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Comparative Effects of PKB-α and PKC-ζ on the Phosphorylation of GLUT4-Containing Vesicles in Rat Adipocytes

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Insulin stimulates glucose transport in muscle and fat cells by promoting the translocation of glucose transporter (GLUT4) to the cell surface. Phosphatidylinositide 3-kinase (PI3-kinase) has been implicated in this process. However, the involvement of protein kinase B (PKB)/Akt and PKC-ζ, those are known as the downstream target of PI3-kinase in regulation of GLUT4 translocation, is not known yet. An interesting possibility is that these protein kinases phosphorylate GLUT4 directly in this process. In the present study, PKB-α and PKC-ζ were added exogenously to GLUT4-containing vesicles purified from low density microsome (LDM) of the rat adipocytes by immunoadsorption and immunoprecipitation for direct phosphorylation of GLUT4. Interestingly GLUT4 was phosphorylated by PKC-ζ and its phosphorylation was increased in insulin stimulated state but GLUT4 was not phosphorylated by PKB-α. However, the GST-fusion proteins, GLUT4 C-terminal cytoplasmic domain (GLUT4C) and the entire major GLUT4 cytoplasmic domain corresponding to N-terminus, central loop and C-terminus in tandem (GLUT4NLC) were phosphorylated by both PKB-α and PKC-ζ. The immunoblots of PKC-ζ and PKB-α antibodies with GLUT4-containing vesicles preparation showed that PKC-ζ was co-localized with the vesicles but not PKB-α. From the above results, it is clear that PKC-ζ interacts with GLUT4-containing vesicles and it phosphorylates GLUT4 protein directly but PKB-α does not interact with GLUT4, suggesting that insulin-elicited signals that pass through PI3-kinase subsequently diverge into two independent pathways, an Akt pathway and a PKC-ζ pathway, and that later pathway contributes, at least in part, insulin stimulation of GLUT4 translocation in adipocytes via a direct GLUT4 phosphorylation.

Key Words: PI3-kinase, PKC-ζ, PKB-α, Phosphorylation, GLUT4, GLUT4-containig vesicles, Translocation, Insulin, GST-fusion proteins

INTRODUCTION

Insulin stimulates glucose transport in adipose tissue and skeletal muscle by increasing the translocation of the insulin responsive glucose transporter, GLUT4, from an intracellular pool to the plasma membrane (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Bell et al, 1993; Kandror & Pilch, 1996). In GLUT4 translocation mechanism, the protein phosphorylation is known to be an important step (Piper et al, 1993) in which PI3-kinase activation is recognized to be required (Cheatham et al, 1994; Okada et al, 1994; Haruta et al, 1995; Kotani et al, 1995; Yang et al, 1996; Czech & Corvera, 1999; Olefsky, 1999). However, downstream effectors of PI3-kinase are still uncertain. Recently, two types of serine-threonine kinase have been shown to act downstream of PI3-kinase. One of these kinases is Akt (also known as protein kinase B) that contains a pleckstrin homolog domain that binds directly to phosphatidylinositol 3, 4-biphosphate, which is one of the products of PI3-kinase activity *in vivo* and stimulates kinases activity *in vitro* (Franke et al, 1995; Klippel et al, 1997). Another downstream target of the insulin-regulated PI3-kinase is protein kinase C (PKC) isoform zeta, PKC-ζ (Czech & Corvera, 1999) that is an atypical PKC (aPKC) family (Nakanishi et al,

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1993; Le Good et al, 1998). Recently insulin is reported to activate aPKC in 3T3/L1 adipocytes (Bandyopadhyay, 1997a; Kotani et al, 1998), rat adipocytes (Standaert et al, 1997), L6 myotubes (Bandyopadhyay, 1997b), and 32D cells (Mendez et al, 1997). The increase in aPKC enzyme activity appears to be largely dependent upon activation of PI3-kinase, and PKC-ζ is activated by phosphatidylserine (PS) and polyphosphoinositides, i.e. phosphatidylinositol 3,4,5-$(PO_4)_3(PIP_3)$ (Nakanishi et al, 1993; Palmer et al, 1995). It has also been reported that inhibitors of PKC-ζ concomitantly inhibit insulin effects on glucose transport in rat adipocytes (Standaert et al, 1997) and L6 myotubes (Bandyopadhyay et al, 1997b), and the stable expression of a kinase-inactive (KI) form of PKC-ζ inhibits insulin-stimulated GLUT4 translocation and glucose transport in transpected 3T3/L1 cells (Bandyopadhyay et al, 1997a) and L6 myotubes (Bandyopadhyay et al, 1997b).

