Tyrosine Phosphorylation of Specific Protein Kinase C Isoenzymes Participates in Insulin Stimulation of Glucose Transport in Primary Cultures of Rat Skeletal Muscle

Liora Braiman, Liat Sheffi-Friedman, Asia Bak, Tamar Tennenbaum, and Sanford R. Sampson

Several reports indicate that protein kinase C (PKC) plays a role in insulin-induced glucose transport in certain cells. The precise effects of insulin on specific PKC isoforms are as yet unknown. Utilizing primary cultures of rat skeletal muscle, we investigated the possibility that insulin may influence the activation state of PKC isoenzymes by inducing their translocation and tyrosine phosphorylation. This, in turn, may mediate insulin effects on glucose transport. We identified and determined the glucose transporters and PKC isoforms affected by insulin and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Insulin and TPA each caused an increase in glucose uptake. Insulin translocated GLUT3 and GLUT4 without affecting GLUT1. In contrast, TPA translocated GLUT1 and GLUT3 without affecting GLUT4. Insulin translocated and tyrosine phosphorylated and activated PKC-β2 and -ζ; these effects were blocked by phosphatidylinositol 3-kinase (PI3K) inhibitors. TPA translocated and activated PKC-α, -β2, and -δ; these effects were not noticeably affected by PI3K inhibitors. Furthermore, wortmannin significantly inhibited both insulin and TPA effects on GLUT translocation and glucose uptake. Finally, insulin-induced glucose transport was blocked by the specific PKC-β2 inhibitor LY379196. These results indicate that specific PKC isoenzymes, when tyrosine-phosphorylated, are implicated in insulin-induced glucose transport in primary cultures of skeletal muscle. *Diabetes* 48:1922–1929, 1999

In the cascade of events leading to the multiple effects of insulin, this hormone activates its receptor tyrosine kinase and stimulates a number of downstream signaling factors, among which are several insulin receptor substrates (IRS-1, IRS-2, IRS-3), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase, and other protein kinases (1). Among the major and important subsequent effects of insulin is the stimulation of glucose uptake into several tissues via translocation of GLUTs from the cytoplasm to the membrane (2). While several of the key participating molecules have been identified, the precise steps between insulin receptor activation and GLUT translocation have not been entirely delineated. One possible component of this cascade is protein kinase C (PKC), which has been shown to be activated by insulin in some preparations (3–6). PKC is a family of serine-threonine kinases that plays an important regulatory role in a variety of biological phenomena (7,8). The family is composed of a number of individual isoforms that belong to three distinct categories: *1*) conventional isoforms (α, β1, β2, γ) activated by Ca^{2+}, phorbol esters, and diacylglycerol liberated intracellularly by phospholipase C; *2*) novel isoforms (δ, ε, η, θ), which are also activated by phorbol esters and diacylglycerol but not by Ca^{2+}; and *3*) atypical isoforms (ζ, λ, ι), which are not activated by Ca^{2+}, phorbol esters, or diacylglycerol. The pattern of isoform distribution varies among different tissues (9). It is generally considered that the enzymes, when quiescent, are located in the cytoplasm and upon activation are translocated to the plasma membrane (10). Activation of PKC isoenzymes has also been found to be associated with their phosphorylation state. For example, it has been shown that several growth factors and PKC activators induce tyrosine phosphorylation of a number of signaling factors, including PKC-δ, in several cell types (11–18).

Skeletal muscle is the major target organ for insulin regulation of blood glucose levels. The preparation of primary skeletal muscle cultures obtained from neonatal rat pups appears to be a useful model for the study of regulation of glucose uptake by insulin. These cells, plated initially as individual myoblasts, align and fuse into multinucleated muscle fibers by day 3–4 in vitro. The mature fibers display resting membrane and action potentials that are nearly identical to those seen in vivo. Similarly, the pattern of appearance and expression of several membrane proteins, such as tetrodotoxin-sensitive Na^+ channels, Ca^{2+}- and voltage-dependent K^+ channels, and the Na^+-K^+ pump, and their physiological expression in this preparation have been shown to resemble closely those obtained in vivo (19–21). Whereas a glucose uptake system for these cells has been described (22), the types of glucose transporters and the effects of insulin are unknown. In an earlier report from this laboratory (23), it was suggested that activation of PKC might be a fundamental early signal in stimulation of the Na^+/K^+ pump by insulin in cul-

From the Otto Meyerhoff Center and Health Sciences Research Center, Bar-Ilan University, Ramat-Gan, Israel.

Address correspondence and reprint requests to S.R. Sampson, PhD, Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel. E-mail: sampsos@mail.biu.ac.il.

Received for publication 30 November 1998 and accepted in revised form 11 June 1999.

DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECL, enhanced chemical luminescence; EM, Eagle's medium; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; TBST, Tris-buffered saline with Tween; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; WM, wortmannin.

tured skeletal muscle. Neither the specific PKC isoforms expressed in this preparation nor the effects of insulin on their cellular localization and phosphorylation state were examined. In this study, we have investigated the possibility that translocation and tyrosine phosphorylation of specific PKC isoforms may play an important role in the effects of insulin on glucose uptake in primary cultures of skeletal muscle. We have identified the PKC isoforms and glucose transporters in this preparation, determined their intracellular distribution and phosphorylation state, and examined effects of insulin and phorbol esters on them. Preliminary results have been reported (24).

RESEARCH DESIGN AND METHODS

Materials. Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence (ECL) was performed with antibodies purchased from Bio-Rad (Hercules, CA) and reagents from Sigma (St. Louis, MO). Antibodies to various proteins were obtained from the following sources: GLUT1, GLUT3, and GLUT4 as a gift from Dr. S. Cushman (National Institutes of Health, Bethesda, MD) and purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PKC antibodies were purchased from Santa Cruz Biotechnology and Transduction Laboratories (Lexington, KY); anti-phosphotyrosine (mouse monoclonal anti–rat IgG) was obtained from UBI (Lake Placid, NY); horseradish peroxidase–conjugated anti–rabbit and anti–mouse IgG were obtained from Bio-Rad. Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), orthovanadate, pepstatin, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma. Insulin (HumulinR, recombinant human insulin) was purchased from Lilly France (Fergersheim, France). Wortmannin (WM) and LY294002 were purchased from Calbiochem (La Jolla, CA). LY379196, a specific inhibitor of PKC-β2, was a gift from Lilly Research Laboratories (Indianapolis, IN).

Preparation of rat muscle cell cultures. Skeletal muscle cultures were prepared from thigh muscles obtained from 1- to 2-day neonatal rats as described (19–23). The muscles were removed from the limbs, washed in phosphate-buffered saline (PBS) to remove excess blood cells, and then transferred to a Ca-free 0.25% trypsin solution containing EDTA (1 mmol/l) for incubation with continuous stirring at 37°C. Cells were collected after serial trypsinization (successive 10-min periods until all tissue was dispersed) and then centrifuged for 5 min at 500*g*. Pellets were resuspended in growth medium and preplated for 20–30 min to reduce the number of fibroblasts. The myoblasts were diluted with growth medium (83% Dulbecco's modified Eagle's medium [DMEM]-high glucose, 15% horse serum, 2% chick embryo extract) to a concentration of 0.8×10^6 cells/ml for plating in collagen-coated 10-cm plastic tissue culture (10 ml/dish) or 24-well plates (400 µl/well). Cultures were prepared weekly and grown in a water-saturated atmosphere of 95% air:5% CO_2 at 37°C. On day 4 in culture, myotubes were transferred to low-glucose (4.5 mmol/l) serum-free DMEM containing 1% bovine serum albumin for 24 h before study.

