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Role of Protein Kinase C in the Translational Regulation of Lipoprotein Lipase in Adipocytes*

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The hypertriglyceridemia of diabetes is accompanied by decreased lipoprotein lipase (LPL) activity in adipocytes. Although the mechanism for decreased LPL is not known, elevated glucose is known to increase diacylglycerol, which activates protein kinase C (PKC). To determine whether PKC is involved in the regulation of LPL, we studied the effect of 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) on adipocytes. LPL activity was inhibited when TPA was added to cultures of 3T3-F442A and rat primary adipocytes. The inhibitory effect of TPA on LPL activity was observed after 6 h of treatment, and was observed at a concentration of 6 nM. 100 nM TPA yielded maximal (80%) inhibition of LPL. No stimulation of LPL occurred after short term addition of TPA to cultures. To determine whether TPA treatment of adipocytes decreased LPL synthesis, cells were labeled with [^{35}S]methionine and LPL protein was immunoprecipitated. LPL synthetic rate decreased after 6 h of TPA treatment. Western blot analysis of cell lysates indicated a decrease in LPL mass after TPA treatment. Despite this decrease in LPL synthesis, there was no change in LPL mRNA in the TPA-treated cells.

Long term treatment of cells with TPA is known to down-regulate PKC. To assess the involvement of the different PKC isoforms, Western blotting was performed. TPA treatment of 3T3-F442A adipocytes decreased PKC α, β, δ, and ϵ isoforms, whereas PKC λ, θ, ζ, μ, ι, and γ remained unchanged or decreased minimally. To directly assess the effect of PKC inhibition, PKC inhibitors (calphostin C and staurosporine) were added to cultures. The PKC inhibitors inhibited LPL activity rapidly (within 60 min). Thus, activation of PKC did not increase LPL, but inhibition of PKC resulted in decreased LPL synthesis by inhibition of translation, indicating a constitutive role of PKC in LPL gene expression.

Lipoprotein lipase (LPL)[1] is a central enzyme in lipid metabolism that is expressed primarily in adipose tissue and muscle (1). The regulation of lipoprotein lipase is complex and regulation may occur at the transcriptional, translational, and post-translational levels (2). LPL activity is decreased in the adipose tissue of patients with diabetes. After treatment to control hyperglycemia in both type I and type II diabetes, there is an increase in LPL activity (3, 4), along with an increase in LPL synthetic rate with no change in LPL mRNA, suggesting regulation at the level of translation (5). Similar observations have been made in experimental models of insulin-deficient diabetic rats. Whereas short term insulin treatment of insulin-deficient rats increased LPL protein more than LPL mRNA, prolonged insulin treatment increased both LPL protein and LPL mRNA levels (6).

Glucose and insulin modulate protein kinase C (PKC) activity in rat adipocytes (7). Hyperglycemia is known to increase cellular diacylglycerol (DAG), which in turn is the natural activator of PKC. Elevated DAG, resulting in PKC activation has been identified in insulin-deficient diabetic adipose tissue (8–10). The role of hyperinsulinemia and hyperglycemia in the activation of PKC isoforms and involvement in insulin resistance has been studied in various animal models of diabetes (11). PKC is present in the soluble cytoplasmic fraction in cells prior to stimulation, and the treatment of cells with the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) resulted in activation and translocation of PKC to the membrane. TPA activates PKC by interacting with the DAG binding site (12), although the degree of stimulation of PKC by TPA is much greater than the induction by DAG. However, prolonged treatment with phorbol ester can down-regulate PKC activity and PKC protein by depleting cellular PKC protein (13, 14).

The eleven related isoforms of PKC have been described and can be classified into three subgroups, depending on the requirement for diacylglycerol, phospholipid, and calcium for activation. These isoenzymes are characterized by differences in their four domains, the regulatory domains, C1 and C2, and the catalytic domains, C3 and C4 (15). Each PKC isoform has a distinct tissue distribution and physiological function. Recent studies indicate that PKC isoforms β and δ are activated preferentially in the vasculature of diabetic animals. However, other PKC isoforms are also increased in the glomeruli and retinal tissues isolated from diabetic animals and in cells cultured in the presence of high glucose (16, 17).

In this study, the effect of PKC activation and depletion by TPA on LPL activity was examined in adipocytes. Although stimulation of PKC did not increase LPL, depletion or inhibition of PKC resulted in a decrease in LPL translation. These data suggest that PKC plays a vital role in the expression of LPL activity in adipocytes.

