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Regulation of GLUT1-mediated glucose uptake by PKC λ –PKC β_{II} interactions in 3T3-L1 adipocytes

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Members of the PKC (protein kinase C) superfamily play key regulatory roles in glucose transport. How the different PKC isoforms are involved in the regulation of glucose transport is still poorly defined. PMA is a potent activator of conventional and novel PKCs and PMA increases the rate of glucose uptake in many different cell systems. In the present study, we show that PMA treatment increases glucose uptake in 3T3-L1 adipocytes by two mechanisms: a mitogen-activated protein kinase-dependent increase in GLUT1 (glucose transporter 1) expression levels and a PKC λ -dependent translocation of GLUT1 towards the plasma membrane. Intriguingly, PKC λ co-immunoprecipitated with PKC β_{II} and did not with PKC β_I . Previously, we have des-

cribed that down-regulation of PKC β_{II} protein levels or inhibiting PKC β_{II} by means of the myristoylated PKC β_{C2-4} peptide inhibitor induced GLUT1 translocation towards the plasma membrane in 3T3-L1 adipocytes. Combined with the present findings, these results suggest that the liberation of PKC λ from PKC β_{II} is an important factor in the regulation of GLUT1 distribution in 3T3-L1 adipocytes.

Key words: glucose uptake, myristoylated protein kinase C β peptide inhibitor, phorbol ester, protein kinase C (PKC), 3T3-L1 adipocyte.

INTRODUCTION

In the recent years, the involvement of the PKC (protein kinase C; EC 2.7.1.37) family in regulating glucose transport at different levels has been identified. However, the underlying mechanisms are poorly defined. Whereas some members of the PKC family are essential for insulin-stimulated glucose transport, others may function as negative-feedback inhibitors of insulin-stimulated glucose transport and, finally, some members of the PKC family can stimulate glucose transport independently of insulin [1–3].

The PKC family consists of at least 11 isoforms that can be divided into three classes [4–6]. The conventional PKC isoforms (α , β_I , β_{II} and γ) and the novel PKC isoforms (δ , ϵ , η and θ) are activated by DAG (diacylglycerol). The atypical PKC isoforms (ζ , λ and ι), which contain a relatively high constitutive activity, can be activated by phosphatidic acid and phosphatidylinositides.

There is much evidence that PKCs play an important role in regulating glucose transport in insulin-sensitive tissues. In particular, the atypical PKC isoforms have received much attention, as PKC ζ and/or PKC λ are essential during insulin-stimulated glucose transport in both adipocytes and muscular cells (reviewed in [1]). In 3T3-L1 adipocytes, transfection of kinase-inactive PKC λ and PKC ζ , which are highly homologous, severely decreased glucose transport and prevented GLUT4 (glucose transporter 4) translocation [7–9]. Since 3T3-L1 adipocytes contain solely PKC λ [8], these observations suggest that in case of 3T3-L1 adipocytes, insulin acts through PKC λ to induce translocation of

GLUT4 from an intracellular vesicular storage pool to the PM (plasma membrane).

PMA is a potent activator of the conventional and novel PKCs and increases the rate of glucose uptake in 3T3-L1 adipocytes, suggesting that the activation of DAG-sensitive PKC isoforms stimulates the rate of glucose uptake [10–13]. However, activation of PKCs results in the exposure of PKC sites sensitive to proteolytic cleavage [14–16]. Consequently, chronic phorbol ester treatment results in the proteolytic degradation, termed down-regulation, of DAG-sensitive PKCs. This is in agreement with our observation that in PMA-treated 3T3-L1 adipocytes, PKC β_{II} is rapidly down-regulated [10]. In addition, inhibition of PKC β_{II} by means of the myristoylated PKC β_{C2-4} peptide inhibitor effectively increases glucose uptake, which strongly suggests that part of the PMA-stimulated glucose uptake in 3T3-L1 adipocytes is due to the loss of PKC β_{II} . In contrast with insulin that predominantly stimulates the translocation of GLUT4 to the PM [17], PMA stimulates glucose transport predominantly by increasing the amount of GLUT1 at the PM [10,11].

The aim of this study was to provide more insight into the mechanism of PKC-regulated glucose uptake in 3T3-L1 adipocytes. In the present study, we demonstrate that in 3T3-L1 adipocytes activation of PKC by PMA increases GLUT1 expression in an MAPKK (mitogen-activated protein kinase kinase)-dependent manner. In addition, the translocation of GLUT1 from intracellular pools to the PM is hampered upon inhibition of PKC λ , and PKC β_{II} is associated with PKC λ . Since loss of PKC β_{II}

Abbreviations used: DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; 2-DOG, 2-deoxyglucose; GLUT, glucose transporter; LDM, low-density membrane; MAPKK, mitogen-activated protein kinase kinase; PKC, protein kinase C; PM, plasma membrane.

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stimulates glucose transport, the latter observation suggests that in the case of 3T3-L1 adipocytes, liberation of PKC λ from PKC β_{II} is an important phenomenon in the regulation of glucose transporter redistribution.