Preparation of cell lysates for immunoprecipitation. Culture dishes (90 mm; Nunc, Roskilde, Denmark) containing the muscle cells were washed with Ca^{2+}/Mg^{2+}-free PBS and then mechanically detached in RIPA buffer (Tris HCl, pH 7.4, 50 mmol/l; NaCl, 150 mmol/l; EDTA, 1 mmol/l; NaF, 10 mmol/l; Triton X-100, 1%; SDS, 0.1%; Na deoxycholate, 1%) containing a cocktail of antiproteases (leupeptin, 20 mg/ml; aprotinin, 10 mg/ml; PMSF, 0.1 mmol/l; DTT, 1 mmol/l) and antiphosphatases (orthovanadate, 200 mmol/l; pepstatin, 2 mg/ml). After scraping, the preparation was centrifuged at 20,000*g* for 20 min at 4°C. The supernatant from this was used for immunoprecipitation.

Immunoprecipitation. To 0.5 ml of cell lysate was added 25 µl of A/G sepharose, and the suspension was rotated continuously through for 30 min at 4°C. The preparation was then centrifuged at 20,000*g* at 4 °C for 10 min, and to the supernatant was added 30 µl of A/G sepharose along with specific antibodies to the individual PKC isoforms (dilution 1:100). Samples were rotated overnight at 4°C. The suspension was then centrifuged at 2,000*g* for 1 min at 4°C, and the pellet was washed twice with Tris-buffered saline with Tween (TBST) (same centrifugation). To this was added 25 µl of sample buffer (0.5 mol/l Tris HCl, pH 6.8; 10% SDS; 10% glycerol; 4% 2-β-mercaptoethanol; 0.05% bromophenol blue). The suspension was again centrifuged at 500*g* (4°C for 10 min), boiled for 5 min, and then subjected to SDS-PAGE.

Cell fractionation. Crude membrane preparations were isolated from muscle cell cultures according to a modification of the method described by Klip et al. (25). Culture dishes (90 mm; Nunc) containing the muscle cells were washed with Ca^{2+}/Mg^{2+}-free PBS and then mechanically detached in Ca^{2+}/Mg^{2+}-free PBS containing 2 mmol/l EDTA with a rubber policeman. The cells were pelleted by centrifugation at 500*g* for 10 min at 4°C. The pelleted cells were resuspended in sonication buffer (Tris HCl, pH 7.4, 50 mmol/l; NaCl, 150 mmol/l; EDTA, 2 mmol/l; EGTA, 1 mmol/l) containing leupeptin, 20 µg/ml; aprotinin, 10 µg/ml; PMSF, 0.1 mmol/l; DTT, 1 mmol/l; orthovanadate, 200 µmol/l; and pepstatin, 2 µg/ml. The suspension was then homogenized in a Dounce glass homogenizer (30 strokes) and centrifuged at 1,100*g* for 5 min. The supernatant was then centrifuged at 100,000*g* for 1 h; the supernatant from this centrifugation step was retained as the cytosol fraction. The pellet was resuspended with the sonication buffer containing 1% Triton and vortexed and sonicated (intermediate setting) four times for 5 s. The preparation was again vortexed until the suspension was uniform and centrifuged at 23,000*g* for 15 min. The supernatant from this step was designated the membrane fraction. The cytosol and membrane fractions were frozen at –70°C until used. The purity of the membrane preparations was confirmed by identification of specific membrane markers (Fig. 1*C*).

PKC activity. Activity of specific PKC isoenzymes was determined in PKC-β2 and -ζ immunoprecipitates from freshly prepared whole-cell lysates from untreated or insulin- or TPA-treated mature muscle cultures. These lysates were prepared in RIPA buffer without NaF. Activity was measured with the SignaTECT Protein Kinase C Assay System (Promega, Madison, WI). In brief, a reaction mixture was prepared that contained PKC biotinylated pseudosubstrate, [γ-^{32}P]ATP (10 µCi/ml), diacylglycerol, and phospholipids; immunoprecipitates were aliquotted on SignaTECT membranes. Following incubation for 10 min at 30°C, the reaction was terminated with termination buffer, scintillation fluid was added, and an aliquot was counted on a β-counter for determination of amount of inorganic phosphate formed.

Western blot analysis. Some 20–25 µg protein were electrophoresed through SDS-polyacrylamide gels (7.5 or 10%) and electrophoretically transferred onto Immobilon-P (Millipore, Bedford, MA) membranes. Following transfer, the mem-

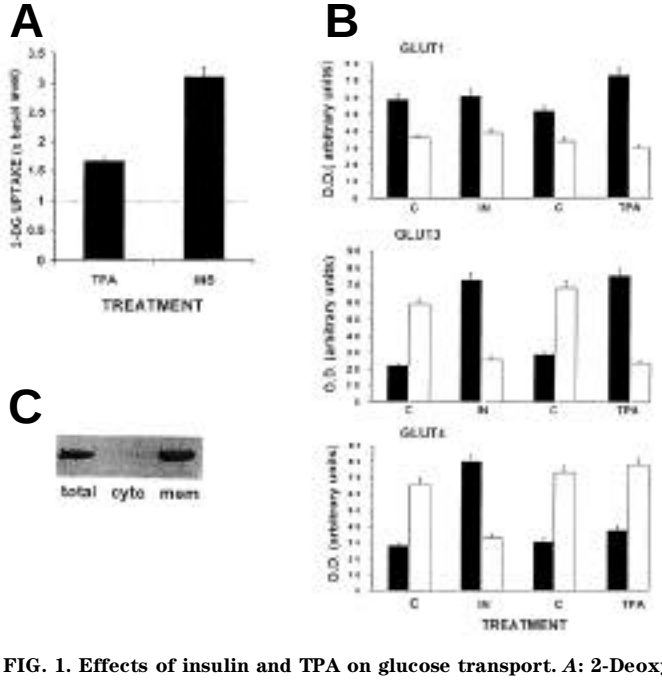

FIG. 1. Effects of insulin and TPA on glucose transport. *A*: 2-Deoxyglucose uptake in 6-day-old myotubes in culture. Cells were transferred to a serum-free low-glucose Eagle's medium (EM) 24 h before study. The uptake of glucose was measured after incubation of the cells with insulin (INS) or TPA for 30 min. Values presented as fold increase above basal level (control, untreated cells). Each bar represents the mean ± SE of triplicate measurements in each of three separate experiments (n = 9; P < 0.002). *B*: Translocation of glucose transporters GLUT1, GLUT3, and GLUT4 by insulin or TPA in 6-day-old cultured myotubes. The figure is a plot of densitometry measurements of Western blots. Cells were transferred to a serum-free low-glucose EM 24 h before study. Cells were incubated for 30 min with insulin or TPA. Plasma membrane fractions (■) were separated from the cytosolic fraction (□) and submitted to SDS-PAGE. The GLUTs were detected by Western blotting. The experiment is representative of nine different experiments obtained from three different extracts. C, control; IN, insulin; O.D., optical density. *C*: Distribution of the α1 subunit of the Na^+/K^+ pump in cultured skeletal muscle. Cells were fractionated into membrane (mem) and cytosolic (cyto) fractions and after SDS-PAGE probed with an antibody to the Na^+/K^+ pump.

