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The protein kinase C pseudosubstrate peptide (PKC19–36) inhibits insulin-stimulated protein kinase activity and insulin-mediated translocation of the glucose transporter glut 4 in streptolysin-O permeabilized adipocytes

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Abstract The effect of insulin on protein kinase activity and plasma membrane translocation of the glucose transporter GLUT 4 has been studied in adipocytes permeabilized by Streptolysin-O. Insulin increased protein kinase activity, and this was completely inhibited by the PKC pseudosubstrate inhibitor peptide (PKC19–36). Insulin-mediated translocation of GLUT 4 was also inhibited by the PKC inhibitor peptide. Both these insulin effects were blocked by a PKC$_\beta$ neutralizing antibody. Our results are consistent with the hypothesis that insulin activates PKC$_\beta$ activity in adipocytes in situ, and that this PKC activation is a component of the system whereby insulin regulates translocation of GLUT 4 to the plasma membrane.

Key words: Insulin; PKC; GLUT 4 translocation; PKC pseudosubstrate peptide inhibitor; mAb-PKC$_\beta$

1. Introduction

The role of protein kinase C (PKC) in insulin signalling has been one of the most controversial problems in the field of insulin action in recent years (Reviews [1–3]). In early studies a role of protein kinase C (PKC) in insulin signalling was indicated since we and others demonstrated insulin stimulation of PKC activity in physiologically relevant tissues, i.e. skeletal muscle [4,5] and adipocytes [6]. Moreover, insulin was found to induce translocation of several isoforms of PKC from cytosol to membrane [7,8]. In contrast, other investigators failed to observe an effect of insulin on PKC activity in adipocytes [9] hepatocytes [10], BC3H-1 cells [11] or several fibroblast cell lines [12]. In the present investigation we have examined whether insulin has a direct effect on protein kinase activity in situ, using Streptolysin-O (SLO)-permeabilized adipocytes to avoid extraction procedures and in vitro assay of PKC. As reported in studies of PKC-mediated phosphorylation of intracellular proteins [13] and PKC activity in T-lymphocytes [14], this technique employs internalization of peptides into the adipocytes during incubation. In the present study we have used as substrate a peptide identical with the 58–72 C-terminal residues of the protein phospholemman (PLM), which has been shown to be a good substrate for PKC in vitro [15]. The specificity of the protein kinase activity responding to insulin has then been examined by use of the synthetic PKC pseudosubstrate (PKC19–36) peptide, which is known as a highly specific inhibitor of PKC [16,17].

Insulin stimulation of glucose transport is primarily mediated by translocation of the glucose transporter GLUT 4 from an intracellular membrane pool (ICM) to the plasma membrane (PM) [18]. A role of PKC in insulin-stimulated GLUT 4 translocation and glucose transport has been suggested since PKC activators like phorbol 12-myristate 13-acetate partly mimic the insulin effect on these processes [19–22], and inhibitors of PKC such as staurosporine, sangivamycin, polymyxine B, sphingosine and H7 have been shown to inhibit insulin-stimulated glucose transport both in muscle and adipose tissue [23–28]. However, these inhibitors lack the specificity required as PKC inhibitors compared with the highly specific peptide inhibitor used in the present study, which has been shown to inhibit glucose uptake in adipocytes [29]. We have therefore also examined whether insulin-stimulated GLUT 4 translocation can be observed in permeabilized adipocytes, and employed the peptide inhibitor to analyse whether PKC may be involved in this phenomenon.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP was from Amersham, Bucks., UK while protein A (^{125}I) was from ICN, Pharmaceutics Inc., Irvine, CA, USA. Streptolysin-O (SLO) was from RBI, (Research Biochemicals International), Natick, MA, USA. Collagenase, leupeptin, ATP and reagents for lactic acid dehydrogenase (LDH) determination were from Sigma, St. Louis, MO, USA. 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 comprising the COOH-terminal amino acid residues 58–72 of phospholemman (PLM) [15] (GTFRSSIRRLSTRRR, in single letter code) was prepared at the Peptide Synthesis Facility, Forskningsparken, University of Oslo, Norway. A PKC pseudosubstrate peptide (PKC19–36) (RFARKGALRQKNVHEVKN) [16] and a PKA inhibitor peptide, PKI [5–24] ('Walsh' inhibitor) were gifts from Dr. A. Czernik, The Rockefeller University, New York. Anti-PKC$_\beta$ monoclonal antibody (mAb) was from Transduction Laboratories, Affinity Research Products Ltd., Mamhead, Exeter, UK. Rabbit anti glucose transporter, insulin regulatable (GLUT 4, IRGT) was from East Acres Biologicals, Southbridge, MA, USA.