Furthermore, the activation of atypical PKC induced by either insulin or bacterial lipopolysaccharide was shown to be inhibited by either a pharmacological inhibitor or a dominant negative mutant of PI3-kinase (Herrera-Velit et al, 1997; Mendez et al, 1997; Standaert et al, 1997). All of these observations indicate that atypical PKC isozymes are downstream effectors of PI3-kinase. However, the specific downstream targets of PI3-kinase for GLUT4 translocation have remained questionable with evidences both for Akt and PKC-ζ (Kohn et al, 1996; Cong et al, 1997; Standert et al, 1997; Kitamura et al, 1998).

Phosphorylation of certain membrane proteins and receptors has been known to facilitate their binding to adapters and subsequent clathrin-coated vesicle formation in their recycling (De Fronzo et al, 1981; Kandror & Pilch, 1994). As for the potential role of protein kinases in insulin-induced GLUT4 redistribution, it is an interesting possibility that these protein kinases phosphorylate GLUT4 and then increase GLUT4 binding to the clathrin-adaptors, AP-2 and AP-1 for clathrin-coated vesicle formation at the plasma membrane and intracellular membrane, respectively.

The present study tested whether these protein kinases phosphorylate GLUT4 directly using both a gulthathiones S-transferase (GST) fusion protein containing the cytosolic portion of GLUT4 and GLUT4 containing vesicles purified from LDM of rat adipocytes.

METHODS

Materials

Collagenase (Type I) was obtained from Worthington (Freehold, NI). Insulin (porcine crystalline) and bovine serum albumin were from Sigma (St. Louis, MI). 1F8 monoclonal antibody was purchased from Biogenesis (Brentwood, NH). PKC-ζ, Akt, PKC-ζ-pseudosubstrate, Mg^{2+}/ATP, ADB II were from Upstate Biotechnology (Lake Placid, NY). PS and PIP_3 were from Matreya Inc. (Pleasant Gap, PA). (γ-^{32}P) ATP was from NEN Life Science Products (Boston, MA). ECL Western Blotting detection reagent was from Amersham Pharmacia Biotech (Buckinghamshire, England). Horseradish-peroxidase (HRP)-protein A was from Zymed (San Francisco, CA). Reacti-gel (GF-2000) was purchased from Pierce (Rockfrod, IO). All other reagents were from sources stated below and were of reagent grade.

Subcellular fractionation and purification of GLUT4-containing vesicles

Adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rat weighing 180~220 g as described (Rampal et al, 1995). Briefly, total membranes were fractionated to plasma membrane enriched fraction (PM), high density microsome (HDM) and low density microsome (LDM) by differential centrifugation (Simpson et al, 1983; Rampal et al, 1995). To get GLUT4-containing vesicles, protein A purified 1F8 antibody (James et al, 1988) as well as nonspecific normal mouse IgG were coupled each to Trisacryl beads (Reacti-Gel, GF-2000) at a concentration of 0.7 mg of antibody /ml of resin according to the manufacturer's instruction. The antibodies coupled on beads were quenched by 2 M Tris, pH 8.0, for 1 h in room temperature; incubated with 2% bovine serum albumin in phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 2 h also at room temperature to block nonspecific binding; and washed five times with 1ml of PBS. LDMs (200 μg of protein) were then incubated with 50 μg of beads overnight at 4°C. The beads were settled down spontaneously in ice bucket, and unbound supernatants were collected for analysis. The beads bound GLUT4-containing vesicles were washed five times with 1 ml of PBS and twice with phosphorylation buffer at 4°C,

and then the beads were used for phosphorylation experiment as described in the following paragraph.

Preparation of GST-GLUT4 fusion protein

Construct for the GLUT4 C-terminal cytoplasmic domain (GLUT4C) or the entire major GLUT4 cytoplasmic domain corresponding to N-terminus, the loop between transmembrane helices 6 and 7 (central loop) and C-terminus in tandem (GLUT4NLC) was cloned into pGEX-3X, a plasmid designed to produce GST fusion proteins. The fusion proteins were purified from cultured DH5 αa cells by adsorption to glutathione-agarose beads. The purified fusion protein was eluted from glutathione-agarose beads and stored in phosphorylation buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 μM $CaCl_2$, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride and 100 μM phenylmethylsulfonyl fluoride (PMSF).

Immunoadsorption of GLUT4-containing vesicles

Purification of GLUT4 protein by immunoprecipitation using Trisacryl beads bound 1F8 monoclonal antibody from 1F8 immunoadsorbed GLUT4-containing vesicles was performed in the presence of 1% Triton X-100 in PBS. The GLUT4-containing vesicles were solubilized in PBS buffer containing 1% Triton X-100 and 10% glycerol for overnight at 4°C with rotary mixer. The supernatant and Trisacryl beads were separated and the beads were washed three times with PBS buffer before use in phosphorylation experiment or SDS/PAGE.