MATERIALS AND METHODS

Cell Culture and Differentiation—3T3-F442A cells were obtained from Dr. Howard Green (Harvard Medical School, Boston, MA). The cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% calf serum (Life Technologies, Inc.). For experiments, the cells were grown to confluence in 12-well cluster plates, and differ-

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[1] The abbreviations used are: LPL, lipoprotein lipase; PKC, protein kinase C; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; PAGE, polyacrylamide gel electrophoresis; FFA, free fatty acid.

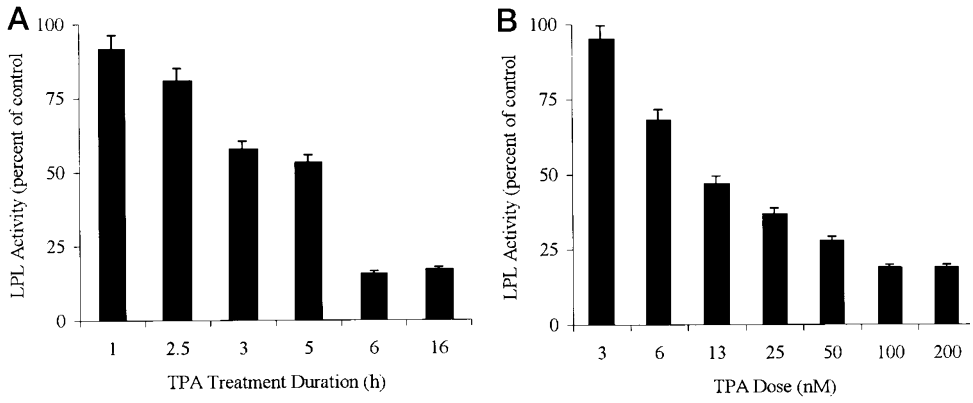

FIG. 1. **Effect of TPA on LPL activity in 3T3-F442A adipocytes.** *A*, cells were grown to confluence in 12-well culture dishes and differentiated for 5–6 days. They were treated with 100 nM TPA for the specified time intervals. Heparin releasable LPL activity was measured as described under "Materials and Methods." LPL activity in control adipocytes was 1.06 ± 0.1 μmol of FFA/hr/mg of protein. *B*, dose response to TPA on LPL activity in 3T3-F442A adipocytes. Differentiated cells were treated with varying doses of TPA ranging from 3 to 200 nM for 6 h, and heparin releasable LPL activity was measured. LPL activity in control adipocytes was 0.63 ± 0.1 μmol of FFA/hr/mg of protein. All data represent the results of three experiments performed in quadruplicate.

entiated by incubation in medium with 10% fetal calf serum containing 100 nM insulin for 5–7 days.

Differentiated adipocytes were treated with the indicated concentration of TPA dissolved in 1 μl of Me_2SO in 1 ml of culture medium. Control cells received the same amount of Me_2SO. PKC inhibitors staurosporine and calphostin C were purchased from Calbiochem. Staurosporine and calphostin C stock solutions were made in Me_2SO at 1,000× concentration. Calphostin C was activated after addition to the culture medium by exposure to light for 10 min as recommended by the manufacturer.

Measurement of LPL Activity—Heparin releasable and extractable LPL were prepared as described previously (18). To measure heparin releasable LPL, the medium was aspirated, and cells were treated with 10 units/ml of heparin in Dulbecco's modified Eagle's medium for 45 min at 37 °C. After collecting this fraction, the cell layer was extracted in a detergent-containing buffer (19). LPL catalytic activity was measured as described previously (20). Samples were incubated with emulsified substrate containing [^{3}H]triolein and human serum as a source of apo C-II for 45 min at 37°. LPL activity has been expressed as nanomoles of free fatty acid (FFA) released/hr/mg of cell protein.

LPL Synthetic Rate—The synthetic rate of LPL was measured in control and TPA-treated adipocytes by pulse labeling the cells for the specified time intervals with [^{35}S]methionine (100 μCi/ml). The unincorporated label was aspirated, and the total cellular proteins were extracted in lysis buffer containing 50 mM phosphate buffer, pH 7.4, 2% deoxycholate, 1% SDS, 20 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 2 mM EDTA. Labeled LPL was immunoprecipitated using LPL specific polyclonal antibodies described earlier (21). Immunoprecipitated samples were analyzed on 10% SDS-PAGE followed by autoradiography as described earlier (22).