2.2. Cell preparation

Adipocytes were isolated from epididymal fat pads from 8–12 rats by the method of Rodbell [30]. Following dissection the fat pads were minced and incubated in 'extracellular' buffer containing 129 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4 with 20 mM HEPES (pH 7.4) containing 2 mM Na-pyruvate and 2% (w/v) BSA. Collagenase was added and incubation continued for 90 min at 37°C. The cells were filtered through nylon screen and

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washed 4 times by flotation and resuspension in 'extracellular' buffer. The cells were then washed once in 'intracellular' buffer containing 120 mM KCl, 4 mM NaCl, 10 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$, 1 mM EGTA, leupeptin (10 μg/ml), 20 mM HEPES buffer (pH 7.4), 2 mM Na-pyruvate and 2% BSA, resuspended in 'intracellular' buffer, and aliquots were removed for incubation with SLO (2 U/ml). In preliminary experiments LDH was rapidly released from the permeabilized adipocytes by this treatment, and after 30 s incubation at 37°C, extracellular LDH approached the level seen following detergent treatment of cells with 1% Triton-X-100 (not shown).

2.3. Determination of protein kinase activity

The assay was done essentially as described by Alexander et al. [14]. Washed cell suspensions (185 μl) in 'intracellular' buffer were incubated with 2 U/ml SLO at 37°C in plastic vials containing (final conc.): 250 μM peptide substrate, 800 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, without or with 1–40 μM PKC19–36 pseudosubstrate peptide inhibitor, and 20 mM HEPES, (pH 7.4) in the absence or presence of 100 nM insulin in a total volume of 300 μl. Reactions were terminated after the times indicated by addition of 120 μl 25% (w/v) trichloroacetic acid in 2 M acetic acid. Following centrifugation for 10 min, aliquots (30 μl) of the supernatants were spotted on P81 phosphocellulose chromatography paper (Whatman), the papers were washed three times in 30% (v/v) acetic acid containing 1% (v/v) phosphoric acid for 30 min, and once in methanol, and radioactivity of the paper squares were determined by Cerenkov counting. Tubes containing no peptide substrate were included in the experiments to estimate 'back ground' phosphorylation.

2.4. Determination of translocation of the insulin-regulated glucose transporter GLUT 4

Washed cell suspensions were suspended in 'intracellular' buffer containing SLO (2 Unit/ml), 2 mM Na-pyruvate, 200 nM adenosine, 5 mM ATP and leupeptin (10 μg/ml). Incubation was done at 37°C for 10 min in plastic vials in the absence or presence of 100 nM insulin and with addition of various concentrations of PKC pseudosubstrate peptide (19–36) or antibodies against PKC_β as reported in the text. Following flotation by centrifugation, the cells were washed once in homogenization buffer containing 250 mM sucrose, 10 mM NaF, 10 mM Na-pyrophosphate, leupeptin (10 μg/ml) in 25 mM Tris-HCl buffer, (pH 7.4), suspended in the same buffer, and subcellular fractionation done by the method of Karnieli et al. [31] to prepare plasma membranes (PM) and total intracellular membranes (ICM). The fractions were suspended in 3% (w/v) SDS 'stop' solution, boiled for 2 min, and after protein determination using color reagent from Pierce Chemical Co., aliquots containing 50 μg protein were subjected to SDS-PAGE. Proteins were blotted on to polyvinylidene difluoride (PVDF) membranes, and immunoblotting analysis was done with antibodies raised against the C-terminal peptide of GLUT 4. Relative amounts of GLUT 4 were revealed by using protein A (^{125}I) and quantification by excising appropriate bands followed by γ-radiation counting.

