Original Articles

Alterations in the Expression and Cellular Localization of Protein Kinase C Isozymes ε and θ Are Associated With Insulin Resistance in Skeletal Muscle of the High-Fat–Fed Rat

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We have tested the hypothesis that changes in the levels and cellular location of protein kinase C (PKC) isozymes might be associated with the development of insulin resistance in skeletal muscles from the high-fat–fed rat. Lipid measurements showed that triglyceride and diacylglycerol, an activator of PKC, were elevated four- and twofold, respectively. PKC activity assays indicated that the proportion of membrane-associated calcium-independent PKC was also increased. As determined by immunoblotting, total (particulate plus cytosolic) PKC α, ε, and ζ levels were not different between control and fat-fed rats. However, the ratio of particulate to cytosolic PKC ε in red muscles from fat-fed rats was increased nearly sixfold, suggesting chronic activation. In contrast, the amount of cytosolic PKC θ was downregulated to 45% of control, while the ratio of particulate to cytosolic levels increased, suggesting a combination of chronic activation and downregulation. Interestingly, while insulin infusion in glucose-clamped rats increased the proportion of PKC θ in the particulate fraction of red muscle, this was potentiated by fat-feeding, suggesting that the translocation is a consequence of altered lipid flux rather than a proximal event in insulin signaling. PKC ε and θ measurements from individual rats correlated with triglyceride content of red gastrocnemius muscle; they did not correlate with plasma glucose, which was not elevated in fat-fed rats, suggesting that they were not simply a consequence of hyperglycemia. Our results suggest that these specific alterations in PKC ε and PKC θ might contribute to the link between increased lipid availability and muscle insulin resistance previously described using high-fat–fed rats. *Diabetes* 46:169–178, 1997

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ANOVA, analysis of variance; aPKC, atypical PKC; cPKC, conventional PKC; DAG, diacylglycerol; nPKC, novel PKC; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; TLC, thin layer chromatography.

NIDDM is characterized by a decrease in the insulin sensitivity of target tissues, resulting in a diminished whole-body response to the hormone. The insulin resistance seen in skeletal muscle, especially red muscle, is of particular importance, since this is a major site of insulin-stimulated glucose uptake, and, hence, the mechanism by which it arises is the focus of much research. Emphasis has recently been placed on the link between the development of insulin resistance and increased lipid availability and metabolism (1), as demonstrated in studies with both human subjects (2) and animal models (3,4). However, the mechanistic basis for this link is currently unknown.

There has been much recent progress in elucidation of the signaling cascade acting downstream of the insulin receptor. A key event is the activation of the insulin receptor tyrosine kinase and resultant phosphorylation of the docking proteins IRS-1, IRS-2, and Shc. This leads to the recruitment of downstream components involved in regulating effector pathways, such as PI3-kinase, *ras*, and the tyrosine phosphatase SHPTP2 (5,6). Disruption of one or more of these steps, or at the level of metabolic enzymes acting further downstream, especially glycogen synthase, could underlie the development of insulin resistance. In this context, interest is focused on the family of protein kinase C (PKC) isozymes (7–9), with the demonstrations that aberrant activity of one or more PKC isozymes can lead to decreased insulin signaling from the insulin receptor. For example, glycogen synthase activity can be reduced by PKC phosphorylation (10), while the insulin receptor tyrosine kinase has been reported to show decreased activity on phosphorylation by PKC in vitro and in vivo (11–14). More recently, PKC overexpression has been found to interfere with insulin-stimulated PI3-kinase activation and phosphorylation of IRS-1 and Shc (15), and it has been suggested that PKC may stimulate tyrosine phosphatase activity (16,17).

The PKC family consists of at least nine isozymes and has been divided into three subfamilies: the conventional PKCs (cPKC α, β, and γ), which are dependent on calcium, diacylglycerol (DAG), and phospholipid for activity; the novel

PKCs (nPKC δ, ε, η, and θ), which are calcium insensitive; and the atypical PKCs (aPKC ζ and ι), which are independent of both calcium and diacylglycerol (18). When intracellular levels of the activators are elevated, PKC isozymes can translocate from the cytosol to membranes and assume an active conformation. The extent to which a PKC isozyme is found in membrane fractions of cell extracts has therefore been commonly used as a measure of the activation of the kinase. As a consequence of prolonged activation, the kinases are downregulated by proteolysis, although individual isozymes differ in their rates of disappearance.

