141–146
- Nishimura, H. and Simpson, I. A. (1994) *Biochem. J.* **302**, 271–277
- Walaas, O., Horn, R. S. and Walaas, S. I. (1997) *FEBS Lett.* **413**, 152–156
- Walaas, S. I., Horn, R. S., Adler, A., Albert, K. A. and Walaas, O. (1987) *FEBS Lett.* **220**, 311–318
- Kotani, K., Ogawa, W., Matsumoto, M., Kitamura, T., Sakaue, H., Hino, Y., Miyake, K., Sano, W., Akimoto, K., Ohno, S. and Kasuga, M. (1998) *Mol. Cell. Biol.* **18**, 6971–6982
- Walaas, O., Walaas, E., Lystad, E., Alertsen, A. R. and Horn, R. S. (1979) *Mol. Cell. Endocrinol.* **16**, 45–55
- Palmer, C. J., Scott, B. T. and Jones, L. R. (1991) *J. Biol. Chem.* **266**, 11126–11130
- Walaas, O., Horn, R. S. and Walaas, S. I. (1991) *Biochim. Biophys. Acta* **1094**, 92–102
- Walaas, S. I., Czernik, A. J., Olstad, O. K., Sletten, K. and Walaas, O. (1994) *Biochem. J.* **304**, 635–640
- Chen, L. S., Lo, C. F., Numann, R. and Cuddy, M. (1997) *Genomics* **41**, 435–443
- Kowdley, G. C., Ackerman, S. J., Chen, Z., Szabo, G., Jones, L. R. and Moorman, J. R. (1997) *Biophys. J.* **72**, 141–145
- Moorman, J. R., Ackerman, S. J., Kowdley, G. C., Griffin, M. P., Mounsey, J. P., Chen, Z., Cala, S. E., O'Brian, J. J., Szabo, G. and Jones, L. R. (1995) *Nature (London)* **377**, 737–740
- Chiles, T. C., O'Brien, W. and Kilberg, M. S. (1987) *Anal. Biochem.* **163**, 136–142
- Olmsted, J. B. (1981) *J. Biol. Chem.* **256**, 11955–11957
- Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038
- Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375–380
- Alexander, D. R., Graves, J. D., Lucas, S. C., Cantrell, D. A. and Crumpton, M. J. (1990) *Biochem. J.* **268**, 303–308
- Goetz, S. E., Nestler, E. J., Chehrizi, B. and Greengard, P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2130–2134
- Karnieli, E., Zarnowski, M. J., Hissin, P. J., Simpson, I. A., Salans, L. B. and Cushman, S. W. (1981) *J. Biol. Chem.* **256**, 4772–4777
- Graff, J. M., Rajan, R. R., Randall, R. R., Nairn, A. C. and Blackshear, P. J. (1991) *J. Biol. Chem.* **266**, 14390–14398
- Robinson, L. J., Pang, S., Harris, D. S., Heuser, J. and James, D. E. (1992) *J. Cell Biol.* **117**, 1181–1196
- Pfeiffer, A. (1996) *Exp. Clin. Endocrinol.* **104**, 17–18

-
- 35 Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993) *Nature (London)* **362**, 318–324
- 36 Südhof, T. C. (1995) *Nature (London)* **375**, 645–653
- 37 Rothman, J. E. (1994) *Nature (London)* **372**, 55–63
- 38 Laurie, S. M., Cain, C. C., Lienhard, G. E. and Castle, J. D. (1993) *J. Biol. Chem.* **268**, 19110–19117
- 39 Martin, L. B., Shewan, A., Millar, C. A., Gould, G. W. and James, D. E. (1998) *J. Biol. Chem.* **273**, 1444–1452
- 40 Timmers, K. I., Clark, A. E., Omatsu-Kanbe, M., Whiteheart, S. W., Bennett, M. K., Holman, G. D. and Cushman, S. W. (1996) *Biochem. J.* **320**, 429–436
- 41 Malide, D., Dwyer, N. K., Blanchette-Mackie, E. J. and Cushman, S. W. (1997) *J. Histochem. Cytochem.* **45**, 1083–1096
- 42 Nichols, R. A., Haycock, J. W., Wang, J. K. and Greengard, P. (1987) *J. Neurochem.* **48**, 615–621
- 43 Walaas, S. I. (1999) *Neurochem. Int.* **34**, 221–233
- 44 Smith, C., Moser, T., Xu, T. and Neher, E. (1998) *Neuron* **20**, 1243–1253
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