branes were subjected to standard blocking and incubation procedures, and were incubated with polyclonal antibodies to specific PKC isoforms, glucose transporters, or phosphotyrosine. The membranes were washed four times for 15 min in TBST 20 1% and then further incubated for 20 min at room temperature with horseradish peroxidase–labeled secondary antibody (goat anti–rabbit or anti–mouse IgG, BioRad) diluted 1:10,000 in blocking buffer without sodium azide. After three washes (1 × 15 min and 2 × 5 min) in TBST, the membranes were treated with ECL reagents for 1 min and then exposed on Kodak X-ray film for the required times (5–30 s) and developed.

Glucose uptake. The total and nonspecific rates of glucose transport were measured in triplicate samples in 24-well plates with the use of 2-[^{3}H]DG (13). After appropriate treatment, cells were washed three times with 0.5 ml PBS, the final wash being replaced immediately with 0.5 ml PBS containing 1 μCi/ml of (^{3}H)2-DG in glucose at a concentration of 2 mmol/l. Cells were then incubated for 15 min at 37°C, washed four times with 0.5 ml cold (4–6°C) PBS, and detached from the wells by addition of 300 μl Triton X-100 (1%) and incubation for 30 min. The contents of each well were transferred to counting vials and 3.5 ml scintillation fluid added to each vial. Samples were counted in a scintillation counter. Nonspecific uptake was determined in the presence of excess (100 mmol/l) D-glucose. Net specific uptake was then calculated as the difference between the total and nonspecific values. Baseline glucose uptake values under control conditions ranged from 14–20 nmol · min^{-1} · mg^{-1} protein.

RESULTS

Effects of insulin and TPA on glucose transport. We first sought to determine if insulin and stimulation of PKC by phorbol esters produce similar effects on glucose uptake in primary cultures of skeletal myotubes. Figure 1*A* compares the effects of insulin and TPA on glucose uptake and glucose transporter distribution in this preparation. Both substances caused glucose uptake to increase beginning within 10 min and maximum effects in both cases being reached within 20–30 min. The level of glucose uptake remained elevated for 3–4 h, after which time the rate of uptake gradually returned to control levels (not illustrated). In all subsequent experiments, measurements were made 20–30 min after addition of insulin. Typically, the effect of insulin was significantly greater than that of TPA, with maximum effects of the former being ~2.5- to 3.5-fold increased over basal levels, whereas those of the latter were in the range of 1.5–2.0 times those of control.

As the transport of glucose into cells is accomplished by activation of glucose transporters, we next identified the glucose transporters involved in the responses to insulin and TPA. We determined in preliminary experiments that primary cultures of skeletal muscle express the GLUT1, GLUT3, and GLUT4 transporters. In these studies, we prepared homogenates of cells that had been treated with either insulin or TPA for 30 min and we isolated membrane and cytosol fractions. Purity of membrane fractions was confirmed by the expression of the α subunit of the Na^+/K^+ pump (Fig. 1*C*). The effects of insulin and TPA on the translocation of these transporters are shown in Fig. 1*B*. Insulin translocated both the GLUT3 and GLUT4 transporters from the cytoplasmic fraction to the plasma membrane, but was without effect on GLUT1. In contrast, TPA induced translocation of both GLUT1 and GLUT3 from the cytoplasm to the plasma membrane and was without effect on GLUT4.

Identification and distribution of PKC isoforms. The findings so far show that both insulin and TPA stimulate PKC activity. Since the PKC family includes a number of isoenzymes, it was important first to identify the isoenzymes expressed in this preparation and to determine which of them might be involved. These studies were done on preparations of cytoplasmic and plasma membrane fractions obtained from lysates of cultures 5–7 days in vitro. Of 11 PKC isoforms (α, β1, β2, γ, δ, ε, η, θ, ζ, λ, and ι) examined, 6 (α, θ, ε, β2, ζ, and δ)

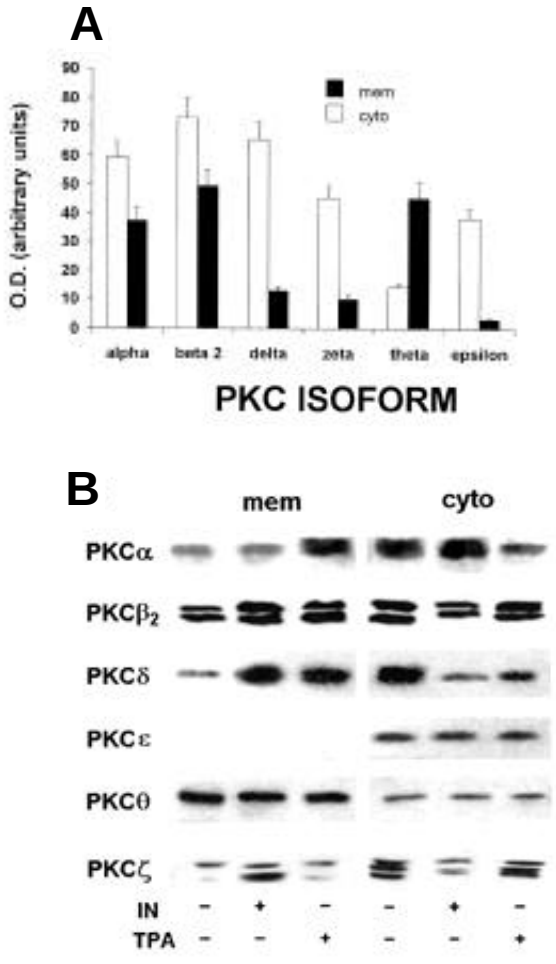

FIG. 2. PKC isoenzyme expression in cultured skeletal muscle. *A*: Distribution of PKC isoforms in 6-day-old cultured myotubes. The graph shows optical density (O.D.) of Western blots representative of nine experiments from three different preparations. ■, distribution of the isoforms in the plasma membrane; □, the isoforms in the cytosolic fraction. *B*: Effect of insulin or TPA on translocation of PKC isoforms in 6-day-old cultured myotubes. Following incubation in serum-free low-glucose EM for 24 h, cells were incubated for 30 min with insulin or TPA. Plasma membrane fractions were separated from the cytosolic fraction and submitted to SDS-PAGE. PKC isoforms were detected by Western blotting. The experiment is representative of nine different blots done on three different extracts. cyto, cytosolic fraction; IN, insulin; mem, plasma membrane fractions.

were identified in primary culture lysates, in agreement with other studies on skeletal muscle in vivo and in culture (26–29). The relative distribution of these isoforms in cytoplasmic and membrane fractions of cell lysates is shown in Fig. 2*A*. Under basal control conditions, expression of PKC isoforms α, β2, ζ, and δ was found to be higher in the cytoplasm than in the plasma membrane. In contrast, expression of PKC-θ was higher in the plasma membrane than in the cytoplasm, and PKC-ε was detected primarily in the cytoplasm and was barely detectable in the plasma membrane fraction.