RNA Extraction and Northern Blotting—RNA was extracted from adipocytes using the method of Chomczynski and Sacchi (23). Equal amounts of total RNA from the various treatment groups were analyzed using 2.2 M formaldehyde, 1% agarose gels. Northern blots were probed with the ^{32}P-labeled hLPL cDNA (24) and glyceraldehyde-3-phosphate dehydrogenase cDNA probe as described by us previously (22, 25). Previous studies involving murine LPL, including 3T3-F442A adipocytes have yielded two RNA species at 3.2 and 3.6 kilobases because of the two alternative sites of polyadenylation (26, 27).

Western Blot Analysis—The cell layer was rinsed in ice-cold phosphate buffered saline and total protein was extracted using the cell lysis buffer described above. The lysates were centrifuged at 1,500 × *g* for 15 min to separate the insoluble debris. Proteins were fractionated using 10% SDS-PAGE. Samples containing 15 μg of total protein were electrophoresed for Western blot analysis and transferred onto nitrocellulose membranes using 300 mA current for 2–3 h. Membranes were treated with 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.2% Tween 20, and 5% nonfat dry milk overnight at 4° C.

Detection of LPL Protein—LPL polyclonal antibody (rabbit) was a generous gift from Dr. Ira Goldberg (Columbia University, New York, NY). Anti-rabbit horseradish peroxidase conjugate was obtained from Sigma Chemical Company. The primary antibody was used at 1:2,000 dilution and the secondary at 1:5,000. Membranes were washed extensively with Tris buffer containing 0.1% Tween 20 to remove the excess of primary or secondary antibody. The LPL protein was detected using chemoluminescence reagents (Amersham Pharmacia Biotech).

Detection of PKC Isoforms—The isoform specific primary and secondary antibodies were purchased from Transduction Laboratories, Lexington, KY. The primary antibodies were used at 1:1,000 dilution and the secondary antibody at 1:5,000 dilution. Membranes were washed, and the PKC protein bands were detected using chemoluminescence.

Data Analysis—All experiments were performed in triplicate with triplicate wells in each, and data are expressed as mean ± S.E.

RESULTS

Effect of TPA Treatment on LPL Activity—3T3-F442A adipocytes were treated with 100 nM TPA for varying time intervals. As shown in Fig. 1, heparin releasable LPL activity was inhibited by TPA treatment. Although there was no significant decrease in activity during the first 60 min of treatment with 100 nM TPA, LPL activity began to diminish after 3 h of treatment and was inhibited by 80% after 6 h of treatment with TPA (control, 1.06 ± 0.1; TPA treated, 0.158 ± 0.025 μmol of FFA/hr/mg of protein). Overnight treatment with TPA had no additional inhibitory effect on LPL activity. More than 75% of the total LPL activity was found in the heparin releasable fraction of 3T3-F442A adipocytes. Total extractable LPL activity was also inhibited 82% in TPA-treated cells (control, 1.6 ± 0.1; TPA treated, 0.280 ± 0.08 μmol of FFA/hr/mg of protein). In addition to studying 3T3-F442A adipocytes, primary cultures of rat adipocytes were examined, and TPA inhibited heparin releasable LPL activity, but to a lesser degree than in 3T3-F442A cells. After overnight treatment of rat adipocytes with 100 nM TPA, control and TPA-treated LPL activity was 44.6 ± 5.1 and 31.1 ± 2.9 nmol of FFA/hr/10^6 cells, respectively.

To study the dose response of TPA, cells were treated with increasing concentrations of TPA. As indicated in Fig. 1*B* the inhibition of LPL activity was dose-dependent and 6 nM TPA inhibited LPL activity by 30%. The inhibition increased with increasing concentration of TPA and was maximal at 100 nM TPA.

To determine whether the decrease in LPL activity in response to TPA treatment resulted from a decrease in LPL mRNA levels, cells were treated with 100 nM TPA for varying time intervals, followed by RNA extraction and Northern blot analysis. The addition of TPA had no inhibitory effect on LPL mRNA levels as shown in Fig. 2. LPL mRNA remained the same at all the time points studied. The same blot was also probed for glyceraldehyde-3-phosphate dehydrogenase mRNA as a control for equal loading of RNA. The expression of glyceraldehyde-3-phosphate dehydrogenase mRNA did not change with TPA treatment.