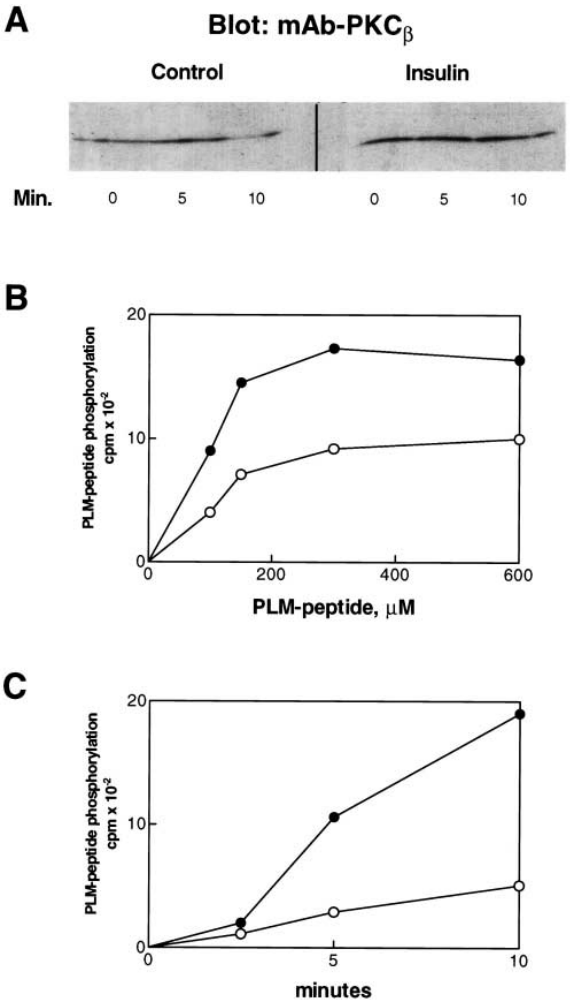

Fig. 1. Insulin stimulates protein kinase activity in SLO-permeabilized adipocytes. (A) PKC was identified in SLO-permeabilized adipocytes by Western immunoblotting with a monoclonal PKC_β neutralizing antibody as a 80-kDa band. Following incubation of the adipocytes in the absence and the presence of insulin, a 100 000×*g* membrane pellet was solubilized in SDS, subjected to SDS-PAGE and transferred to PVDE blot as described in Section 2. (B) Insulin stimulates PLM-peptide phosphorylation in SLO-permeabilized adipocytes in a concentration-dependent manner. Assays were performed following 5 min incubation at 37°C without – ○ – or with – ● – 100 nM insulin. (C) Time course of PLM-peptide phosphorylation in the absence – ○ – or the presence – ● – of 100 nM insulin at 250 μM PLM-peptide concentration. The details are described in Section 2.

3. Results

3.1. Protein kinase activity

Following incubation of SLO-permeabilized adipocytes in the absence or presence of insulin, PKC_β was identified in a 100 000×*g* membrane fraction by Western immunoblotting with a PKC_β monoclonal antibody as an 80-kDa band, the amount of which was increased after incubation of the cells with insulin (Fig. 1A). SLO-treated adipocytes were also able to catalyze phosphorylation of the PLM-peptide under basal conditions, and the extent of this phosphorylation was increased following incubation with insulin (Fig. 1B). Following a lag period of about 2 min, which may be attributed to activation of the insulin signalling cascade pathway, this effect of insulin was seen during 2–10 min incubation, and represented an 1–3-fold increase in PLM-peptide phosphorylation above control (Fig. 1C). Insulin is known to stimulate the activity of a series of serine/threonine kinases [32,33] and in order to detect whether a putative insulin-stimulated PKC was responsible, the PKC-specific peptide inhibitor was employed. When the PKC (19–36) peptide was included in the assay at a concentration of 40 μM the level of phosphorylation induced by insulin was reduced to control level and there was also a partial inhibition of the control level of PLM peptide phosphorylation (Fig. 2A), in agreement with the presence of PKC in the control membrane as shown in Fig. 1A. The inhibition of the insulin-stimulated PLM peptide phosphorylation was concentration-dependent, with an apparent IC_{50} of 5 μM PKC (19–36) peptide (Fig. 2B). Moreover, a monoclonal PKC_β neutralizing antibody present during incubation blocked insulin-stimulation of PLM-peptide phosphorylation (Fig. 2C). These results strongly indicate that the stimulation

of PLM peptide phosphorylation by insulin solely is mediated by activation of PKC in adipocytes in situ.