Effects of insulin and TPA on translocation and tyrosine phosphorylation of PKC isoforms. The different time courses of PKC stimulation by insulin and TPA suggest that each substance may differentially affect different isoenzymes. Stimulation of PKC isoenzymes is known to involve translocation from intracellular to membrane sites and

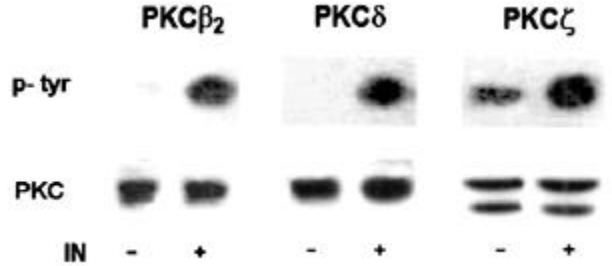

FIG. 3. Tyrosine phosphorylation of PKC isoforms translocated by insulin. Effect of insulin on phosphorylation of PKC isoforms in 6-day-old cultured myotubes. After incubation in serum-free low-glucose EM for 24 h, cells were incubated for 30 min with insulin. The isoforms were immunoprecipitated by the appropriate anti-PKC antibody and submitted to SDS-PAGE. Western blotting was performed with anti-phosphotyrosine. Equal loading of PKC protein was confirmed by anti-PKC Western blots. The experiment is representative of nine different blots obtained from three different extracts. IN, insulin.

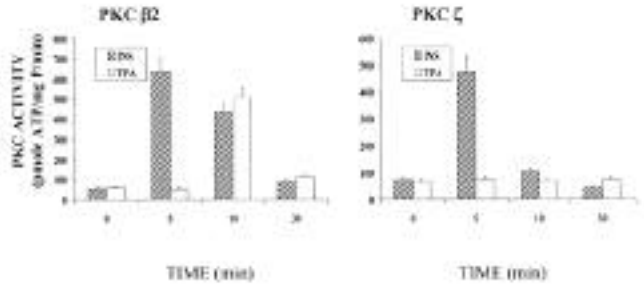

FIG. 4. Effects of insulin and TPA on activity of PKC isoenzymes β2 and ζ. Immunoprecipitates of the PKC isoenzymes were prepared from whole-cell lysates of 6-day-old muscle cell cultures. After incubation in serum-free low-glucose EM for 24 h, cells were incubated for the designated times with insulin (INS) or TPA. The cultures were then washed with PBS and prepared for measurements of PKC activity as described in METHODS. Each bar represents the mean ± SE of triplicate measurements in each of three experiments (n = 9; P < 0.005).

changes in phosphorylation state. We have, therefore, investigated effects of insulin and TPA on these properties. Cultures of 6-day-old myotubes were treated with insulin or TPA for 30 min, and results were compared with those obtained from untreated control cultures. The effects of insulin on the various isoforms are shown in the middle columns of Fig. 2*B*. Insulin caused translocation of isoforms β2, δ, and ζ from the cytoplasm to the plasma membrane, but it had no effect on PKC-α, -θ, and -ε. In contrast, TPA (column TPA) translocated PKC isoforms α, β2, and δ but was without effect on the θ, ε, and ζ isoforms. Thus, PKCs β2 and δ were each translocated by both insulin and TPA, while PKC-ζ was translocated exclusively by insulin and PKC-α exclusively by TPA.

The insulin-induced translocation of the β2, δ, and ζ isoforms indicates that the hormone may activate them. In addition to translocation, tyrosine phosphorylation has been shown to reflect the activation state of PKC isoenzymes (15–18). Therefore, we studied the phosphorylation state of the insulin-translocated isoenzymes by immunoprecipitating the appropriate PKC isoforms from control and insulin-stimulated cultures and probing the blots with anti-phosphotyrosine antibody to determine if the enzymes were indeed phosphorylated. As shown in Fig. 3, PKC-β2 and -ζ appear to display a low degree of phosphorylation under resting basal conditions, whereas no phosphorylation of PKC-δ could be detected in the basal state. Treatment with insulin increased tyrosine phosphorylation of PKC-β2 and -ζ and appeared to induce tyrosine phosphorylation of PKC-δ.

Effects of insulin on activity of specific PKC isoforms. Tyrosine phosphorylation of PKC-δ has been shown to be associated with changes in its activation state. To determine if insulin-induced tyrosine phosphorylation of the various PKC isoenzymes alters their activity, we immunoprecipitated each isoform and measured its activity. Figure 4 shows that insulin caused a four- to fivefold increase in activity of both PKC-β2 and PKC-ζ by 5 min after addition; activity returned toward control values after 30 min. TPA increased activity of PKC-β2 with the same times course as that caused by insulin, but it was without effect on PKC-ζ.

Effects of PI3K inhibitors on glucose transport. The results taken together indicate that insulin translocates certain PKC isoforms and specifically changes their phosphorylation state. One possible pathway for these effects is via stimulation of PI3K, which can act either directly or indirectly by liberation of diacylglycerol and other phospholipids known to stimulate conventional and novel PKC isoenzymes (30,31). Accordingly, we investigated this possibility by examining effects of WM, a known inhibitor of PI3 kinase, on insulin- and TPA-induced glucose transport. Figure 5*A* illustrates the effects of WM on translocation of glucose transporters by insulin and TPA. As can be seen, treatment with WM blocked the translocation of both GLUT3 and GLUT4 by insulin and the translocation of GLUT1 and GLUT3 by TPA. Furthermore, as can be seen in Fig. 5*B*, the abrogation of GLUT translocation by WM was associated with reduction in insulin and TPA-induced glucose uptake without a change in basal glucose uptake. Similar results were obtained with another PI3K inhibitor LY294002 (not illustrated).

Effects of PI3K inhibitors on PKC translocation and phosphorylation. Next we examined the influence of WM on insulin- and TPA-induced effects on the various PKC isoenzymes. The results of studies on the effect of WM are shown in Fig. 6*A*. As can be seen, WM significantly reduced, if not completely abolished, the insulin-induced translocation of PKC isoforms β2 and ζ, whereas translocation of PKC-δ was not blocked. In contrast, WM did not appear to affect TPA-induced translocation of PKC isoforms α, β2, and δ. Similarly, as shown in Fig. 6*B*, WM blocked tyrosine phosphorylation of the PKC isoforms in control and insulin-treated cultures. However, as in the case of translocation, insulin-induced tyrosine phosphorylation of PKC-δ was not reduced by WM treatment. Identical results were obtained with LY294002, which also blocked insulin-induced activation of PKC-β2 and -ζ (not shown).