MATERIALS AND METHODS

Materials

DMEM (Dulbecco's modified Eagle's medium) was purchased from ICN Biomedicals (Aurora, OH, U.S.A.) and foetal calf serum was purchased from PPA Laboratories (Linz, Austria). All other culture reagents were purchased from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). The rabbit PKC λ antibody was from Transduction Laboratories (Hamburg, Germany). The mouse PKC β_1 antibody and rabbit PKC β_{II} antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The myristoylated PKC β (amino acid sequence: FARKGALRQ) and myristoylated PKC λ (amino acid sequence: JSIYRRGARRWRKL) peptide inhibitors were purchased from the Department of Immunohematology and Blood Transfusion (Leiden University Medical Center, Leiden, The Netherlands). BSA, 3-isobutyl-1-methylxanthine, dexamethasone, 2-DOG (2-deoxyglucose), PMA, U0126 and PD98059 were obtained from Sigma (St. Louis, MO, U.S.A.). Rapamycin was obtained from Calbiochem (Darmstadt, Germany). Recombinant human insulin was from Eli Lilly and Co (Nieuwegein, The Netherlands). Dithiothreitol was purchased from Research Organics (Santa Cruz, CA, U.S.A.) and 1-[¹⁴C]-2-DOG (specific activity, 2.0 GBq/mmol) was purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). All other chemicals were of analytical grade.

Adipogenesis of 3T3-L1 adipocytes

The 3T3-L1 cells (A.T.C.C., Manassas, VA, U.S.A.) were fully differentiated into adipocytes as described previously [10].

Glucose uptake assay

Fully differentiated 3T3-L1 adipocytes, subcultured in 12-well plates, were incubated in DMEM without serum or in serum-free DMEM with 10 μ M U0126 and 20 μ M PD98059 or 0.1 μ M rapamycin for 30 min. Subsequently, in the continuous presence of the inhibitors, cells were treated either for 7 h with 0.1 μ M PMA in serum-free medium or for 90 min with 100 μ M myristoylated PKC β peptide inhibitor in reaction buffer containing 138 mM NaCl, 1.85 mM CaCl₂, 1.3 mM MgSO₄, 4.8 mM KCl, 0.2% (w/v) BSA and 50 mM Hepes adjusted to pH 7.4. Cells were washed twice with PBS and the adipocytes were refreshed with 760 μ l of reaction buffer containing 333 kBq of 1-[¹⁴C]-2-DOG and 40 μ M 2-DOG. After 5 min of incubation at 37 °C, 2-DOG uptake was terminated by washing the cells three times with ice-cold PBS containing 10 mM glucose. Subsequently, cells were lysed in 1% (w/v) SDS and 0.2 M NaOH. The incorporated radioactivity was measured by liquid-scintillation spectrometry.

Real-time reverse transcriptase-PCR analysis of GLUT1 and GLUT4

Fully differentiated 3T3-L1 adipocytes, subcultured in 12-well plates, were incubated in DMEM without serum or in serum-free DMEM with 10 μ M U0126 and 20 μ M PD98059 for 30 min. In the continuous presence of the inhibitors, cells were subsequently treated for 7 h with 0.1 μ M PMA in serum-free medium. In addition, adipocytes were treated for 90 min with 100 μ M myristoylated PKC β peptide inhibitor in a reaction buffer from the glucose uptake assay (see above). After treatment, cells were washed twice with PBS and total RNA was isolated using the

RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentrations were determined from the spectrophotometric absorption at 260 nm using the Genequant (Amersham, Eindhoven, The Netherlands). Total RNA (1.0 μ g) was denatured for 10 min at 70 °C and immediately cooled on ice. Reverse transcription was performed using the Reverse Transcription System (Promega Benelux, Leiden, The Netherlands) according to the manufacturer's instructions. After annealing of random hexamers for 10 min at 20 °C, cDNA synthesis was performed for 60 min at 42 °C followed by an enzyme inactivation step for 5 min at 95 °C. The GLUT1 forward primer was 5'-catcctattgccaggtgttt-3' and the reversed primer was 5'-gaagacgacactgagcagcaga-3'; the GLUT4 forward primer was 5'-cttcttgagattggccctgg-3' and the reversed primer was 5'-aggtgaagatgaagaagccaagc-3'. All samples were normalized for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression (forward primer 5'-ggcatccacagtctctgg-3' and the reversed primer 5'-accacagtccatgccatcatgcca-3'). By means of SyBR Green Master Mix (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), amplifications were performed by denaturation at 95 °C for 10 min, 40 cycles of 15 s at 95 °C and 60 s at 60 °C on an ABI Prism 7700 Sequence detection system (PE Applied Biosystems).