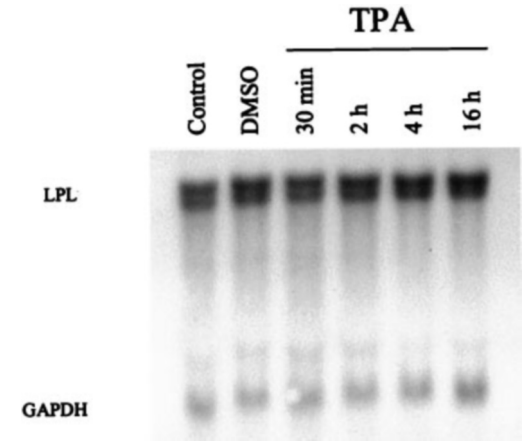

FIG. 2. **Effect of TPA treatment on LPL mRNA in 3T3-F442A adipocytes.** The cells were treated with 100 nM TPA for the specified time interval. Total RNA was extracted and analyzed using Northern blot analysis. Densitometric analysis of the autoradiograms indicates no change in the intensity of LPL and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) bands. The data shown represent one of two experiments.

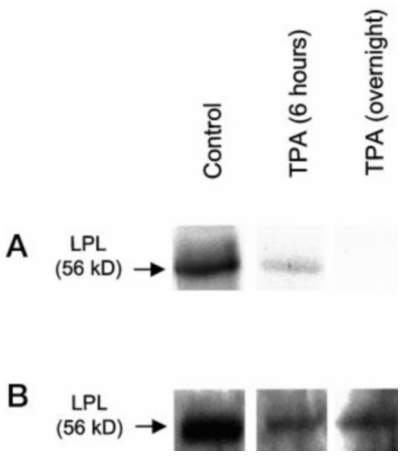

FIG. 3. **Effect of TPA on LPL synthesis in 3T3-F442A adipocytes.** *A*, the cells were treated with TPA (100 nM) for 6 h and pulse labeled with [^{35}S]methionine. Equal trichloroacetic acid precipitable counts were immunoprecipitated using anti-LPL antibodies and analyzed on 10% SDS-PAGE. The data shown represents one of three experiments. *B*, Western blots were performed after TPA treatment on total cell lysates. The data shown represent one of three experiments.

Because TPA treatment did not decrease LPL mRNA, the decrease in LPL activity following TPA treatment could result from either a decrease in synthetic rate of LPL or a change in LPL posttranslational processing. To determine whether TPA treatment decreased LPL synthetic rate, control and TPA-treated adipocytes were pulse labeled with [^{35}S]methionine for 30 min, and LPL was immunoprecipitated. As shown in Fig. 3*A*, LPL synthetic rate was decreased by 75–80% with 6 h of TPA treatment as compared with the untreated control. To assess total LPL immunoreactive mass, total cell lysates from control, and TPA-treated cells were analyzed for LPL protein by Western blot analysis. As shown in Fig. 3*B*, LPL mass was decreased in both 6- and 16-h TPA-treated lysates.

The decreased [^{35}S]methionine incorporation described above could have been due to a decrease in LPL translation, or to an increase in LPL degradation. To determine whether there were any effects of LPL degradation, we performed [^{35}S]methionine pulse labeling for 10, 20, 30, and 40 min, followed by immunoprecipitation of LPL. As shown in Fig. 4, [^{35}S]methionine incorporation into LPL was linear in both control and TPA-treated cells, and there was a 50–60% decrease in LPL synthesis in the TPA-treated cells at all time intervals. Thus, the decrease in LPL synthesis in the TPA-treated cells at the shorter (10 and 20 min) pulse labeling times, and the linear incorporation into both control and TPA-treated cells, indicate that the decrease in LPL activity resulted from a decrease in LPL translation, and not from an increase in LPL degradation.

To study the effect of prolonged TPA treatment on the expression of PKC isoforms, 3T3-F442A adipocytes were treated with TPA, and total cell lysates were analyzed using Western blots. As shown in Fig. 5, TPA treatment decreased the expression of PKC α, β, δ, and ϵ within 6 h of treatment, and overnight treatment resulted in a further decrease, such that isoforms α, δ, and ϵ were undetectable. PKC isoforms μ, λ, and ζ remained unchanged with TPA treatment. Isoforms ι and γ were not detectable in 3T3-F442A adipocytes.