Effects of PKC inhibitors on insulin-induced glucose transport. The results so far demonstrate that insulin activates PKC-β2 and -ζ, and that this is associated with an increase in glucose transport. We next determined if blockade of PKC activation by insulin would affect insulin effects on glucose uptake. We first examined effects of chelerytrin, a relatively selective PKC inhibitor, and found that both insulin-induced glucose uptake and GLUT4 translocation were blocked (not illustrated). We have also examined the effects of selective inhibition of PKC-β2 with the use of LY379196 (31). This compound specifically inhibits PKC-β2 at a concentration of 30 nmol/l, which is one twenty-fifth of that required to block PKC-δ and one-sixtieth of that required to block PKC-ζ. As shown in Fig. 7, LY379196 did not alter basal glucose

uptake (106% of control) but reduced insulin-stimulated glucose uptake by ~80%. In addition, this β2 inhibitor completely blocked translocation of GLUT3 and reduced insulin-induced translocation of GLUT4 (not shown).

DISCUSSION

The primary cultures of skeletal muscle cells used in these experiments express essentially all of the properties of skeletal muscle in vivo. Of particular importance in this study is the identification of each of the PKC isoforms described for skeletal muscle in vivo in other reports, namely PKC-α, -β2, -δ, -ε, -θ, and -ζ (25–28). This renders this preparation to be a reliable system for the study of the possible role of PKC isoenzymes in insulin receptor signaling.

Although our results with regard to insulin-induced translocation of GLUT3 and GLUT4 are similar to those reported by others on L6 cells (33), our study does not support an association between insulin stimulation and acute translocation of GLUT1 (34). The differences might be explained by the use of an immortalized skeletal muscle cell line as opposed to the primary cultures of skeletal muscle used in this study. Thus, we would concur with Wertheimer et al. (35), who suggested that the GLUT1 transporter belongs to the family of glucose-regulated stress proteins. They reported that GLUT1 transporter is expressed at very low levels in freshly excised skeletal muscle and the levels increase with time of incubation in vitro. Therefore, with prolonged life in vitro, tissues expressing the GLUT1 transporter may induce conditions that cause it to be insulin-responsive. These conditions may not exist fully in primary cultures, which are much closer to an in vivo state than are cell lines. The lack of effect of insulin on GLUT1 is consistent

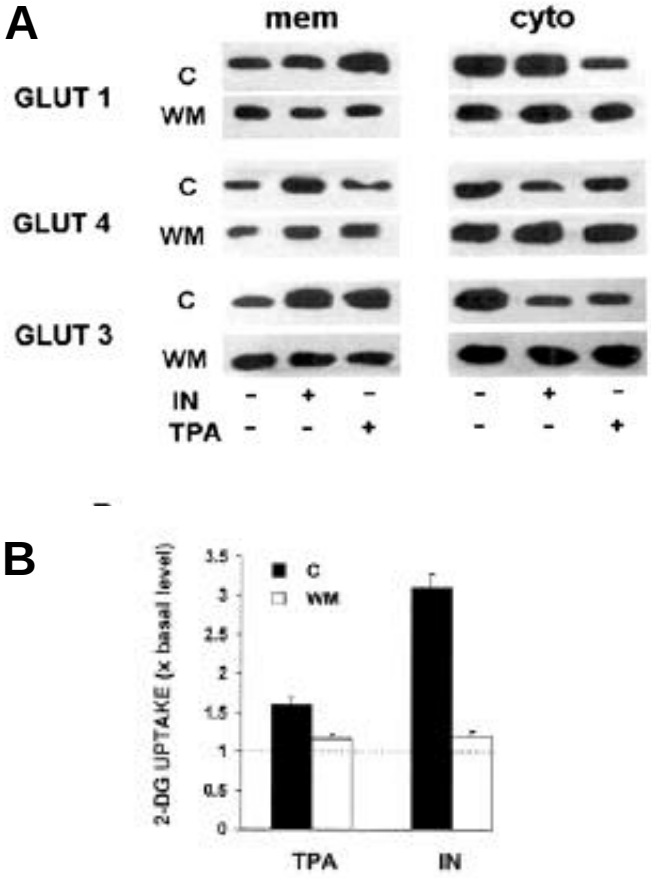

FIG. 5. Effects of WM on insulin- or TPA-induced glucose transport in cultured skeletal muscle. *A*: Expression and translocation of glucose transporters GLUT1, GLUT3, and GLUT4. Cells were transferred to a serum-free low-glucose EM 24 h before study. Cells were incubated for 30 min with WM, washed with PBS, and then incubated for 30 min with insulin or TPA. Plasma membrane fractions were separated from the cytosolic fraction and submitted to SDS-PAGE. The GLUTs were detected by Western blotting. The experiment is representative of nine different blots obtained from three different extracts. C, control; cyto, cytosolic fraction; IN, insulin; mem, plasma membrane fractions. *B*: Glucose uptake induced by insulin (IN) or TPA in 6-day-old cultured myotubes. The uptake of glucose was measured after incubation of the cultures for 30 min with insulin or TPA alone (■) or insulin or TPA after preincubation with WM (10^{-7} mol/l) for 30 min. Values are presented as fold increase above basal level (control [C], untreated cells). Each bar represents the mean ± SE of triplicate measurements in each of three experiments ($n = 9$; $P < 0.005$).

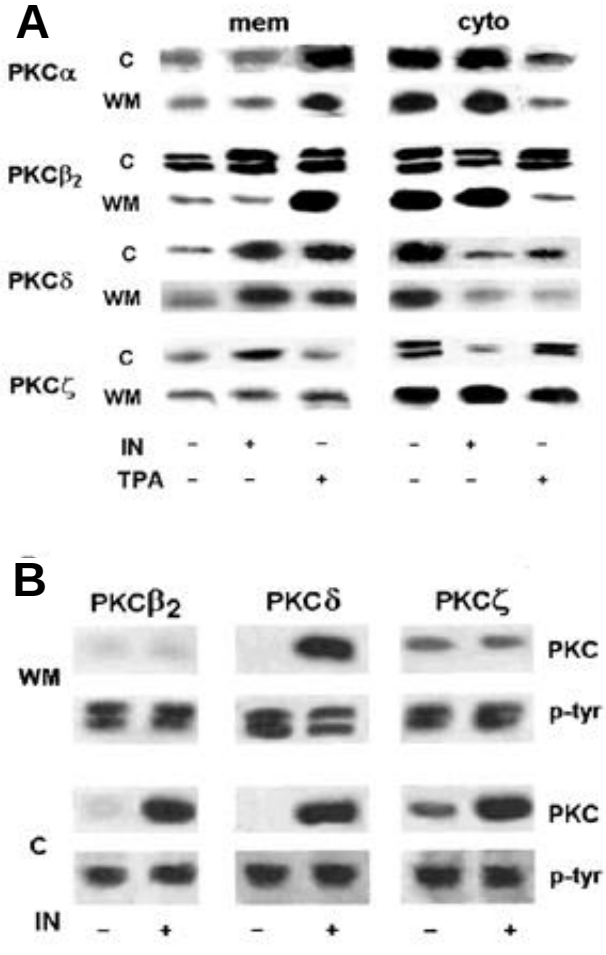

FIG. 6. Effect of WM on translocation and phosphorylation of PKC by insulin or TPA. The study was performed on 6-day-old cultured myotubes after incubation in serum-free low-glucose EM for 24 h. Cells were incubated for 30 min with WM, washed with PBS, and then incubated for 30 min with insulin or TPA. *A*: PKC translocation; plasma membrane fractions were separated from the cytosolic fraction and submitted to SDS-PAGE. The PKC isoforms were detected by Western blotting. The experiment is representative of nine different blots obtained from three different extracts. C, control; cyto, cytosolic fraction; IN, insulin; mem, plasma membrane fractions. *B*: Cell lysates were immunoprecipitated with anti-PKC antibodies as described in METHODS. The immunoprecipitates were submitted to SDS-PAGE, and the blots were probed with an antiphosphotyrosine antibody. Equal loading of PKC protein was confirmed by anti-PKC Western blots.