In vitro phosphorylation of GLUT4-containing vesicles

A reaction mixture for phosphorylation consists of 50 μl of beads immunoadsorbed GLUT4-containing vesicles, 50 μl of phosphorylation buffer, 5 μl of PS (0.5 mg/ml) and 5 μl of PIP_3, dipalmitoyl (0.5 mg/ml), 10 μl of human recombinant PKC-ζ (10 ng/μl), and [γ-^{32}P] ATP (50 μl, 3,000 Ci/mmol). In the case of PKB phosphorylation experiment, 20 μl of PKB-α recombinant protein expressed in Sf9 cells were added instead of PKC-ζ. The phosphorylation assay was started by adding [γ-^{32}P] ATP into the reaction mixture and incubated for 30 min at room temperature with vigorous shaking. Beads were then precipitated and washed two times with phosphorylation buffer. The immunoadsorbed materials were eluted with the same volume of double concentrated Laemmli sample buffer without 2-mercaptoethanol containing 0.125 M Tris-HCl, 4% sodium dodecylsulfate (SDS), 20% glycerol and appropriate amount of pyronin-X. The eluted solutions were then added by 1% mercaptoethanol and electrophoresed onto 10% acrylamide/glycine gel with 130 volts. Gels were transferred to nitrocellulose (NC) membrane with 105 volts for 1.5 h. The NC membranes were wrapped with Saran wrap and exposed overnight in a storage phosphor screen cassette, and phosphor images were quantitated in a PhosphorImager (Storm, Molecular Dynamics). Alternately, phosphorylated proteins were electrophoresed, and dried gels were used for phosphor image scanning in the PhosphorImager. Phosphorylation was assessed by measuring ^{32}P incorporation of GLUT4 protein by exogenously administered PKB-α and PKC-ζ.

In vitro phosphorylation of GST-GLUT4 fusion proteins

Phosphorylation of GST-GLUT4 fusion proteins (20 μg) was carried out in a 150 μl of reaction mixture containing 10 μl of PS (0.5 mg/ml), 5 μl of PIP_3 (0.5 mg/ml), 10 μl of human recombinant PKC-ζ (10 ng/μl), 30 μl of [γ-^{32} P] ATP (50 mM, 3,000 Ci/mmol), and phosphorylation buffer. Phosphorylation assay was started by adding [γ-^{32}P] ATP into the reaction mixture and incubated for 30 min at room temperature with shaking. After incubation, the glutathione agarose beads were washed with phosphorylation buffer and phosphorylated GLUT4 proteins were eluted with Laemmli sample buffer containing 1% mercaptoethanol for 2 h with vigorous shaking. The eluted solutions were electrophoresed on 10% acrylamide/glycine gel with 150 volts, and transferred to NC membrane with 105 volts for 1.5 h. NC membrane was dried, wrapped with Saran wrap, and put in a storage phosphor screen cassette overnight. Phosphorylation was determined by phosphor image quantitated in a Phosphor Imager. In order to see whether phosphorylation of GST-GLUT4 fusion proteins is dependent on protein amounts, different doses of proteins (2.5, 5.0, 10 and 20 μg) were applied to phosphorylation experiment and the effect of pseudosubstrate of PKC-ζ on the GLUT4 phosphorylation was also measured.

RESULTS

Phosphorylation of GLUT4-containing vesicles by PKC-ζ

In order to see insulin effects on the phosphorylation of GLUT4-containing vesicles, glucose flux experiment was done using an aliquot of adipocytes that were used in the preparation of GLUT4-containing vesicles just before used. Fig. 1 shows the 3-O-methyl glucose equilibrium exchange in the adipocytes. As shown here 3-O-methyl glucose flux increased above 5-fold in insulin treated cells. Then these adipocytes were used in the perparation of GLUT4-containing vesicles for the phosphorylation experiment. GLUT4-containing vesicles were immunoabsorbed from LDM of rat adipose cells treated with or without insulin, and PKC-ζ was administered exogenously to the material adsorbed on the beads in the presence of (γ-^{32}P) ATP as described in "Methods".

Under these conditions, radioactive phosphate was incorporated into GLUT4 protein in the vesicles and several other proteins were also phosphorylated (Fig. 2). Although phosphorylated proteins vary to some extent in different experiments, GLUT4 phosphorylation was consistently detected and increased on the vesicles immunoabsorbed from LDM of insulin-treated adipocytes based on both the phosphor image intensity and radioactivity counting of strips of NC membranes (Fig. 3 & Table 1). Moreover, GLUT4 phosphorylation was inhibited in the presence of PKC-ζ-pseudosubstrate and the GLUT4 phosphorylation was also increased in insulin-treated adipocytes (Fig. 4). According to a recent report (Kupriyanova & Kandror, 1999) all vesicular proteins with exceptions for GLUT4 can be solubilized in PBS containing 1% Triton X-100 and recovered in the Triton elute, whereas GLUT4 is resistant to Triton elution and can be removed from immunobeads only with SDS-containing Laemmli sample buffer. To further