3.2. GLUT 4 translocation

In accordance with previous work [34] the biochemical system mediating insulin regulation of GLUT 4 translocation was found to be retained in SLO-permeabilized adipocytes. In the presence of insulin the content of GLUT 4 was increased by 3–4 fold in PM, and decreased by 30% in ICM

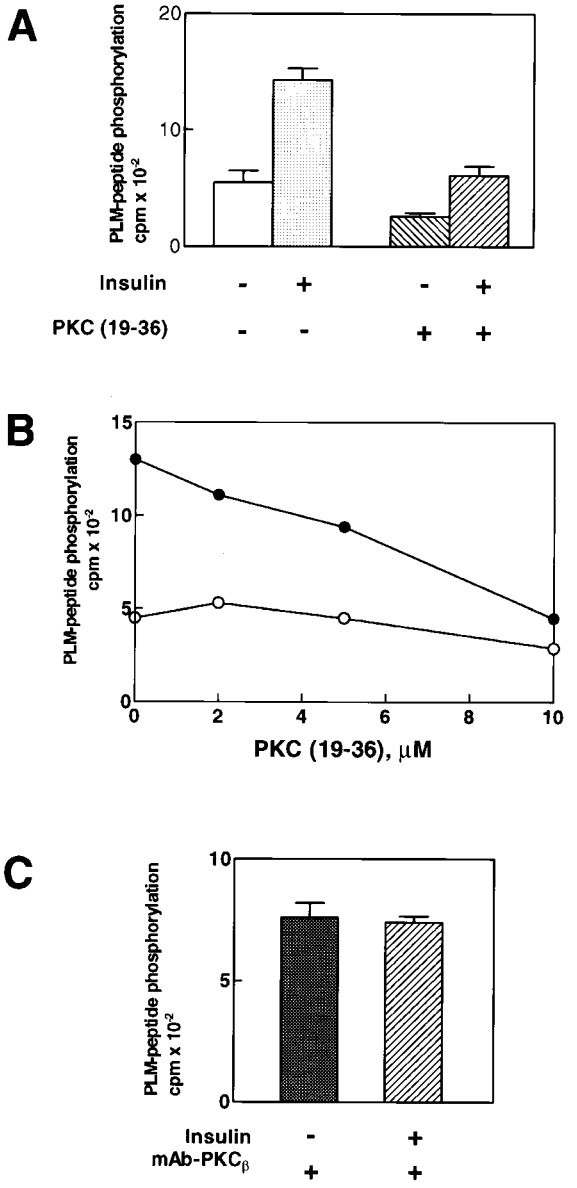

Fig. 2. The PKC-peptide (19–36) and a monoclonal PKC_β neutralizing antibody inhibit insulin-stimulated PLM-peptide phosphorylation in SLO-permeabilized adipocytes. (A) The PKC-peptide (19–36) at a concentration of 20 μM inhibits insulin-stimulated PLM-peptide phosphorylation. Assays carried out following 10 min incubation at 37°C and with 250 μM PLM-peptide concentration ($N=5$). (B) PKC-peptide (19–36) inhibits insulin-stimulated PLM-peptide phosphorylation in a concentration-dependent manner. Incubation without – ○ – or with – ● – insulin. The data are representative of four individual experiments with similar results. (C) A monoclonal PKC_β neutralizing antibody at a concentration of 5 μg/ml blocked insulin-stimulated PLM-phosphorylation ($N=4$).