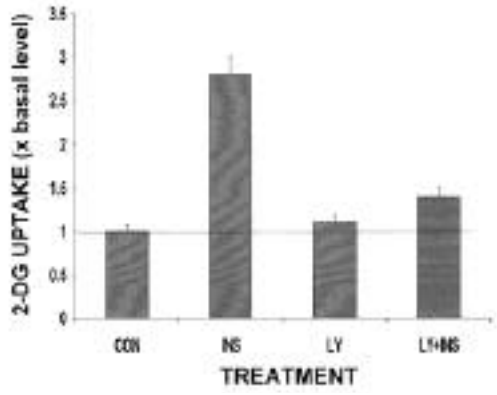

FIG. 7. Effects of LY379196 on insulin-induced glucose transport in 6-day-old cultured myotubes. The uptake of glucose was measured after incubation of the cultures for 30 min with insulin alone or insulin after preincubation with LY379196 (3×10^{-8} mol/l) for 20 min. Values presented as fold increase above basal level (control, untreated cells). Each bar represents the mean ± SE of triplicate measurements in each of three experiments (n = 9; P < 0.005). CON, control; INS, insulin for 30 min; LY, LY379196 for 20 min; LY+INS, insulin for 30 min after pretreatment with LY379196 for 20 min.

with the results of Mueckler and colleagues (36,37), who reported that muscles overexpressing GLUT1 developed glucose transport insulin resistance and suggested that GLUT1 participates mainly in basal glucose uptake.

In this study, we have shown that insulin rapidly and strongly stimulates activity of specific PKC isoenzymes. On the basis of our findings regarding translocation and tyrosine phosphorylation of PKC isoenzymes, we identified PKC isoforms β2, ζ, and δ, which are resident in primary cultures of rat skeletal muscle, as possible candidates involved in insulin-induced glucose transport. We found that translocation and tyrosine phosphorylation of PKC isoenzymes β2 and ζ by insulin were associated with their stimulation and that WM blocked the effects of insulin both on activation of these PKC isoforms and on insulin-induced glucose uptake and insulin-induced GLUT translocation. We further showed that the specific PKC-β2 inhibitor LY379196 inhibited insulin-induced glucose uptake, and this was associated with abrogation of insulin-induced translocation of GLUT3 and impaired insulin-induced translocation of GLUT4. This latter finding strengthens the conclusion that PKC-β2 is involved in insulin-induced glucose transport.

The findings regarding PKC-ζ are in relatively good agreement with other studies that appear to implicate this PKC isoenzyme in insulin signal transduction in certain cells, including skeletal muscle, fibroblasts, and adipocytes (5,6). The involvement of the PKC-β2 isoform in insulin-induced glucose uptake, however, has not been incontrovertibly established. On the one hand, it was shown that insulin activates and translocates PKC-β2 in several cell types, including BC3H-1 murine muscle cell line (38), 3T3/L1 cells (5), and L6 myotubes (39). Also in support of involvement of PKC-β2 in insulin-induced glucose transport are studies showing that transfection of L6 myotubes with certain COOH-terminal deletion mutants of this isoenzyme inhibited insulin-induced glucose uptake, and that co-transfection of full-length PKC-β2 rescued the inhibition of insulin-stimulated glucose uptake (40). On the other hand, against a role for this isoenzyme in insulin signaling are studies on L6 myotubes that showed that the PKC-β inhibitor LY379196 reduced insulin-induced glucose transport only at higher concentrations that are expected to affect other isoenzymes (6). This is in direct opposition to our findings (regarding both glucose transport and insulin-induced translocation of GLUT3 in particular) and to those of Chalfant et al. (40), who reported that another specific PKC-β2 inhibitor (CG53353) inhibited insulin-induced glucose uptake in L6 myotubes by 50–60%. The reasons for these contradictory findings on L6 myotubes are not apparent. However, it should be pointed out that the L6 muscle cell line lacks a number of important membrane proteins compared with the primary muscle cell cultures used in this study (19–22). Therefore, the possibility must be considered that the pattern of expression of various PKC isoforms and their responses to various stimuli may differ between L6 cells and primary muscle cells. Furthermore, different preparations and growth conditions of any cell line, including rate of proliferation or confluency of cells in culture, or the degree of differentiation and aging, could affect the results obtained in cell lines maintained in culture.

Another argument raised against the possible involvement of PKC-β2, a diacylglycerol-regulated isoform, in insulin-stimulated glucose transport is that phorbol ester–induced down-regulation of certain PKC isoforms fails to alter either basal or insulin-stimulated glucose transport (6,25). This downregulation assumes that PKC activation depends entirely on translocation of a given isoenzyme to the plasma membrane. There are, however, certain limitations to this notion (41). Many PKC isoenzymes can be detected in the particulate fraction of cells independent of the activation state. Moreover, PKC stimulation need not occur exclusively by translocation to the plasma membrane; translocation to the nuclear zone, as well as association with cytoskeletal components on activation, is well documented. Finally, products of lipid hydrolysis, such as free *cis*-unsaturated fatty acids, may activate PKC directly in the cytosol. It should be pointed out that PKC-ζ, which is one of the major isoforms affected by insulin in both skeletal muscle and 3T3/L1 cells, is not affected by phorbol esters. Our data indicate that both PKC-β2 and PKC-ζ are activated by insulin and participate in effects on glucose uptake.

Another unique aspect of this study is the finding that insulin can regulate tyrosine phosphorylation of specific PKC isoenzymes. Our results support a role for tyrosine phosphorylation of PKCs β2 and ζ, as we have shown here in response to insulin. The importance of tyrosine phosphorylation to the state of activity of PKC isoenzymes is unclear. However, tyrosine phosphorylation has been shown to be associated indirectly with the activation state of a number of protein kinases, including PKC-δ (42) in a variety of cell types (10–18).