To examine earlier times after addition of TPA, 3T3-F442A adipocytes were treated with 100 nM TPA, and total cell lysates were analyzed using Western blots. As shown in Fig. 6, there were small decreases in all PKC isoforms at 2 h and much greater decreases at 4 and 6 h of TPA treatment. In addition, we observed an increase in the expression of isoforms α and δ after 30 min of TPA treatment (data not shown). As described in Fig. 1, there was no significant change in LPL activity until 2.5–3 h after TPA treatment. Thus, the decrease in PKC isoforms, as demonstrated by Western blotting, occurred at the same time as the decrease in LPL activity.

Because prolonged TPA treatment of cells is known to deplete PKC (28), we examined the effect of directly inhibiting PKC activity using PKC inhibitors. The addition of staurosporine (1 μM) inhibited LPL activity by 50%, compared with control cells. In contrast to TPA, which required several hours to inhibit LPL, the inhibitory effect of staurosporine was observed within 60 min of addition (data not shown). To further demonstrate the effect of PKC inhibition on LPL, calphostin C, which is a more specific inhibitor of PKC, was added to 3T3-F442A adipocytes. As shown in Fig. 7*A*, LPL activity was inhibited by calphostin C, and this inhibition occurred quickly. Following the addition of 1 μM calphostin C, LPL activity was inhibited by 30% in 30 min and 75% in 2 h. Inhibition of LPL activity by calphostin C increased with increasing concentrations with maximal inhibition of LPL by 2 μM calphostin C (Fig. 7*B*).

DISCUSSION

LPL hydrolyzes the triglyceride core of lipoproteins and is subject to regulation by a number of different hormones (2). The physiologic regulators of LPL include catecholamines, which inhibit adipose lipid accumulation during periods of active lipolysis (*e.g.* fasting), and insulin, which stimulates LPL in concert with an inhibition of lipolysis (*e.g.* after a meal). A number of studies have demonstrated that the regulation of LPL is complex. Under some conditions, levels of LPL mRNA are regulated, whereas other times there is regulation of LPL translation, or posttranslational processing (2). Regulation of LPL translation has been demonstrated in response to several conditions, including glucose (29), thyroid hormone (30, 31), epinephrine (32–34), and in response to improved diabetes control (5, 6). In all of these instances, there was no change in LPL mRNA expression, but the synthetic rate of LPL was altered, resulting in decreased LPL activity. In this study we report that expression of LPL activity in adipocytes is decreased by depletion of PKC, and that this inhibition of LPL takes place at the level of translation.

To determine whether PKC was involved in the regulation of LPL activity in adipocytes, we studied the effect of TPA, which can mimic in part the effect of DAG, the natural ligand for PKC (35). The activation of PKC is an immediate response

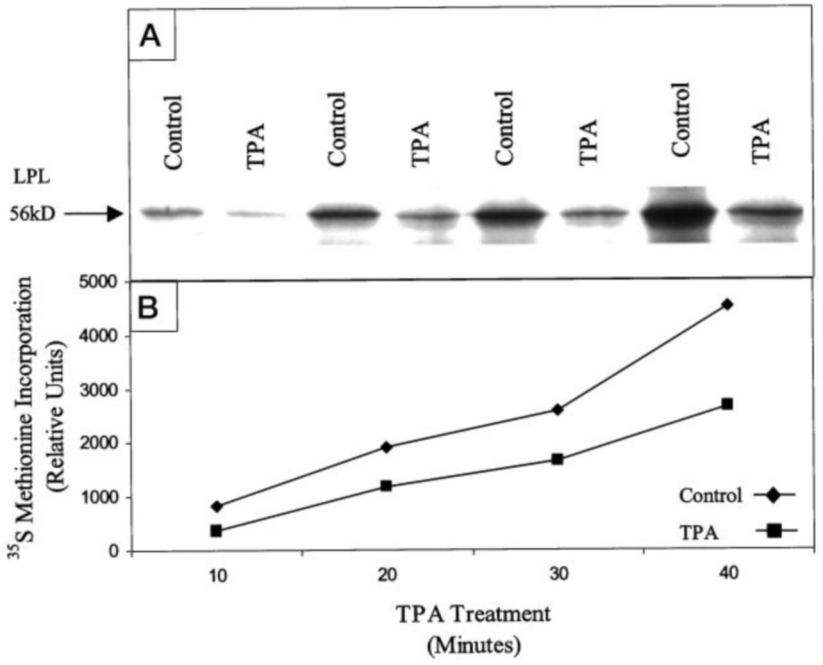

FIG. 4. **Effect of TPA on LPL synthetic rate in 3T3-F442A adipocytes.** The cells were treated with TPA (100 nM) for 16 h and pulse labeled with [^{35}S]methionine for the indicated time intervals. Equal TCA precipitable counts were immunoprecipitated using anti-LPL antibodies and analyzed on 10% SDS-PAGE. *A*, autoradiograph of the immunoprecipitated LPL protein; *B*, densitometric analysis of the image.