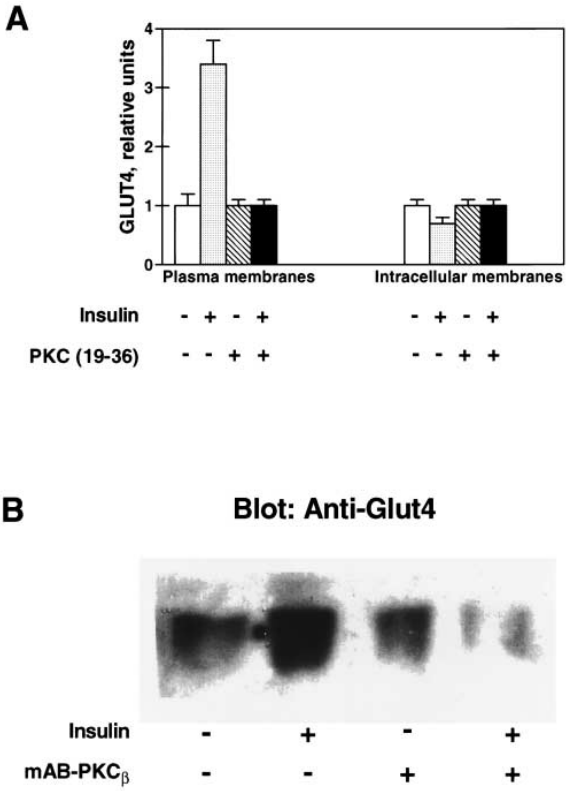

Fig. 3. Insulin-mediated translocation of GLUT 4 from ICM to PM is inhibited by PKC-peptide (19–36) and by a monoclonal PKC_β neutralizing antibody. (A) SLO-permeabilized adipocytes were incubated for 10 min at 37°C in the absence or the presence of 100 nM insulin, and GLUT 4 in PM and ICM isolated as described in Section 2. In the presence of 20 μM PKC-peptide (19–36) insulin-mediated translocation of GLUT 4 was blocked. (B) SLO-permeabilized adipocytes were incubated in the absence or the presence of insulin, and without or with monoclonal PKC_β neutralizing antibody (5 μg/ml) for 10 min. GLUT 4 in PM was isolated and blotted on to PVDF membranes as described in Section 2.

(Fig. 3A). This insulin-induced GLUT 4 translocation was completely prevented in the presence of 40 μM PKC (19–36) peptide. The specificity of this inhibitory effect was confirmed since 40 μM of the PKA inhibitor peptide PKI [5–24] had no effect (not shown). Inhibition of the insulin mediated increase of GLUT 4 in PM by the PKC inhibitor peptide was concentration-dependent with an apparent IC_{50} of 2 μM (Fig. 4). Similarly, the insulin mediated decrease in GLUT 4 in ICM was inhibited at an apparent IC_{50} concentration of 4 μM (Fig. 5). These results strongly indicate that PKC is involved in the insulin signalling system for stimulation of GLUT 4 translocation. Further support for this statement was obtained since the insulin-mediated translocation of GLUT 4 was strongly inhibited by a monoclonal PKC_β neutralizing antibody (Fig. 3B).

4. Discussion

The involvement of PKC in mediating insulin effects has remained a controversial problem for several years. Such problems may partly be caused by techniques used for extraction of membrane and cytosolic fractions followed by in vitro determination of PKC activity. The PKC enzyme family contains in the C_1 domain an autoinhibitory pseudosubstrate

sequence which keeps the enzyme in the inactive form [35], and which is removed by the lipid PKC activators phosphatidylserine and diacylglycerol [36]. Extraction conditions comprising detergent treatment and chromatography might therefore affect both partition of PKC in membranes and cytosol and the basal state of activity, and in vitro determination of PKC activity may therefore not necessarily reflect true PKC activity in situ in the cell. The cell permeabilization technique used in the present investigation, and which allows measurement of the activity of membrane-stabilized PKC in situ by internalization of specific substrates and inhibitors, appears to eliminate these problems.

Our study shows that adipocytes contain an insulin-stimulated protein kinase which phosphorylates a PLM-peptide which is known as a good substrate for PKC in vitro [15]. The phosphorylation of this PLM-peptide was blocked by the PKC pseudosubstrate peptide (PKC19–36). This compound is known as a highly specific inhibitor of PKC, with inhibition of phosphorylation of a peptide derived from glycogen synthase occurring at an IC_{50} concentration of 0.2 μM [16], and with inhibition of PKC activity in cytosolic cell extracts in vitro occurring at an apparent IC_{50} concentration of 1 μM, while other protein kinases were not inhibited at concentrations below 1 mM [17]. In our study the insulin-stimulated PLM-peptide phosphorylation was inhibited by the PKC inhibitor peptide at concentrations comparable with inhibition of PKC activity in cytosolic cell extracts in vitro, displaying an apparent IC_{50} concentration of 5 μM. Hence, our results strongly indicates that adipocytes contain an insulin-sensitive