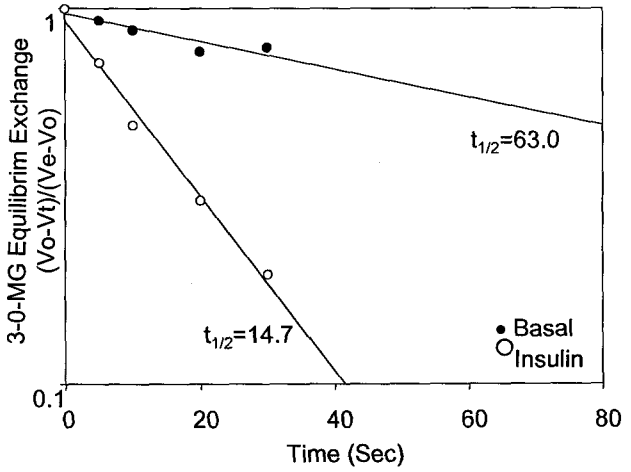

Fig. 1. Time course of the equilibrium exchange of 3-O-methyl glucose (3-OMG) by isolated adipocytes without (●) and with (○) 34 nM insulin. The concentration of 3-OMG was 10 mM and the flux was measured at 37°C. Results were plotted based on a model of tracer exchange in a closed, two component system (Vinten et al, 1976) where Vt, Ve and Vo denote cellular radioactivities for a given time, at the complete equilibration, and at the start of experiment, respectively. The curves represent this model computer-fitted to the data.

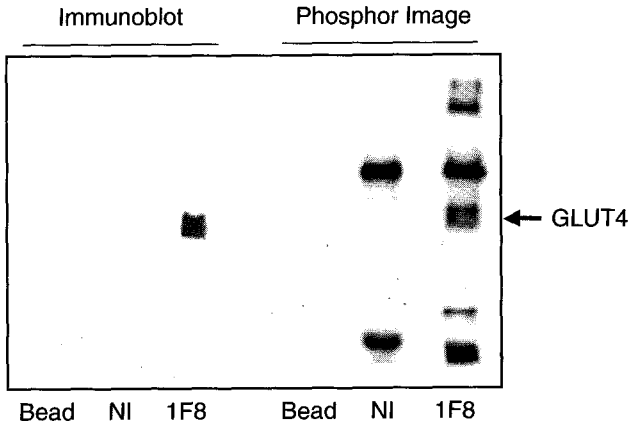

Fig. 2. Phosphorylation of GLUT4 protein purified from LDM of rat adipocytes by PKC-ζ *in vitro*. GLUT4-containing vesicles were isolated by immunoadsorption using 1F8 antibody and phosphorylated using [γ-^{32}P] ATP as described in "Methods". A reaction mixture for phosphorylation consists of 50 μl of PS (0.5 mg/ml) and 5 μl of PIP_3-dipalmitoyl (0.5 mg/ml), 10 μl of human recombinant PKC-ζ (10 ng/l), and [γ-^{32}P] ATP (50 μl, 3,000 Ci/mmol). Phosphorylation assay was started by adding into [γ-^{32}P] ATP into the reaction mixture and incubated for 30 min at room temperature with vigorous shaking. Beads were then washed and eluted with Laemmli sample buffer, electrophoresed, transferred to nitrocellulose membrane, immunoblotted with GLUT4 antibody (the blot is shown on the left). After immunoblot was complete, the membrane was exposed in a phosphorImager overnight, and the resulting autoradiogram is shown on the right. Bead: Trisacryl beads only, NI: immunoadsorpted with mouse IgG, 1F8: immunoadsorpted with 1F8.

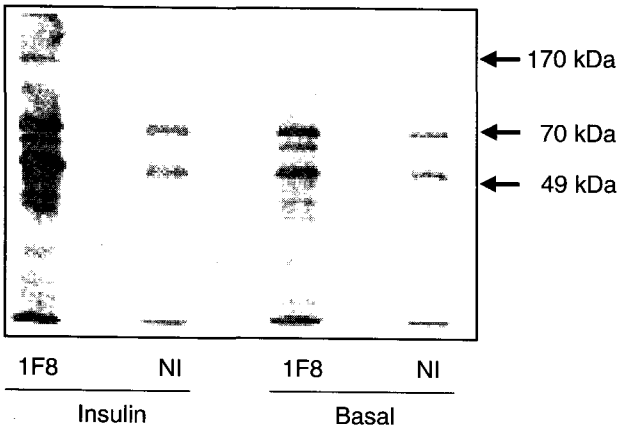

Fig. 3. Effects of insulin on the phosphorylation of GLUT4-containing vesicles. Phosphorylation was performed as in Fig. 2 legend. Phosphor image densities of phosphorylated GLUT4-containing vesicles prepared from adipocytes treated with or without insulin were compared each other. NI: immunoadsorpted with mouse IgG, 1F8: immunoadsorpted with 1F8.