Western-blot analysis of GLUT1 and GLUT4 in different cell fractions

Differentiated 3T3-L1 adipocytes, grown in T₇₅ flasks, were treated for 5.5 h with or without 0.1 μ M PMA in serum-free medium and subsequently for 90 min in reaction buffer from the glucose uptake assay (see above) with or without 20 μ M myristoylated PKC λ peptide inhibitor. After washing with PBS, adipocytes from a T₇₅ flask were scraped and the PM fraction and the LDM (low-density membrane) fraction were obtained; the amounts of GLUT1 and GLUT4 were determined by Western blotting as described previously [10,18]. The bands were scanned using the documentation and analysis system AlphaImager™ 1220 (Biozym, Landgraaf, The Netherlands) and the integrated density values were calculated with the computer program AlphaEase v5.1 (Biozym).

Immunoprecipitation and detection of PKC β_{II} and PKC λ

Differentiated 3T3-L1 adipocytes, grown in 9 cm dishes, were scraped in 750 μ l of lysis buffer containing 1 mM Na₃VO₄, 150 mM NaCl, 5 mM NaF, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Nonidet P40, 0.5% (w/v) sodium deoxycholate, 1 tablet of complete mini protease inhibitor cocktail per 50 ml and 50 mM Tris adjusted to pH 7.4. Cell lysates were tumbled for 30 min at 4 °C and subsequently centrifuged at 13 000 g for 10 min at 4 °C. The supernatant was precleared with a 10 μ l slurry of ProtA beads for 60 min at 4 °C after which the supernatant (approx. 1 mg) was mixed with 5 μ g of non-immunized rabbit serum or PKC-isotype-specific antibodies for 90 min at 4 °C. Immunocomplexes were collected by incubating with a 10 μ l slurry of beads (ProtA for rabbit polyclonal antibodies and ProtG for mouse monoclonal antibodies) for 90 min at 4 °C. Beads were washed three times with lysis buffer and once with a buffer containing 1 mM EDTA and 50 mM Tris adjusted to pH 7.4. Subsequently, beads were resuspended in sample buffer and subjected to SDS/PAGE after which the proteins were transferred on to a PVDF membrane (Immobilon P; Millipore, Bedford, MA, U.S.A.) by Western blotting. Membranes were washed twice with PBS and blocked for 2 h at room temperature in a buffer containing 0.25% (v/v) Tween 20, 150 mM NaCl, 5% (w/v) milk powder and 50 mM Tris adjusted to pH 7.4 (TBS-M buffer). Subsequently, membranes were incubated for 1 h at room temperature in TBS-M

buffer containing the PKC-isotype-specific antibodies. The antibodies were diluted 1:1000 for the detection of PKC β_1 , PKC β_{II} or PKC λ . Next, membranes were washed several times with TBS-M buffer followed by an incubation for 1 h with goat anti-rabbit (for detection of PKC β_{II}) or goat anti-mouse (for detection of PKC β_1 or PKC λ) antibodies conjugated to horseradish peroxidase (1:10 000). Bands were visualized using ECL[®] (Amersham Biosciences).

Data analysis

The results presented are means \pm S.D. for 3–9 individual experiments and statistical significance was determined by ANOVA. For significance tests ($P < 0.05$), individual groups were compared as described by Tukey–Kramer.

RESULTS

Characteristics of PMA- and myristoylated PKC β peptide inhibitor-stimulated glucose transport

The rate of glucose uptake was increased 3.7 ± 0.4 -fold ($n = 6$) after 7 h incubation of 3T3-L1 adipocytes with $0.1 \mu\text{M}$ PMA (Figure 1A). Inhibition of MAPKK in these cells with either U0126 or PD98059 significantly decreased PMA-stimulated glucose transport compared with PMA-treated cells not exposed to the inhibitors. However, in the presence of U0126 and PD98059, PMA treatment still significantly increased the rate of glucose uptake [2.0 ± 0.3 -fold ($n = 6$) and 2.4 ± 0.8 -fold ($n = 6$) respectively]. Treatment of 3T3-L1 adipocytes with rapamycin, which inhibits the mammalian target of rapamycin, did not affect PMA-stimulated glucose transport. In contrast, inhibition of PKC λ by incubation of the cells with the myristoylated PKC λ peptide inhibitor ($100 \mu\text{M}$) during the final 90 min of PMA treatment prevented stimulation of the rate of glucose transport by PMA.

Incubation of 3T3-L1 adipocytes with $100 \mu\text{M}$ myristoylated PKC β peptide inhibitor for 90 min increased the rate of glucose uptake 4.1 ± 1.5 -fold ($n = 9$) (Figure 1B). Neither of the two MAPKK inhibitors nor rapamycin affected the increase in the rate of glucose uptake by the myristoylated PKC β peptide inhibitor. A co-incubation of the cells with the myristoylated PKC λ peptide inhibitor and the myristoylated PKC β peptide inhibitor at their effective concentrations was not possible as it lysed the cells.