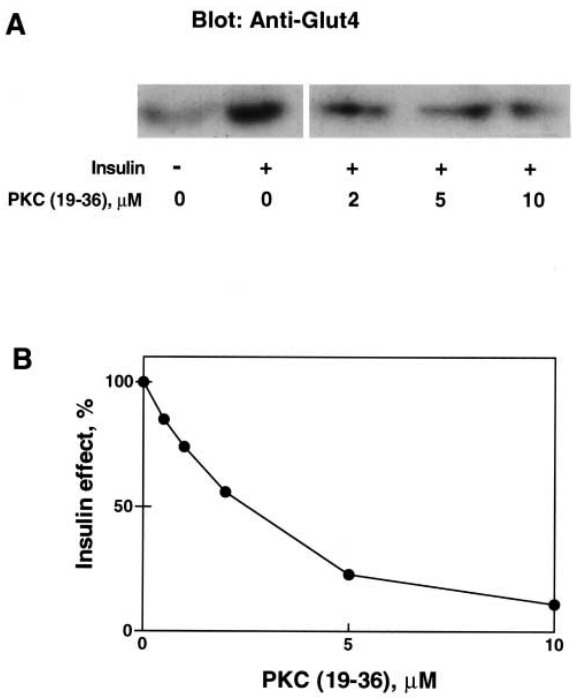

Fig. 4. Concentration-dependent inhibition of insulin-mediated increase of GLUT 4 in PM in SLO-permeabilized adipocytes by PKC-peptide (19–36). SLO-permeabilized adipocytes were incubated as described in Fig. 3A and GLUT 4 in PM isolated as described in Section 2. The GLUT 4 bands on the autoradiogram (A) were cut out and quantitated by γ-radiation counting (B). The data are representative of four individual experiments with similar results. The ^{125}I radioactivity incorporated into the GLUT 4 band was 245 cpm (control) and 808 cpm (insulin).

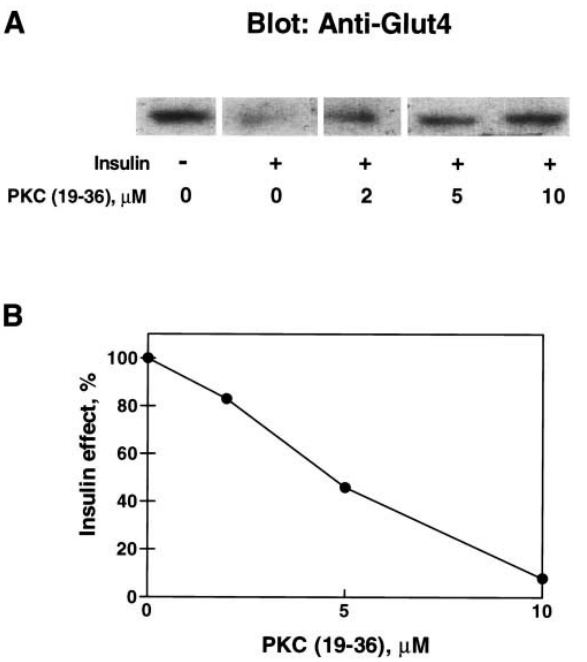

Fig. 5. Concentration-dependent inhibition of insulin-mediated decrease of GLUT 4 in ICM in SLO-permeabilized adipocytes by PKC-peptide (19–36). Following incubation as described in Fig. 3, GLUT 4 in ICM was isolated as described in Section 2. The GLUT 4 bands on the autoradiogram (A) were cut out and quantitated by γ-radiation counting (B). The data are representative of three individual experiments with similar results. The ^{125}I radioactivity incorporated into the GLUT 4 band was 533 cpm (control) and 263 cpm (insulin).

PKC and that this enzyme can phosphorylate PKC-substrates in situ. This is in agreement with our previous work on intact muscle where insulin increased phosphorylation of phospholemman at serine-63 and serine-68, both sites phosphorylated by PKC in vitro [37,15].