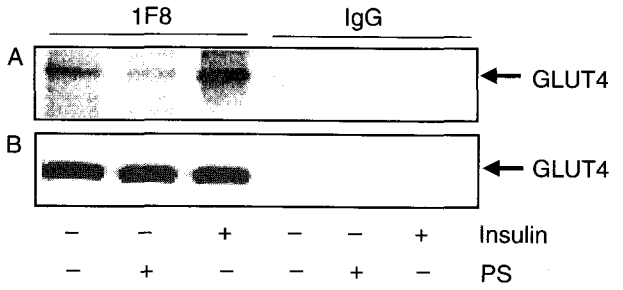

Fig. 4. Effects of PKC-ζ pseudosubstrate on the PKC-ζ induced phosphorylation of GLUT4 protein. Phosphorylation was performed as desrcibed in the presence or absence of 100 μM PKC-ζ pseudosubstrate (Upstate Biotechnology). In the case of absence, same volume of phosphorylation buffer was added. A. Phosphor Image of the phosphorylated GLUT4-containing vesicles. B. Immunoblot with GLUT4 antibody (polyclonal) of the same NC membrane used for Phosphor image scanning. PS: PKC-ζ pseudosubstrate, IgG: immunoadsorpted with mouse IgG, 1F8: immunoadsorpted with 1F8.

Table 1. Radioactivity of 32P incorporated to GLUT4-containing vesicle

		GLUT4 (cpm)	≅ 70 kDa (cpm)	≅ 165 kDa (cpm)
NI	Bas	578	410	138
	Ins	1185	1027	321
1F8	Bas	1076	875	204
	Ins	2166	1227	541

Numerical Value represents a mean value of two experiments, respectively. NI: non-immune group, 1F8: immune group used 1F8 antibody, Bas: basal state, Ins: insulin stimulated state.

identify GLUT4, phosphorylated GLUT4-containing vesicles were solubilized in 1% Triton X-100 and immunoprecipitated with Trisacryl bead bound 1F8 monoclonal antibody. As expected, phosphorylated GLUT4 was immunoprecipitated with 1F8 antibody from Triton elute. The phosphorylation increased at GLUT4 immunoprecipitated from the GLUT4-containing vesicles treated with insulin. This was confirmed by phosphor image of gel and immunoblot of the transferred NC membrane with GLUT4 antibody (Fig. 5).

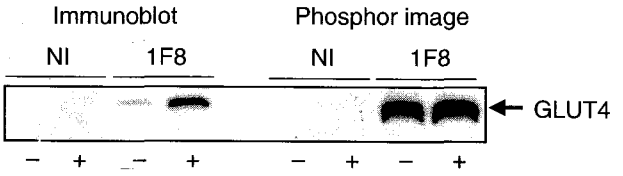

Fig. 5. Phosphorylation of GLUT4 immunoprecipitated in the presence of 1% Triton X-100 in the vesicles. GLUT4-containing vesicles were purified from adipocytes treated with or without insulin, phosphorylated as described above. After phosphorylation, GLUT4-containing vesicles were solubilized in 1% Tx-100 in PBS and material bound to the bead coupled with 1F8 was eluted with Laemmli sample buffer and run on the 1D SDS/PAGE. The gel was transferred to NC membrane for 2h with 105V and the NC membrane put into a storage phosphor screen overnight. Phosphor image was scanned and then the NC membrane was blotted with GLUT4 antibody. NI: immunoprecipitated from IgG immunoadsorpted materials, 1F8: immunoprecipitated from 1F8 immunoadsorpted materials.

Phosphorylation of GLUT4-containing vesicles by PKB-α

GLUT4-containing vesicles immunoabsorbed with 1F8 antibody from LDM of rat adipocytes treated with or without insulin were used to phosphorylate by adding PKB-α exogenously as described above.