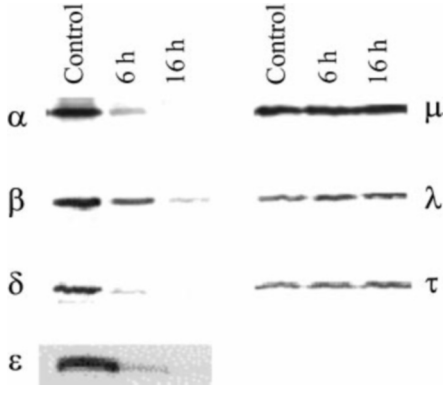

FIG. 5. **Depletion of PKC isoforms in response to prolonged TPA treatment.** Cells were treated with 100 nM TPA for 6 or 16 h. 20 μg of total cell protein was analyzed using Western blot analysis for the presence of various PKC isoforms using specific antibodies (see "Materials and Methods").

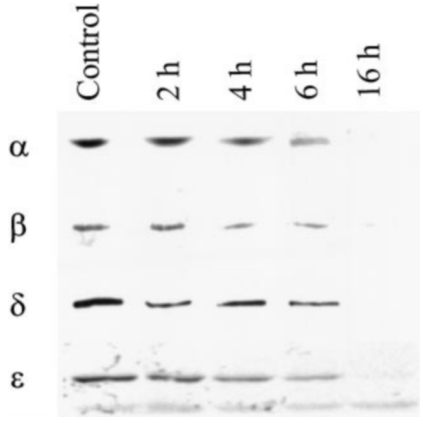

FIG. 6. **Depletion of PKC isoforms in response to TPA treatment.** Cells were treated with 100 nM TPA for 2, 4, 6, and 16 h. 20 μg of total cell protein were analyzed using Western blotting for the presence of PKC isoforms using specific antibodies.

to TPA, and occurs within 30 min (36, 37). We observed that TPA treatment caused no significant change in LPL activity during this time interval. The inhibitory effect of TPA on LPL activity, however, was evident after longer treatment with TPA. Long term treatment with TPA is known to decrease PKC in several cell types (38). Phorbol ester-induced down-regulation of PKC has been documented in several studies and is mainly due to increased rate of degradation (39, 40). For example, Shea *et al.* (41) demonstrated that with prolonged TPA treatment of human neuroblastoma cells, the rate of degradation of PKC-α is faster than the rate of synthesis, which resulted in the depletion of PKC protein. To demonstrate that the inhibitory effects of TPA on LPL were caused by PKC depletion, we directly inhibited PKC with specific inhibitors. This resulted in inhibition of LPL activity. We also demonstrated the depletion of several PKC isoforms by Western blotting. Thus, depletion of PKC or direct inhibition using PKC inhibitors inhibited LPL activity, indicating that physiological levels of active PKC play a vital role in the regulation of LPL activity. Because stimulation of PKC did not stimulate LPL, our data suggest that PKC must play a constitutive role to maintain LPL synthesis.

In our previous studies (32, 33), we have demonstrated that LPL translation is regulated by an RNA-binding protein that is stimulated by catecholamines, and binds to the 3′-untranslated region of the LPL mRNA. Other examples of regulatory RNA-binding proteins have involved phosphoproteins (42), and thus it is possible that PKC may alter LPL translation through the phosphorylation of a protein intermediate. Phorbol esters trigger phosphorylation and activation of RAF1, which is a 75-kDa phosphoprotein with intrinsic kinase activity and is an important physiological substrate for PKC α (43). Activated RAF functions as a kinase kinase and triggers signaling proteins including cytosolic enzymes like S6 kinase.