Effects of treatment with PMA or myristoylated PKC β peptide inhibitor on the expression of GLUTs

Treatment of the adipocytes with $0.1 \mu\text{M}$ PMA for 7 h increased the amount of mRNA encoding GLUT1 approx. 6-fold when compared with untreated cells (GLUT1/GAPDH: 0.015 ± 0.003 in control versus 0.087 ± 0.014 in PMA-treated cells; $n = 3$) (Figure 2A). Prolonged PMA treatment did not change the amount of mRNA encoding GLUT4. Incubation of 3T3-L1 adipocytes for 90 min with $100 \mu\text{M}$ myristoylated PKC β peptide inhibitor had no significant effect on GLUT1 and GLUT4 mRNA levels. Similarly as with the mRNA analyses, PMA treatment of adipocytes increased the amount of GLUT1, but not GLUT4, at the protein level, whereas incubation of cells with the myristoylated PKC β peptide inhibitor did not change either GLUT1 or GLUT4 protein levels (Figure 2B).

PMA increases the amount of GLUT1 through activation of MAPKK

To investigate whether prolonged PMA treatment increases the expression of GLUT1 through signalling by activation of

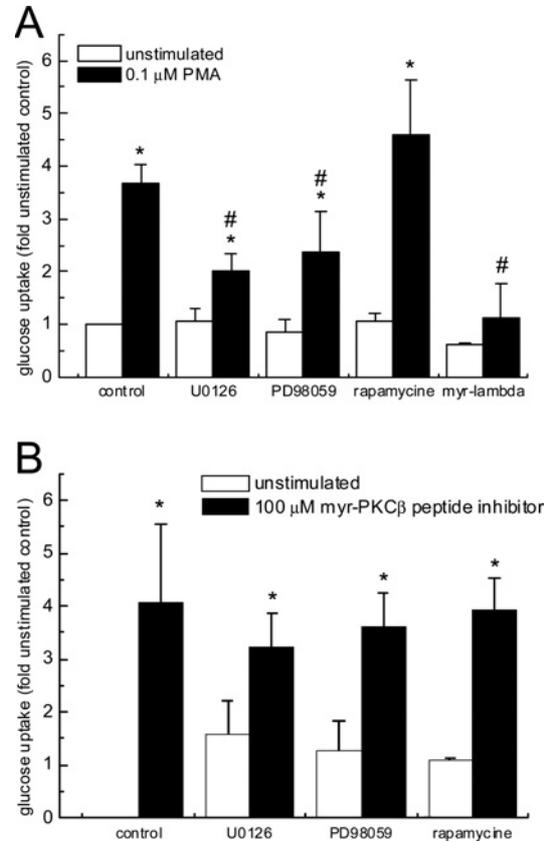


Figure 1 Long-term PMA treatment and incubation with the myristoylated PKC β peptide inhibitor increases glucose uptake in 3T3-L1 adipocytes

The rate of glucose uptake was determined for cells treated with PMA (A) or with the myristoylated PKC β peptide inhibitor (B). For PMA, the rate of glucose uptake in unstimulated cells was $808 \pm 137 \text{ fmol} \cdot \text{min}^{-1} \cdot \text{well}^{-1}$ ($n = 6$). For the myristoylated PKC β peptide inhibitor, the rate of glucose uptake in unstimulated cells was $480 \pm 267 \text{ fmol} \cdot \text{min}^{-1} \cdot \text{well}^{-1}$ ($n = 9$). Basal glucose uptake was set at 100% to which all other values were related. The values presented are means \pm S.D. for 3–9 experiments; *significantly different from corresponding untreated cells; #significantly different from 7 h of $0.1 \mu\text{M}$ PMA-treated cells. Incubation with different inhibitors is shown at the bottom of each graph; control, no inhibitor present; myr-lambda, myristoylated PKC λ peptide inhibitor.

MAPKK, 3T3-L1 adipocytes were pretreated with the MAPKK inhibitors U0126 and PD98059 after which the cells were exposed to $0.1 \mu\text{M}$ PMA for 7 h in the continuous presence of the inhibitors. Figure 3 shows that the increase in PMA-mediated mRNA (Figure 3A) and protein (Figure 3B) levels for GLUT1 were completely prevented when the activity of MAPKK was blocked. Although inhibition of PKC λ by incubating the PMA-treated cells for 90 min with the myristoylated PKC λ peptide inhibitor prevented stimulation of the rate of glucose transport (Figure 1A), the increase in PMA-mediated GLUT1 mRNA and protein levels was not lowered after 90 min incubation with the myristoylated PKC λ peptide inhibitor (Figure 3).

Myristoylated PKC λ peptide inhibitor decreased the amount of GLUT1 at the PM

Incubation of PMA-treated 3T3-L1 adipocytes with the myristoylated PKC λ peptide inhibitor lowered the increase in glucose uptake (Figure 1) but not the increase in GLUT1 expression (Figure 3). Therefore we investigated whether the peptide inhibitor affected the cellular redistribution of GLUT1. To this end,