Strikingly, GLUT4 protein in the vesicles was not

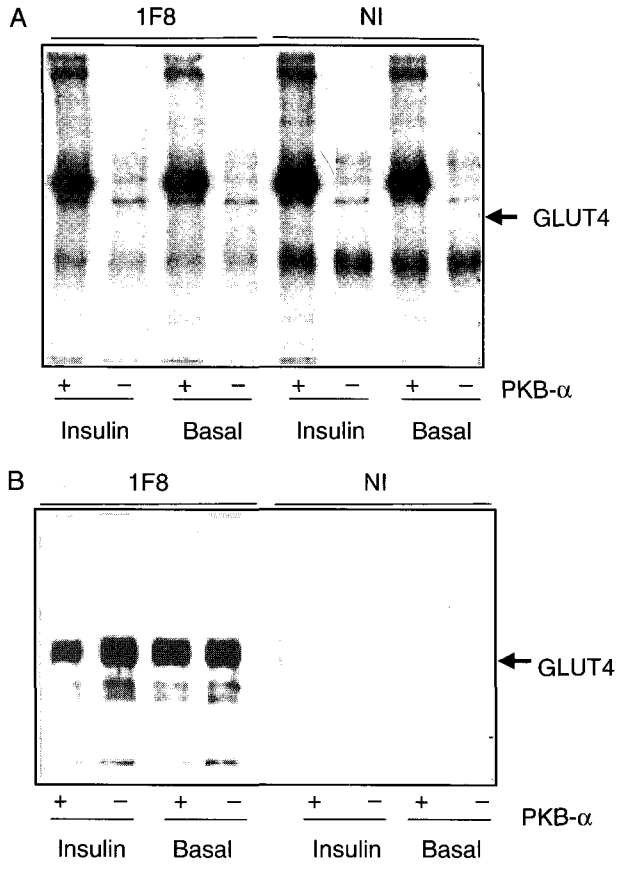

Fig. 6. Phosphorylation of GLUT4-containing vesicles by PKB-α. Phosphorylation experiment was performed as in Fig. 2 legend using PKB-α instead of PKC-ζ. In this experiment, PKB-α (Upstate Biotechnology) at concentration of 0.03 μg/ml was added. A. Phosphor image of the phosphorylated GLUT4-containing vesicles. B. Immunoblot with GLUT4 antibody (polyclonal) of the same NC membrane used for phosphor image scanning.

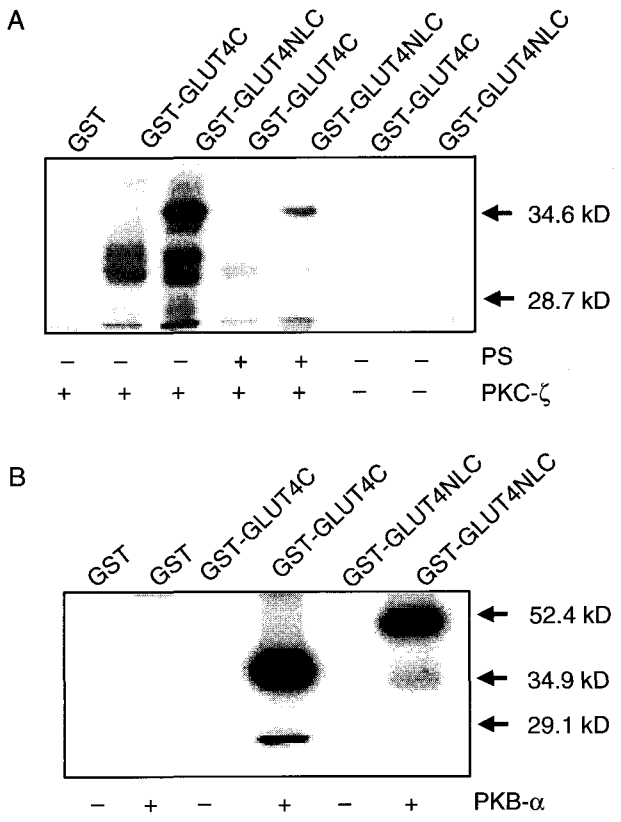

Fig. 7. Phosphorylation of GST-GLUT4 fusion proteins by PKC-ζ and PKB-α. GST-GLUT4C, GST-GLUT4NLC and GLUT4 mutant fusion proteins were prepared as described in "Methods". Phosphorylation of GST-GLUT4 fusion proteins (30 μg of each sample) was carried out in a 150 μl of reaction mixture containing 10 μl of PS (0.5 mg/ml), 5 μl of PIP_3 (0.5 mg/ml), 2.5 μl of human recombinant PKC-ζ (10 ng/l), 10 μl of [γ-^{32}P] ATP into the reaction mixture and incubated for 30 min further in the presence of 10 mM cold ATP. After phosphorylation, the glutathione agarose beads were washed with phosphorylation buffer A. A part of the eluted samples (10 μg of each sample) was electrophoresed on 10% SDS/PAGE and transferred to nitrocellulose membrane. The membrane was wrapped with Saran wrap and exposed overnight in a storage phosphor screen, and phosphorImage was quantitated in a PhosphorImager. A. Phosphor image of GST-GLUT4 fusion protein by PKC-ζ. B. Phosphor image of GST-GLUT4 fusion protein by PKB-α. Abbreviations used are denoted in "Methods".

phosphorylated, but several other proteins were phosphorylated and increased on the vesicles immunoabsorbed from LDM of insulin-treated adipocytes based on phosphor image and immunoblot of GLUT4 antibody with NC membrane after phosphor imaging (Fig 6).