Previous studies have shown that vesicle-mediated translocation of GLUT 4 to PM persists in permeabilized adipocytes. Insulin stimulated GLUT 4 translocation in α-toxin treated adipocytes [38], and in SLO-permeabilized 3T3-L1 adipocytes insulin-mediated GLUT 4 translocation was seen if a source of ATP was added [34]. This was confirmed in our work on SLO-permeabilized adipocytes, with 3–4-fold increase of insulin-stimulated GLUT 4 translocation to PM, determined by differential centrifugation method [31]. Recent studies indicate that phosphatidyl-inositol-3-kinase is of central importance in the GLUT 4 vesicle translocation process [39–41]. Other studies have shown that in addition to a phosphatidylinositol-3-kinase (Vps34) a serine protein kinase (Vps15) is an essential component in regulating transmembrane protein sorting in yeast [42]. Therefore, we have investigated if protein kinase activity also may be involved in GLUT 4 translocation in adipocytes. Our work demonstrates that insulin-mediated translocation of GLUT 4 from an ICM pool to PM can be seen in the permeabilized adipocytes, and that this effect is inhibited by the PKC pseudosubstrate inhibitor at similar concentration levels as shown for inhibition of insulin stimulated PKC activity in the adipocytes. The specificity of the effect of the PKC-peptide (19–36) inhibitor was confirmed since a PKI inhibitor peptide (5–24) had no effect on insulin stimulation of protein kinase activity or GLUT 4 transloca-

tion (not shown). Moreover, the insulin-stimulation of protein kinase activity as well as the insulin-mediated GLUT 4 translocation was blocked by a PKC_{β} neutralizing monoclonal antibody. These results indicate a requirement for insulin stimulation of PKC activity in the insulin signalling pathway of translocation of GLUT 4. In conclusion, our work are consistent with the hypothesis that insulin can stimulate PKC activity in adipocytes in situ, and that this activation of PKC is a component in the regulation of GLUT 4 vesicle translocation which occurs in response to insulin.