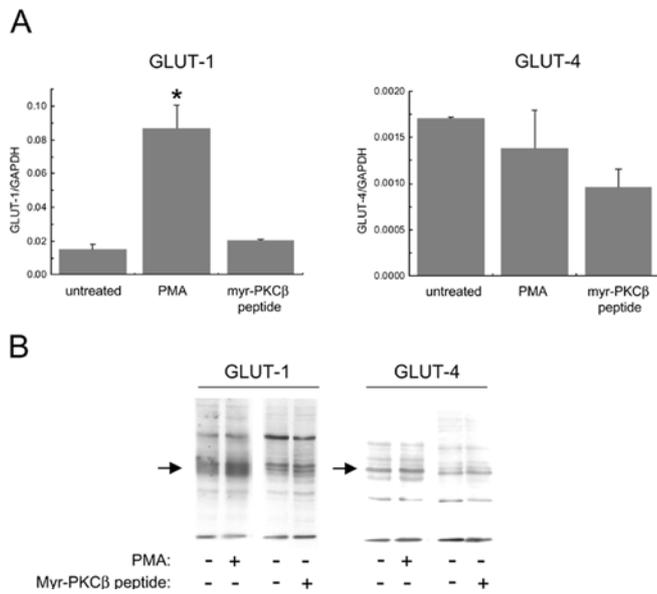


Figure 2 Long-term PMA treatment increases GLUT1 mRNA and protein levels in 3T3-L1 adipocytes

Cells were treated for 7 h with 0.1 μ M PMA or for 90 min with 100 μ M myristoylated PKC β peptide inhibitor and the amounts of GLUT1 and GLUT4 were determined from mRNA (**A**) and protein levels (**B**). For mRNA analyses, the values presented are means \pm S.D. for three experiments; *significantly different from untreated cells. For protein analyses, one representative result from three separate experiments is presented. Arrows indicate bands representing the glucose transporter. myr-PKC β peptide, myristoylated PKC β peptide inhibitor.

untreated and PMA-treated 3T3-L1 adipocytes were incubated for 90 min with or without myristoylated PKC λ peptide inhibitor (20 μ M). By means of differential centrifugation the intracellular vesicles (LDM) were separated from the PM and, subsequently, the relative amounts of GLUT1 in these different fractions were measured. The densitometric analyses are indicated below in parentheses and indicate the relative changes (median; $n = 3$) compared with the amount of GLUT1 in the fractions of untreated cells. Note that, during the glucose uptake, cells could be treated with 100 μ M myristoylated PKC λ peptide inhibitor, whereas this had to be lowered to 20 μ M when the adipocytes were subjected to differential centrifugation to maintain the integrity of the LDM and PM fractions (results not shown). Figure 4 shows that the addition of myristoylated PKC λ peptide inhibitor for 90 min increased the amount of GLUT1 in the LDM fraction (1.17-fold) and decreased the amount of GLUT1 in the PM fraction (0.79-fold). Similar to our earlier report [10], the amount of GLUT1 increased in both the LDM (1.26-fold) and PM (1.21-fold) fractions of cells treated for 7 h with 0.1 μ M PMA. When PMA-treated cells were incubated with the myristoylated PKC λ peptide inhibitor during the last 90 min of PMA treatment, the amount of GLUT1 in the PM fraction decreased (1.08-fold) and the amount of GLUT1 in the LDM fraction increased (1.64-fold) compared with the respective amounts in PMA-treated 3T3-L1 adipocytes not exposed to the peptide inhibitor.

Co-immunoprecipitation of PKC β_{II} and PKC λ

Previously, we reported that depletion or inhibition of PKC β_{II} promotes the translocation of GLUT1 to the PM [10]. Since this translocation of GLUT1 to the PM requires PKC λ activity (Figure 4), we investigated whether there is an interaction between PKC β_{II} and PKC λ . Figure 5 shows that beads coupled with

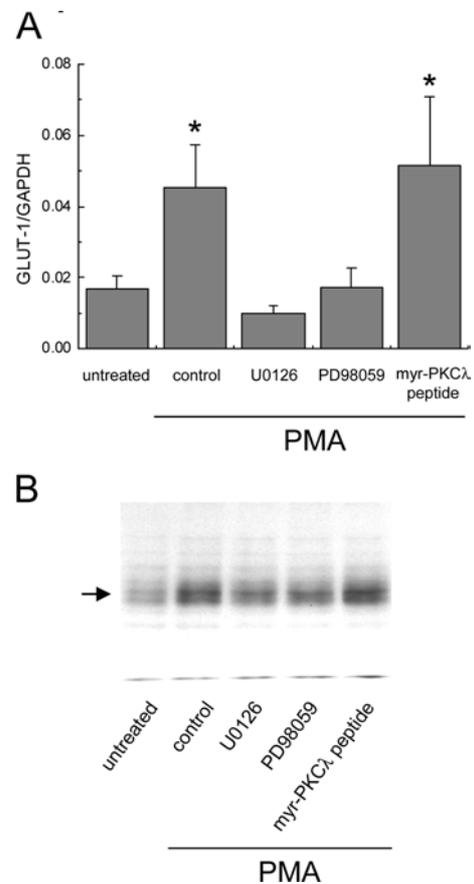


Figure 3 Inhibition of MAPKK prevents PMA-stimulated increases in GLUT1 mRNA and protein levels in 3T3-L1 adipocytes

Untreated and PMA-treated cells were harvested and the amount of GLUT1 was investigated from mRNA levels (**A**) and protein levels (**B**). For mRNA analyses, the values presented are the means \pm S.D. for four experiments; *significantly different from untreated cells. For protein analyses, one representative result of two separate experiments is shown. The arrow indicates a band representing the glucose transporter. Treatment with inhibitors is indicated below the graph; control, not exposed to inhibitor; myr-PKC λ peptide, myristoylated PKC λ peptide inhibitor.