Phosphorylation of GST-GLUT4 fusion protein by PKC-ζ or PKB-α

The GST-fusion proteins, GLUT4 C-terminal cytoplasmic domain (GLUT4C) and the entire major GLUT4 cytoplasmic domain corresponding to N-terminus, central loop and C-terminus in tandem (GLUT4NLC), were phosphorylated by both PKB-α and PKC-ζ (Fig. 7). The reasons for this discrepancy why whole molecule (GLUT4) is phosphorylated by PKC-ζ but not by PKB-α and GST-fusion proteins are phosphorylated by both is obscure. However, it might be due to different orientation of the protein molecules for phosphorylation sites.

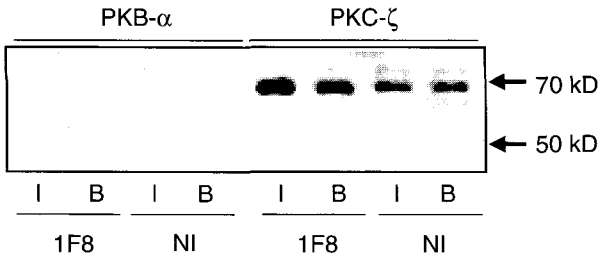

Fig. 8. Co-localization of GLUT4-containing vesicles with PKC-ζ but not with PKB-α. GLUT4-containing vesicles was subjected to SDS/PAGE and immunoblotted with PKC-ζ antibody (Upstate Biotechnology, 1 : 100 dilution) and PKB-α antibody (Upstate Biotechnology, 1 : 100 dilution) as company recommended. I: insulin treated sample B: basal sample 1F8: immunoadsorbed GLUT4-containing vesicles with 1F8 antibody NI: immunoadsorbed GLUT4-containing vesicles with mouse IgG as control.

Co-localization of GLUT4-containing vesicles with PKC-ζ or PKB-α

To determine whether intrinsic protein kinase Co-localized with GLUT4-containing vesicles, specific antibodies for PKB-α or PKC-ζ were immunoblotted with proteins separated by SDS/PAGE of GLUT-4 containing vesicles immunoadsorbed with 1F8 antibody from LDM of rat adipocytes treated with or without insulin. As expected, PKC-ζ was detected in GLUT4-containing vesicles prepared from both the rat adipocytes treated (Ins) and not treated (Bas) with insulin (Fig. 8).

The association of PKC-ζ with GLUT4-containing vesicles increased by insulin administration (Fig. 8, right panel). However, PKB-α was not found in GLUT4-containing vesicles obtained from both insulin treated cells and untreated cells (Fig. 8, left panel). Of course there were no reactions in non-immune group immunoadsorbed with non-immune serum, mouse IgG, instead of 1F8 during preparation procedure of GLUT4-containing vesicles from LDM of rat adipocytes as control.

DISCUSSION

An ample evidence suggest that stimulation of glucose transport by insulin is mediated mainly by a pathway triggered by PI3-kinase (Hara et al, 1994; Okada et al, 1994; Chang et al, 1997; Frevert & Kahn, 1997, Sakaue et al, 1997). Both Akt and atypical PKC isozymes operate downstream of PI3-kinase (Alessi et al, 1997; Franke et al, 1997; Stokoe et al, 1997), and, since PKB and PKC-ζ have been reported to interact physically, it has seen of interest to determine whether insulin-stimulated GLUT4 transport is mediated through both Akt and PKC-ζ in the same signaling pathway or whether they transmit signals through different pathways in respect that insulin stimulates GLUT4-translocation through a PI3-kinase dependent mechanism (Standeart et al, 1997), in which GLUT4 phosphorylation step might be involved. To address this important question, we have investigated the direct phosphorylating effects of these kinases on GLUT4-containing vesicles, particularly, comparing the effects of PKC-ζ with PKB-α on GLUT4 protein phosphorylation.

In the present study, we prepared adipocytes treated with or without insulin, and confirmed insulin stimulating effect on glucose influx (Fig. 1) before subcellular fractionation. Insulin effect on glucose flux was not shown maximally because the adipocytes might be due to relatively aged cells. The adipocytes were then used to get GLUT4-containing vesicle for all of the phosphorylation experiments done below. 1F8 immunoadsorbed GLUT4-containing vesicles showed several phosphorylated proteins besides GLUT4 protein by PKC-ζ in vitro (Fig. 2). They might be some proteins colocalized with GLUT4 in the vesicles as "cargo proteins". The protein composition of GLUT4-contianing vesicles is now well characterized. They are insulin regulated aminopeptidase (IRAP), vesicle-associated membrane protein-2 (VAMP2), cellubrevin, secretory carrier-associated membrane proteins (SCAMPs), low molecular mass GTP-binding proteins, phosphatidylinositol kinases, and several others (reviewed in Kupriyanova & Kandror, 1999). Among them higher molecular proteins such as 165 kD and lower molecular proteins such as 35 kD were phosphorylated remarkably. With respect to GLUT4 phosphorylation, PKC-ζ increased the phosphorylation in GLUT4-containing vesicles prepared from rat adipocytes treated with insulin based on both the phosphor image intensity and radioactivity of phosphor imaged strips of NC membrane. The effects was further confirmed at immunoprecipitated GLUT4 protein with 1F8 monocolonal antibody in the presence of 1% Triton X-100. These results clearly show that GLUT4 is phosphorylated by PKC-ζ and insulin increases GLUT4 phosphorylation by