Also of interest is the ability of WM to block insulin-induced effects on translocation and phosphorylation of PKC-β2 and -ζ and on glucose uptake in primary cultures of skeletal muscle. This supports the hypothesis that these isoforms play an important role in the mediation of insulin's effect on glucose transport. Furthermore, in agreement with others (31), WM and LY294002 blocked both glucose uptake and translocation of GLUT1 and GLUT3 induced by TPA, whereas it did not noticeably affect TPA-induced translocation of PKC-α, -β2, and -δ. These compounds are considered to block selectively PI3K upstream from the PKC isoenzymes stimulated by TPA. Our finding that TPA-induced glucose uptake was also blocked by these inhibitors indicates that they

may either act downstream from PKC activation in addition to its upstream effect on PI3K, or that constitutive PI3K activity is necessary for translocation of glucose transporters at some unknown site. Whether this is accomplished by direct linkage with PI3K or through the intervention of other signaling proteins remains to be determined (42).

The results obtained with WM appear to uncover an interesting divergence among effects of insulin on the different PKC isoenzymes. In contrast to the ability of WM to block insulin-induced translocation and tyrosine phosphorylation of PKC-β2 and -ζ, WM had no effect on translocation or tyrosine phosphorylation of PKC-δ. This indicates that insulin most probably acts via PI3K to translocate and tyrosine-phosphorylate the β2 and ζ isoforms but not PKC-δ.

While our results appear to suggest that PKC-δ may not be involved in insulin-induced glucose transport, several studies indicate that this isoenzyme has an important role in insulin and other signaling pathways (15,41–47) in both NIH3T3 fibroblasts and 32D myeloid progenitor cells in vitro. Alternatively, our results might suggest a unique interaction between PKC-δ and insulin receptor in the mediation of insulin effects. Further studies addressing the particular function of this isoenzyme in insulin signaling in skeletal muscle are in progress.

Interestingly, insulin activated both PKC-β2 and PKC-ζ and translocated GLUT3 and GLUT4, whereas TPA activated PKC-β2 and translocated GLUT1 and GLUT3. Moreover, PI3K inhibition blocked translocation of both transporters. In addition, specific inhibition of PKC-β2 completely blocked insulin-induced translocation of GLUT3. These data would appear to implicate PKC-β2 specifically in translocation of GLUT3. We would like to suggest, therefore, that activation of both PKC-β2 and PKC-ζ is required for complete translocation of GLUT3 and GLUT4 and for insulin-induced glucose transport. Thus, blockade of either one of these isoenzymes would result in an impaired glucose transport process. The specific sites of action of these isoenzymes in insulin-induced glucose transport have yet to be determined.

ACKNOWLEDGMENTS

This study was supported in part by the Sorrell Foundation, the Ben and Effie Raber Research Fund, the Harvett-Aviv Neuroscience Research Fund, and the Otto Meyerhoff Center. S.R.S. holds the Louis Fisher Chair in Cellular Pathology.

REFERENCES

1. White MW, Kahn RC: The insulin signaling system. *J Biol Chem* 269:1–4, 1994
2. Klip A, Ramlal T, Bilan PJ, Marette A, Liu Z, Mitsumoto Y: What signals are involved in the stimulation of glucose transport by insulin in muscle cells? *Cell Signal* 5:519–529, 1993
3. Acevedo-Duncan M, Cooper DR, Standaert ML, Farese RV: Immunological evidence that insulin activates protein kinase C in BC3H-1 myocytes. *FEBS Lett* 244:174–176, 1989
4. Avignon A, Standaert ML, Yamada K, Mischak H, Spencer B, Farese RV: Insulin increases mRNA levels of protein kinase C alpha and beta in rat adipocytes and protein kinase C alpha, beta and theta in rat skeletal muscle. *Biochem J* 308:181–187, 1995
5. Bandyopadhyay G, Standaert ML, Zhao L, Yu B, Avignon A, Galloway L, Karnman P, Moscat J, Farese RV: Activation of protein kinase C (α, β and ζ) by insulin in 3T3/L1 Cells. *J Biol Chem* 272:2551–2558, 1997
6. Bandyopadhyay G, Standaert NL, Galloway L, Moscat J, Farese RV: Evidence for involvement of protein kinase C (PKC)-ζ and noninvolvement of diacylglycerol-sensitive PKCs in insulin-stimulated glucose transport in L6 myotubes. *Endocrinol* 138:4721–4731, 1997
7. Azzi A, Boscoboinik D, Henessey C: The protein kinase C family. *Eur J Biochem* 208:547–557, 1992
8. Haller H, Lindsschau C, Luft FC: Role of protein kinase C in intracellular signaling. *Ann N Y Acad Sci* 733:313–320, 1994
9. Wetsel WC, Kahn WA, Merchenthaler I, Rivera H, Halpern AE, Phung HM, Negro-Vialr A, Hannun Y: Tissue and cellular distribution of the extended family of the protein kinase C isoenzymes. *J Cell Biol* 117:121–133, 1992
10. Einspahr KJ, Abraham RT, Dick CJ, Leibson PJ: Protein tyrosine phosphorylation and p561ck modification in IL-2 or phorbol ester-activated human natural killer cells. *J Immunol* 145:1490–1497, 1990
11. Nel AE, Pollack S, Landreth G, Ledbetter JA, Hultin L, Williams K, Katz R, Akerley B: CD-3-mediated activation of MAP-2 kinase can be modified by ligation of the CD4 receptor: evidence for tyrosine phosphorylation during activation of this kinase. *J Immunol* 145:971–979, 1990
12. Gold MR, Law DA, DeFranco AL: Stimulation of protein tyrosine phosphorylation by the B-lymphocyte antigen receptor. *Nature* 345:810–813, 1990
13. Rossomando AJ, Payne DM, Weber MJ, Sturgill TW: Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. *Proc Nat Acad Sci U S A* 86:6940–6943, 1989
14. Thomas SM, DeMarco M, D'Arcangelo G, Halegoua S, Brugge JS: Ras is essential for nerve growth factor– and phorbol ester–induced tyrosine phosphorylation of MAP kinases. *Cell* 68:1031–1040, 1992
15. Li W, Miscchak H, Yu J-C, Wang L-M, Mushinski JF, Heidara MA, Pierce JH: Tyrosine phosphorylation of protein kinase C-δ in response to its activation. *J Biol Chem* 269:2349–2352, 1994
16. Soltoff SP, Toker A: Carbachol, substance P, and phorbol ester promote the tyrosine phosphorylation of protein kinase Cδ in salivary gland epithelial cells. *J Biol Chem* 270:13490–13495, 1995
17. Denning MF, Dlugosz AA, Howett MK, Yuspa SH: Expression of an oncogenic rasHa gene in murine keratinocytes induces tyrosine phosphorylation and reduced activity of protein kinase C delta. *J Biol Chem* 271:26079–26081, 1993
18. Zang Q, Lu Z, Curto M, Barile N, Shalloway D, Foster DA: Association between v-Src and protein kinase C in v-Src-transformed fibroblasts. *J Biol Chem* 272:13275–13280, 1997
19. Brodie C, Sampson SR: Nerve growth factor and fibroblast growth factor influence post-fusion expression of Na-channels in cultured rat skeletal muscle. *J Cell Physiol* 144:492–497, 1990
20. Vigdor-Alboim S, Sampson SR: Characterization and regulation of apamin binding K+ channels in skeletal muscle. *Muscle Nerve* 19:331–337, 1996
21. Brodie C, Sampson SR: Serum factor induces selective increase in Na-channel expression in cultured skeletal muscle. *J Cell Physiol* 148:48–53, 1990
22. Brodie C: Regulation by thyroid hormones of glucose transport in cultured rat myotubes. *J Neurochem* 55:186–191, 1990
23. Sampson SR, Brodie C, Alboim S: Role of protein kinase C in insulin activation of the Na/K pump in cultured skeletal muscle. *Am J Physiol* 266: C751–C757, 1994
24. Sampson SR, Friedman L, Wiksman L: Protein kinase Cs β2, δ, and ζ mediate insulin-induced glucose uptake in cultured skeletal muscle: Proceedings of the 16th IDF Congress, Helsinki, 1997. *Diabetologia* 40 (Suppl. 1):152, 1997
25. Klip A, Ramlal T, Young D, Holloszy JO: Insulin–induced translocation of glucose transporters in rat hindlimb muscle. *FEBS Lett* 224:224–230, 1987
26. Osada S, Mizuno K, Suzuki K, Kuroki T, Ohno S: A new member of the protein kinase C family, nPKC theta predominantly expressed in skeletal muscle. *Mol Cell Biol* 12:3930–3938, 1987
27. Cooper D, Watson JE, Dao ML: Decreased expression of protein kinase C alpha, beta and epsilon in soleus muscle of Zuo (fa/fa) rats. *Endocrinology* 133:2241–2247, 1993
28. Avignon A, Standaert ML, Yamada K, Mischak H, Spencer B, Farese RV: Insulin increases mRNA levels of protein kinase C alpha and beta in rat adipocytes and protein kinase C alpha, beta and theta in rat skeletal muscle. *Biochem J* 308:181–187, 1995
29. Hong D, Huan J, Ou B, Yeh J, Saido TC, Cheeke PR, Fordberg NE: Protein kinase C isoforms in muscle cells and their regulation by phorbol ester and calpain. *Biochim Biophys Acta* 1267:45–54, 1995
30. Nave BT, Siddle K, Shepherd PR: Phorbol esters stimulate phosphatidylinositol 3,4,5-triphosphate production in 3T3-L1 adipocytes: implications for stimulation of glucose transport. *Biochem J* 318:203–205, 1996
31. Shepherd PR, Nave BT, O'Rahilly S: The role of phosphoinositide 3-kinase in insulin signaling. *J Mol Endocrinol* 17:175–184, 1996
32. Jirousek MR, Gillig JR, Heath WF, Gonzalez CM, McDonald JH III, Neel DA, Rito CJ, Stramm LE, Singh U, Melikan-Badalian A, Baevsky M, Ballas LM, Hall SE, Winneroski LL, Faul MM: (S)-13-[(Monomethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno- 1H,13H- dibenzo[E,K]pyrrolo-[3,4-H][1,4,13] oxadiaza-cyclohexadecin-1,3(2H)-dione (LY333531) and related analogues: isozyme-selective inhibitors of protein kinase Cβ (PKCβ). *J Med Chem* 39:2664–2671, 1996
33. Wilson CM, Mitsumoto Y, Maher F, Klip A: Regulation of cell surface GLUT1,