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References

[1] Grunberger, G. (1991) Cell. Signal. 3, 171–177.
[2] Farese, R.V., Standaert, M.L., Arnold, T., Yu, B., Ishizuka, T., Hoffman, J., Vila, M. and Cooper, D.R. (1992) Cell. Signal. 4, 133–143.
[3] Considine, R.V. and Caro, J.F. (1993) J. Cell. Biochem. 52, 8–13.
[4] Walaas, S.I., Horn, R.S., Adler, A., Albert, K.A. and Walaas, O. (1987) FEBS Lett. 220, 311–318.
[5] Cooper, D.R., Konda, T.S., Standaert, M.L., Davis, J.S., Pollet, R.J. and Farese, R.V. (1987) J. Biol. Chem. 262, 3633–3639.
[6] Egan, J.J., Saltis, J., Wek, S.A., Simpson, I.A. and Londos, C. (1990) Proc. Natl. Acad. Sci. USA 87, 1052–1056.
[7] Walaas, O., Parker, P., Horn, R. and Walaas, I. (1992) Acta Endocrinol. 126, suppl. 3, 11.
[8] Yamada, K., Avignon, A., Standaert, M.L., Cooper, D.R., Spencer, B. and Farese, R.V. (1995) Biochem. J. 308, 177–180.
[9] Glynn, B.P., Colliton, J.W., McDermott, J.M. and Witters, L.A. (1986) Biochem. Biophys. Res. Commun. 135, 1119–1125.
[10] Vaartjes, W.J., de Haas, C.G.M., Chang, K., Sherman, W.R. and Turk, J. (1986) Biochem. Biophys. Res. Commun. 138, 1328–1333.
[11] Spach, D.H., Nemenoff, R.A. and Blackshear, P.J. (1986) J. Biol. Chem. 261, 12750–12753.
[12] Blackshear, P.J., Haupt, D.M. and Stumpo, D.J. (1991) J. Biol. Chem. 266, 10946–10952.
[13] Alexander, D.R., Hexham, J.M., Lucas, S.C., Graves, J.D., Cantrell, D.A. and Crumpton, M.J. (1989) Biochem. J. 260, 893–901.
[14] Alexander, D.R., Graves, J.D., Lucas, S.C., Cantrell, D.A. and Crumpton, M.J. (1990) Biochem. J. 268, 303–308.
[15] Walaas, S.I., Czernik, A.J., Olstad, O.K., Sletten, K. and Walaas, O. (1994) Biochem. J. 304, 635–640.
[16] House, C. and Kemp, B.E. (1987) Science 238, 1726–1728.
[17] Meier, K.E., Licciardi, K.A., Haystead, T.A. and Krebs, E.G. (1991) J. Biol. Chem. 266, 1914–1920.
[18] Holman, G.D. and Cushman, S.W. (1994) Bioessays 16, 753–759.
[19] Mühlbacher, C., Karnieli, E., Schaff, P., Obermaier, B., Mushack, J., Rattenhuber, E. and Häring, H.U. (1988) Biochem. J. 249, 865–870.
[20] Vogt, B., Mushack, J., Seffer, E. and Häring, H.U. (1991) Biochem. J. 275, 597–600.
[21] Gibbs, E.M., Calderhead, D.M., Holman, G.D. and Gould, G.W. (1991) Biochem. J. 275, 145–150.
[22] Saltis, J., Habberfield, A.D., Egan, J.J., Londos, C., Simpson, I.A. and Cushman, S.W. (1991) J. Biol. Chem. 266, 261–267.
[23] Holman, G.D., Kozka, I.J., Clark, A.E., Flower, C.J., Saltis, J., Habberfield, A.D., Simpson, I.A. and Cushman, S.W. (1990) J. Biol. Chem. 265, 1872–1879.
[24] Robertson, D.G., DiGirolamo, M., Merrill Jr., A.H. and Lambeth, J.D. (1989) J. Biol. Chem. 264, 6773–6779.
[25] Standaert, M.L., Buckley, D.J., Ishizuka, T., Hoffman, J.M., Cooper, D.R., Pollet, R.J. and Farese, R.V. (1990) Metabolism 39, 1170–1179.
[26] Tanti, J.F., Rochet, N., Gremeaux, T., Van Obberghen, E. and Le Marchand-Brustel, Y. (1989) Biochem. J. 258, 141–146.
[27] Christensen, R.L., Shade, D.L., Graves, C.B. and McDonald, J.M. (1987) Int. J. Biochem. 19, 259–265.
[28] Nishimura, H. and Simpson, I.A. (1994) Biochem. J. 302, 271–277.
[29] Standaert, M.L., Sasse, J., Cooper, D.R. and Farese, R.V. (1991) FEBS Lett. 282, 139–142.
[30] Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
[31] Karnieli, E., Zarnowski, M.J., Hissin, P.J., Simpson, I.H., Salans, L.B. and Cushman, S.W. (1981) J. Biol. Chem. 256, 4772–4777.
[32] Czech, M.P., Klarlund, J.K., Yagaloff, K.A., Bradford, A.P. and Lewis, R.E. (1988) J. Biol. Chem. 263, 11017–11020.
[33] Walaas, O., Walaas, E., Lystad, F., Alertsen, Aa.R. and Horn, R.S. (1979) Mol. Cell. Endocrinol. 16, 45–55.
[34] Robinson, L.J., Pang, S., Harris, D.S., Heuser, J. and James, D.E. (1992) J. Cell. Biol. 117, 1181–1196.
[35] Newton, A.C. (1995) J. Biol. Chem. 270, 28495–28498.
[36] Orr, J.W., Keranen, L.M. and Newton, A.C. (1992) J. Biol. Chem. 267, 15263–15266.
[37] Walaas, O., Horn, R.S. and Walaas, S.I. (1991) Biochim. Biophys. Acta 1094, 92–102.
[38] Baldini, G., Hohman, R., Charron, M.J. and Lodish, H.F. (1990) J. Biol. Chem. 266, 4037–4040.
[39] Cheatham, B., Vlahos, C., Cheatham, L., Wang, L., Blenis, J. and Kahn, C.R. (1994) Mol. Cell. Biol. 14, 4902–4911.
[40] Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) J. Biol. Chem. 269, 3568–3573.
[41] Heller-Harrison, R.A., Morin, M., Guilherme, A. and Czech, M.P. (1996) J. Biol. Chem. 271, 10200–10204.
[42] Stack, J.H., Herman, P.K., Schu, P.V. and Emr, S.D. (1993) EMBO J. 12, 2195–2204.