PKC-ζ. The inhibition of PKC-ζ induced phosphorylation of GLUT4 by PKC-ζ-pseudosubstrate was also seen (Fig. 4). PKC-ζ sensitive phosphorylation of GLUT4 was confirmed using GST-GLUT4 fusion protein constructed with GLUT4 C-terminal cytoplasmic domain (GLUT4C) or the entire major GLUT4 cytoplasmic domain corresponding to N-terminus, central loop and C-terminus in tandem (GLUT4NLC) (Fig. 7). Previous studies also have shown that insulin evokes rapid increase in the activity of immunoprecipitable protein kinase C-ζ (Bandyopadhyay et al, 1997a & 1997b; Mendez et al, 1997; Standaert et al, 1997), inhibitors of PKC-ζ concomitantly inhibit insulin effects on glucose transport (Bandyopadhyay et al, 1997b; Standaert et al, 1997), and expression of a kinase-inactive form of PKC-ζ inhibits insulin-stimulated GLUT translocation and glucose transport (Bandyopahyay et al, 1997 a & b). The results provide convincing evidence that PKC-ζ is a potential downstream effector of PI3-kinase for GLUT4 translocation. In contrast to PKC-ζ, however, PKB-α did not phosphorylate GLUT4 in 1F8 immunoadsorbed GLUT4-containing vesicles and there was no insulin effect at all (Fig. 6). Strangely the GST-fusion proteins were phosphorylated by PKB-α, however, the reason of different action of PKB-α between on the GLUT4-containing vesicles and the GST-fusion protein is still obscure. Taking into account of that the majority of evidence currently confirmed implicate the protein kinase B/Akt as a primary target of inositol 3-phosphates, and associated regulatory proteins such as PDK-1, supporting its role as an insulin signaling intermediate (Kohn et al, 1996a; Kohn et al, 1996b; Franke et al, 1997; Bronzinick & Birnbaum, 1998; Le Good et al, 1998; Stephens et al, 1998), the results shown here are completely controversial. Regarding to downstream of PI3-kinase, so far, insulin-elicited signals that pass through PI3-kinases subsequently diverge into two independent pathways, an Akt pathway (Kohn et al, 1996a; Tanti et al, 1997; Calera et al, 1998; Czech & Corvera, 1999) and PKC pathway (Bandyopadhyay et al, 1997; Standaert et al, 1997; Kotani et al, 1998; Standaert et al, 1998; Bandyopadhyay et al, 1999; Standaert et al, 1999). The later pathway contributes, at least in part, to insulin stimulation of glucose transport and GLUT4-translocation. In this regard, although GLUT4 translocation is stimulated by PKC-ζ, it was still unclear whether or not GLUT4 phosphorylation step is required. The results in the present study shows that GLUT4 phosphorylation by PKC-ζ is increased in the insulin-treated adipocytes. However, several previous reports showed that the phosphorylation state of glucose transporter does not seem to be involved in the transporter translocation (Gibbs et al, 1986; Joost et al, 1987; Lawrence et al, 1990). It is difficult to explain clearly the reason for this discrepancy. With respect to possible explanation, it can be postulated that: (a) GLUT4 phosphorylation process may not be required for GLUT4 translocation and (b) PKC-ζ but not PKB-α can be associated with GLUT4-containing vesicles because Western blot of GLUT4-containing vesicles with PKC-ζ shows co-localization but with PKB-α does not show. From above results, it is clear that PKC-ζ but not PKB-α interacts with GLUT4-containing vesicles, and it phosphorylates directly GLUT4 protein. These results, therefore, suggest that insulin-elicited signals that pass through PI3-kinase subsequently diverge into two independent pathways, an Akt pathway and a PKC-ζ pathway, and that the later pathway contributes insulin stimulation of GLUT4 translocation in adipocytes via a direct GLUT4 phosphorylation.

Further studies are needed to more fully define whether or not a direct GLUT4 phosphorylation by PKC-ζ is crucial process in insulin stimulation of glucose transport and GLUT4 translocation.

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