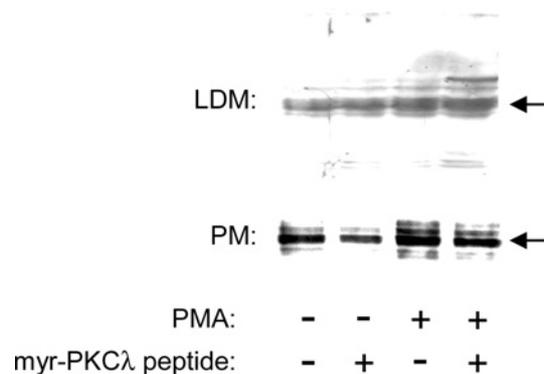


Figure 4 Translocation of GLUT1 from intracellular vesicles to the PM in PMA-treated 3T3-L1 adipocytes is prevented by the myristoylated PKC λ peptide inhibitor

Cells were subjected to differential centrifugation to yield LDM and PM fractions. Proteins (20 μ g) of these fractions were separated by means of SDS/PAGE and subjected to Western blotting. One representative result of three separate experiments is presented. Arrows indicate bands representing GLUT1. Myr-PKC λ peptide, myristoylated PKC λ peptide inhibitor. The treatment condition (-, without, +, with) is indicated below the graph.

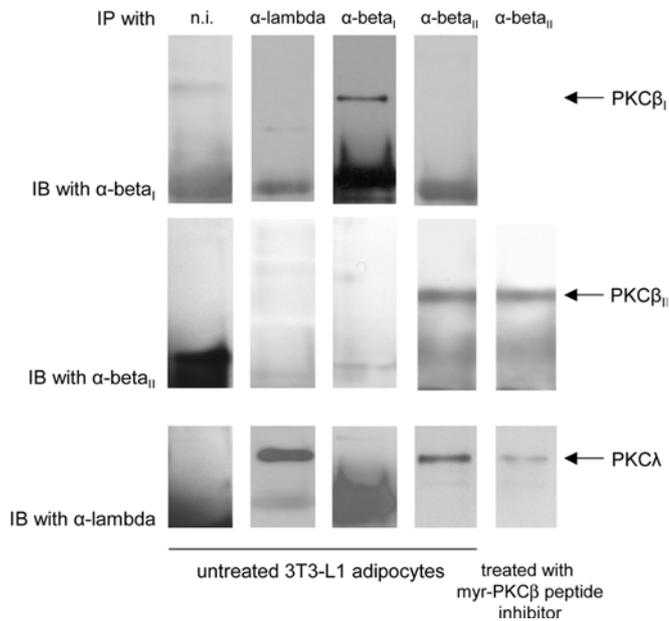


Figure 5 Immunoprecipitation of PKC β_{II} co-immunoprecipitates PKC λ in 3T3-L1 adipocytes

A lysate of 3T3-L1 adipocytes treated with or without 100 μ M myristoylated PKC β peptide inhibitor was incubated with beads coupled with either non-immunized rabbit antiserum (n.i.), anti-PKC λ , anti-PKC β_I or anti-PKC β_{II} . Subsequently, the beads were precipitated and subjected to SDS/PAGE followed by Western blotting. Membranes were probed with anti-PKC β_I (top panels), anti-PKC β_{II} (middle panels) or anti-PKC λ (lower panels). One representative result from three separate experiments is shown, except for the right most lane, which was performed twice. Arrows indicate bands representing the PKC isoforms. IP, immunoprecipitation; IB, immunoblotting.

non-immunized rabbit antiserum did not precipitate PKC β_I , PKC β_{II} or PKC λ from a lysate of 3T3-L1 adipocytes. Immunoprecipitation of beads coupled with anti-PKC λ antibody immunoprecipitated only PKC λ and not PKC β_I or PKC β_{II} . Immunoprecipitation of beads coupled with anti-PKC β_I antibody immunoprecipitated only PKC β_I and not PKC β_{II} or PKC λ . When a lysate of 3T3-L1 adipocytes was incubated with beads coupled with anti-PKC β_{II} antibody, it did not immunoprecipitate PKC β_I but did PKC β_{II} , and co-immunoprecipitated PKC λ . When 3T3-L1 adipocytes were treated for 90 min with 100 μ M myristoylated PKC β peptide inhibitor before immunoprecipitation of PKC β_{II} , the amount of PKC λ that was co-immunoprecipitated was decreased when compared with the amount of PKC λ that was co-immunoprecipitated with PKC β_{II} in a lysate of untreated 3T3-L1 adipocytes.