GLUT3 and GLUT4 by insulin and IGF in L6 myotubes. *FEBS Lett* 368:19–22, 1995
34. Sargeant R, Mitsumoto Y, Maher F, Klip A: Hormonal regulation of glucose transporters in muscle cells in culture. *J Endocrinol Invest* 16:147–162, 1995
35. Wertheimer E, Sasson S, Cerasi E, Ben-Neriah Y: The ubiquitous glucose transporter GLUT1 belongs to the glucose regulated protein family of stress-inducible proteins. *Proc Natl Acad Sci U S A* 86:2525–2529, 1991
36. Gulve EA, Ren, JM, Marshall BA, Gao J, Hansen PA, Holloszy JO, Mueckler M: Glucose transport activity in skeletal muscles from transgenic mice over-expressing GLUT1: increased basal transport is associated with a defective response to diverse stimuli that activate GLUT4. *J Biol Chem* 269: 18366–18370, 1994
37. Buse MG, Robinson KA, Marshall BA, Mueckler M: Differential effect of GLUT1 or GLUT4 overexpression on hexosamine biosynthesis by muscle of transgenic mice. *J Biol Chem* 271:23197–23202, 1994
38. Cooper DR, de Ruiz Galaretta CM, Fanjul LF, Mojsilovic L, Standaert ML, Pollet RJ, Farese RV: Insulin but not phorbol ester treatment increases phosphorylation of vinculin by protein kinase C in BC3H-1 myocytes. *FEBS Lett* 214:122–126, 1987
39. Srinivasan M, Begum N: Stimulation of protein phosphatase-1 activity by phorbol esters: evaluation of the role of protein kinase C in insulin action. *J Biol Chem* 269:16662–16667, 1994
40. Chalfant CE, Ohno S, Konno Y, Fisher AA, Bisnauth LD, Watson JE, Cooper DR: A carboxy terminal deletion mutant of protein kinase C β_2 inhibits insulin-stimulated 2-deoxyglucose uptake in L6 rat skeletal muscle cells. *Mol Endocrinol* 10:1273–1281, 1996
41. Quest AFG: Regulation of protein kinase C: a tale of lipids and proteins. *Enzyme Protein* 49:231–261, 1996
42. Li W, Pierce JH: Protein kinase C-delta, an important signaling molecule in the platelet-derived growth factor beta receptor pathway. *Curr Top Microbiol Immunol* 211:55–65, 1996
43. Formisano P, Oriente F, Miele C, Caruso M, Auricchio R, Vigliotta G, Condorelli G, Beguinot F: In NIH3T3 fibroblasts, insulin receptor interaction with specific protein kinase C isoforms controls receptor intracellular routing. *J Biol Chem* 273:13197–13202, 1998
44. Li W, Yu J-C, Michieli P, Beeler JF, Ellmaore N, Heidaran MA, Pierce J: Stimulation of the platelet-derived growth factor β receptor signaling pathway activates protein kinase C-δ. *Mol Cell Biol* 14:6727–6735, 1994
45. Li W, Michieli P, Alimandi M, Lorenzi MV, Wu Y, Wang L-H, Heidaran MA, Pierce JH: Expression of an ATP binding mutant of PKC-δ inhibits Sis-induced transformation of NIH3T3 cells. *Oncogene* 13:731–737, 1996
46. Varley CL, Brown BL, Groome N, Dobson PRM: Interleukin-1b-induced expression of protein kinase C (PKC)-δ and ε in NIH3T3 cells. *Cytokine* 9: 577–581, 1997
47. Li W, Chen XH, Kelley CA, Alimandi M, Zhang J, Chen Q, Bottaro DP, Pierce JH: Identification of tyrosine 187 as a protein kinase C δ phosphorylation site. *J Biol Chem* 271:26404–26409, 1996
48. Lu Z, Hornia A, Jiang Y-W, Zang Q, Ohno S, Foster DA: Tumor promotion by depleting cells of protein kinase C delta. *Mol Cell Biol* 17:3418–3428, 1997