The role of PKC in the phosphorylation and regulation of neuromodulin and neurogranin, and other nuclear RNA-binding proteins which regulate translational initiation, splicing and ribosomal assembly has been described recently (44). PKC phosphorylation of these proteins inhibits their binding to their target RNA *in vitro*. It is possible that PKC may play a similar role in the regulation of LPL activity. PKC could be involved in the phosphorylation of regulatory proteins that are involved in the translation of LPL. Depletion of PKC may dephosphorylate the binding protein and inhibit LPL activity.

PKC plays a vital role in other aspects of gene expression and

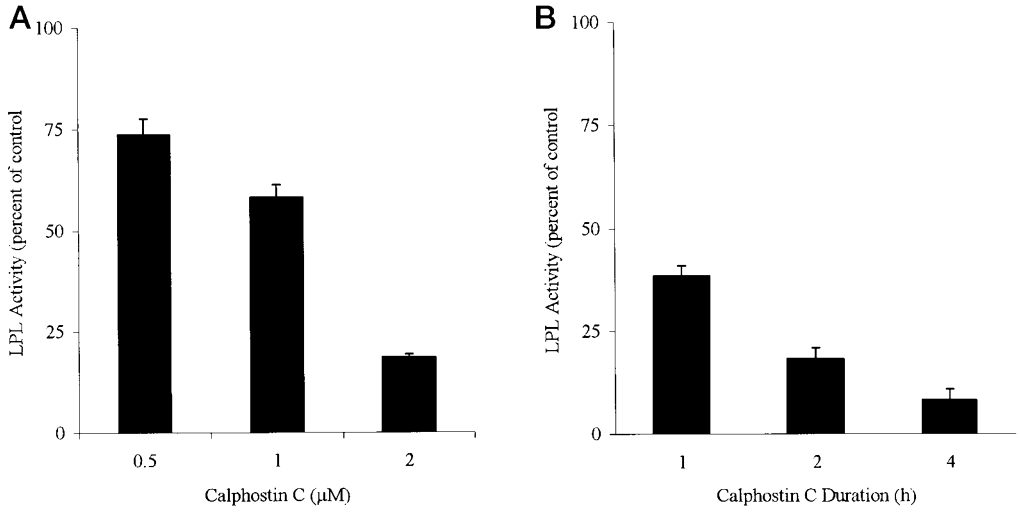

FIG. 7. **Effect of PKC inhibitors on LPL activity.** *A*, the cells were treated with increasing concentrations of calphostin C for 4 h and heparin releasable LPL activity was measured. Control LPL activity was 3.15 ± 0.23 μmol of FFA/hr/mg of protein. *B*, the cells were treated with 1 μM calphostin C for varying time intervals, and heparin releasable LPL activity was measured as described under "Materials and Methods." Control LPL activity was 2.57 ± 0.17 μmol of FFA/hr/mg of protein. All experiments were performed in triplicate and expressed as the mean ± S.E.

insulin signaling in 3T3-L1 adipocytes (45). Depletion of PKC by prolonged treatment of adipocytes with TPA resulted in an activation of GTPase-activating protein and inhibition $p21^{ras}$ GTP loading (substitution of GTP for GDP on $p21^{ras}$), which results in an alteration of insulin action. Another mechanism by which PKC alters insulin action may involve serine phosphorylation on the C-terminal domain of the insulin receptor, which results in a decrease in insulin receptor tyrosine kinase activity, and a decrease in insulin signaling (46).

In this study, we demonstrated that PKC depletion results in a decrease in LPL translation. PKC is an important regulatory protein involved in catalyzing specific substrate phosphorylation in eukaryotic cells (35), and may be relevant to the changes in LPL that occur with diabetes. LPL activity is decreased in the adipose tissue of patients and animals with both insulin-deficient and insulin-resistant diabetes, and improved diabetes control increased LPL activity (4–6, 47). Elevated blood glucose levels stimulates the production of DAG in many cells, including adipocytes (36, 48). One would expect that glucose-mediated increases in DAG would activate PKC, and hence maintain LPL expression. Perhaps such is the case under normal fasting/feeding conditions where elevations in blood glucose are modest and transient. With diabetes, however, where blood glucose is chronically elevated, it is possible that the elevated DAG, or perhaps some related mechanism, results in a down-regulation of PKC and hence an inhibition of LPL expression.

In summary, we have demonstrated that the depletion of PKC in adipocytes resulted in a decrease in LPL translation, although the stimulation of PKC did not affect LPL. These data suggest that PKC is necessary for the normal constitutive expression of LPL and may provide an important link to the signal transduction events that regulate this important enzyme.

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