DISCUSSION

The present study examined how different PKC isoforms are involved in promoting glucose uptake in 3T3-L1 adipocytes. We observed that an increase in PMA-stimulated glucose transport was partially attenuated on inhibition of MAPKK. In addition, the increase in PMA-stimulated glucose transport was associated with an increase in GLUT1 mRNA and protein levels. This PMA-induced increase in GLUT1 expression was prevented by inhibition of MAPKK. Furthermore, incubation with the myristoylated PKC λ peptide inhibitor completely attenuated PMA-stimulated glucose transport but did not affect PMA-stimulated increase in GLUT1 levels. However, incubation of PMA-treated 3T3-L1 adipocytes with the PKC λ peptide inhibitor decreased the amount of GLUT1 at the PM and increased the amount of GLUT1 at

the intracellular vesicles. Finally, in 3T3-L1 adipocytes, immunoprecipitation of PKC β_{II} co-immunoprecipitated PKC λ . These results demonstrate that PMA treatment increases glucose uptake in 3T3-L1 adipocytes by two mechanisms, namely increase in GLUT1 expression levels and translocation of GLUT1 towards the PM. Moreover, given our previous findings on how inactivation of PKC β_{II} stimulates GLUT1-mediated glucose transport [10], the present results suggest that PKC β_{II} and PKC λ are associated and that dissociation of PKC λ is an important mechanism to initiate GLUT1 translocation from intracellular vesicles to the PM.

Several studies have demonstrated that PMA, known to stimulate PKC, increases the rate of glucose transport in 3T3-L1 adipocytes [11–13,19]. In the present study, treatment of 3T3-L1 adipocytes for 7 h with 0.1 μ M PMA increased the rate of glucose uptake by approx. 4-fold. When cells were treated with PMA in the presence of the MAPKK inhibitors U0126 and PD98059, the rate of glucose uptake was still significantly increased but its magnitude was nearly halved when compared with PMA-treated cells not incubated with the inhibitors. Inhibition of the mammalian target of rapamycin, a serine/threonine kinase homologous with phosphatidylinositol 3'-kinase, by rapamycin did not decrease the rate of glucose uptake in PMA-treated adipocytes, whereas in 18 h insulin-treated L6 muscle cells, glucose transport was increased due to increased GLUT1 expression in a rapamycin-sensitive manner [20,21]. These results show that part of the stimulatory effect of PMA on glucose uptake in 3T3-L1 adipocytes is mediated by MAPKK, a component of the Ras-MAPK pathway [22]. Since this pathway is typically activated by growth factors and mitogens to alter transcription factor activity, we measured the expression levels of GLUT1 and GLUT4. No differences were found in the level of GLUT4, whereas the amount of GLUT1 both at the mRNA and the protein level markedly increased in PMA-treated adipocytes when compared with untreated cells. The increases in GLUT1 expression levels were prevented when MAPKK was inhibited either by U0126 or PD98059. Taken together, these results show that PMA stimulates the Ras-MAPK pathway to promote GLUT1 expression. Our results are in agreement with previous reports, which demonstrate that introducing active mutants of Ras, SHP2, Raf-1 or MAPKK into 3T3-L1 adipocytes, which increase the activity of the Ras-MAPK pathway, increases the expression of GLUT1 but not of GLUT4 [23–26]. In Cos-7 cells, PMA-sensitive PKC isoforms activate the Ras-MAPK pathway at the level of Raf [27]. It should be mentioned that PMA is capable of stimulating the Ras-MAPK pathway without the requirement of PKC (reviewed in [28,29]). As in 3T3-L1 adipocytes, PMA-induced activation of MAPK is inhibited by the PKC inhibitors Gö-6976, bisindolylmaleimide I and chelerythrine chloride [30]; it is most probable that PMA stimulates the expression of GLUT1 in our cells through the stimulation of conventional and novel PKCs, which in turn increase the activity of Raf.

Treatment of 3T3-L1 adipocytes by PMA very effectively increases the rate of glucose uptake. However, inhibition of PKC, most probably PKC β_{II} , by incubating 3T3-L1 adipocytes with the myristoylated PKC β peptide inhibitor also stimulates an increase in glucose uptake, as it promotes the translocation of GLUT1 from intracellular compartments to the PM [10]. Stimulation of glucose uptake in adipocytes by the PKC β peptide inhibitor is not attenuated by inhibition of MAPKK, indicating that the translocation of GLUT1 to the PM is not dependent on the Ras-MAPK pathway. Similarly, a significant part of the PMA-stimulated increase in glucose uptake is also not sensitive to MAPKK inhibition. Persistent activation of PKC by PMA causes down-regulation of PMA-sensitive PKCs [14–16] and,

accordingly, PKC β_{II} is completely down-regulated after 7 h of 0.1 μ M PMA treatment, whereas the other PMA-sensitive PKCs (PKC β_I , PKC γ , PKC δ and PKC θ) are still present [10]. Taken together, this is a strong indication that the MAPKK-insensitive part of PMA-stimulated glucose transport is due to loss of PKC β_{II} .

The increased rate of glucose uptake in PMA-treated 3T3-L1 adipocytes was completely prevented when cells were incubated with the myristoylated PKC λ peptide inhibitor. This is not explained by a decreased GLUT1 expression as PMA-mediated increase in GLUT1 levels was not lowered when PMA-treated cells were exposed to 90 min incubation with the myristoylated PKC λ peptide inhibitor. Therefore we analysed GLUT1 expression in the LDM fraction (representing the intracellular vesicles) and the PM fraction in cells that were exposed to the PKC λ peptide inhibitor. The amount of GLUT1 decreased at the PM and tended to increase in the intracellular vesicles when control cells were treated with the PKC λ peptide inhibitor. As reported previously, PMA treatment of 3T3-L1 adipocytes increases GLUT1 levels in both the LDM and the PM fraction [10]. However, in the presence of the PKC λ peptide inhibitor, GLUT1 levels in the PM fraction were no longer increased compared with the levels in untreated adipocytes. In addition, it appears that the GLUT1 proteins are arrested in the intracellular vesicles as the highest amount of GLUT1 was measured in the LDM fraction of PMA-treated adipocytes in which PKC λ was inhibited. Taken together, these results demonstrate the importance of PKC λ in the translocation of GLUT1 from intracellular vesicles to the PM. There are numerous reports demonstrating the importance of atypical PKCs in insulin-stimulated translocation of GLUT4 from intracellular vesicles to the PM (reviewed in [1]). Our results show that PKC λ is also involved in the translocation of GLUT1, which strengthens the idea that atypical PKCs are critical for activating glucose transport responses.

Given the pivotal role of PKC λ in GLUT1-mediated glucose transport, which is stimulated on loss of function of PKC β_{II} [10], we wondered whether the two PKC isoforms are closely associated. Indeed, immunoprecipitation of PKC β_{II} from a lysate of 3T3-L1 adipocytes co-immunoprecipitated PKC λ . Note, however that immunoprecipitation of PKC λ did not co-immunoprecipitate PKC β_{II} . The reason for this could be differences in stoichiometry, i.e. there is relatively more PKC λ not associated with PKC β_{II} than there is PKC β_{II} not associated with PKC λ . In contrast with immunoprecipitation of PKC β_{II} , immunoprecipitation of PKC β_I did not co-immunoprecipitate PKC λ . These results demonstrate, for the first time, that PKC λ is associated with PKC β and, more specifically, that PKC λ is associated with PKC β_{II} and not with PKC β_I . Since the GLUT1-mediated rate of glucose uptake is increased on inhibition of PKC β_{II} , these results suggest that the liberation of PKC λ from PKC β_{II} initiates the translocation of GLUT1 to the PM. The two PKC β isoforms are identical except for their C-terminal V5 region [31]. Interestingly, when we treated the adipocytes with the PKC β peptide inhibitor, which acts as a pseudosubstrate, less PKC λ was precipitated, indicating that the inhibitor competes with PKC λ for binding to PKC β_{II} at the substrate binding domain. Although PKC β_I and PKC β_{II} share an identical substrate-binding domain, PKC β_I does not associate with PKC λ .

In human U937 monocytic cells, MOLT-4 T-lymphoblastoid cells and in NIH-3T3 fibroblasts, PKC β_I and PKC β_{II} are differently localized [32–34], and PKC β_{II} , and not PKC β_I , co-localizes with actin microfilaments and the cytoskeleton. Moreover, the ability of PKC β_{II} to associate with the cytoskeleton was inhibited on disruption of the actin-depolymerizing agent, cytochalasin D. Interestingly, insulin-stimulated GLUT4 translocation is also

inhibited by cytochalasin D [35], as insulin induces cortical actin remodelling and this dynamic actin rearrangement is imperative for insulin-stimulated glucose transport [36]. During this cortical actin remodelling, PKC λ is recruited to these actin structures [37]. On the basis of our findings that PKC β_{II} associates with PKC λ and that in different models PKC β_{II} localizes with actin structures, it is tempting to speculate that at the cortical actin structures PKC β_{II} binds and, hence, inhibits PKC λ , and that dissociation of PKC λ from PKC β_{II} is important to promote translocation of glucose transporters to the PM. In addition, in several cell models, including 3T3-L1 adipocytes, insulin promotes the alternative splicing of PKC β into PKC β_{II} , rather than PKC β_I , which further strengthens the suggestions of an involvement of PKC β_{II} in regulating glucose uptake [38–40]. It remains to be determined whether the dissociation of PKC λ from PKC β_{II} also plays a role in (insulin-stimulated) GLUT4 translocation to the PM.

In summary, our results demonstrate that PMA stimulates GLUT1 expression in a Ras-MAPK-dependent manner and translocation of GLUT1 from intracellular vesicles to the PM requires PKC λ . In 3T3-L1 adipocytes, PKC λ is associated with PKC β_{II} . Given that inhibition of PKC β_{II} stimulates GLUT1-mediated glucose transport, these results suggest that release of PKC λ from PKC β_{II} is an important mechanism in the regulation of glucose transporter distribution.

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