In this study, we have determined alterations in the levels and cellular localization of five PKC isozymes, as well as changes in triglyceride, DAG, and total PKC activity in skeletal muscle of the high-fat–fed rat, which is a well-characterized model of insulin resistance (3,4). We examined muscle taken from insulin-treated and untreated rats fed either a high-fat or control starch diet. Both red and white muscles, which differ in normal insulin sensitivity (19), were investigated to analyze further the relationship between PKC and insulin action. Our results suggest that changes in PKC isozymes (particularly PKCs ε and θ) might provide a mechanistic explanation for the correlation of increased lipid availability and insulin resistance previously documented in this model.

RESEARCH DESIGN AND METHODS

Materials. Rabbit anti-peptide antibodies against PKCs α, β, δ, ε, and ζ were from Gibco BRL, Life Technologies (Mulgrave, Australia). Rabbit anti-peptide antibody against PKC θ was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody MC-2a against PKC β was from Seikagaku (Tokyo, Japan). Mouse monoclonal antibody P17720 against PKC β was from Transduction Laboratories (Lexington, KY). Horseradish peroxidase–linked donkey anti-rabbit antibody was from Jackson Immuno Research Laboratories (West Grove, PA). Goat anti-mouse antibody linked to horseradish peroxidase was from Caltag (San Francisco, CA). Other biochemicals were mostly from Sigma or BDH Laboratory Supplies (Poole, Dorset, U.K.).

Experimental animals, dietary treatment, and euglycemic-hyperinsulinemic clamp studies. All surgical and experimental procedures performed for this study were approved by the Animal Experimentation Ethics Committee (Garvan Institute) and were in accordance with the National Health and Medical Research Council of Australia guidelines on animal experimentation. Procedures were carried out as described previously (20). Briefly, male Wistar rats weighing ~250 g were fed isocaloric rations (350 kJ/day) of either a high-starch or high-fat diet for 3 weeks up to the study day. The composition of the fat diet was 59% fat, 21% protein, and 20% carbohydrate (21); the starch diet consisted of 10% fat, 21% protein, and 69% carbohydrate (4). One week before the study, rats were fitted with jugular and carotid cannulas (4). Insulin action was assessed under conditions of the euglycemic-hyperinsulinemic glucose clamp. Porcine insulin was infused at 1.8 nmol · kg^{-1} · h^{-1}, and arterial blood glucose was maintained at basal levels by a variable infusion of 30% glucose (22). For basal studies, no insulin or glucose infusion was administered. Estimates of the rate of glucose uptake by individual muscles, based on tissue accumulation of phosphorylated 2-[^{14}C(U)]deoxy-D-glucose, and plasma glucose and insulin levels were determined as described previously (23).

Tissue extraction. Red gastrocnemius and red and white quadriceps muscles were collected rapidly after animals were sacrificed by pentobarbitone overdose, frozen with liquid N_2-cooled tongs, and stored at –80°C. Muscles were dismembranated under liquid N_2 and extracted in ice-cold homogenizing buffer (400 µl of 20 mmol/l MOPS, pH 7.5, 250 mmol/l mannitol, 1.2 mmol/l EGTA, 1 mmol/l dithiothreitol, 2 mmol/l phenylmethylsulphonyl fluoride [PMSF], 200 µg/ml leupeptin, and 2 mmol/l benzamidine per 100 mg muscle). This and all subsequent procedures were carried out at 4°C. The extract was centrifuged at 100,000*g* for 45 min and the supernatant, termed the cytosolic fraction, removed and stored in liquid N_2. The 100,000*g* pellet was washed once by resuspension in homogenizing buffer and recentrifugation, and protein solubilized by resuspension in 400 µl per 100 mg of 20 mmol/l MOPS, pH 7.5, 0.5% (wt/vol) decanoyl-*N*-methyl-glucamide, 2 mmol/l EDTA, 5 mmol/l EGTA, 1 mmol/l dithiothreitol, 2 mmol/l PMSF, 200 µg/ml leupeptin, and 2 mmol/l benzamidine. After 1 h at 4°C, the extract was centrifuged again at 100,000*g* for 45 min, and the supernatant, containing solubilized protein, retained as the membrane fraction. The detergent-insoluble fraction remaining was freeze-dried and weighed; this measurement was used to correct loading variations between samples subjected to immunoblotting and densitometry. In some experiments, the detergent-insoluble fraction was further extracted by resuspension in 400 µl per 100 mg of 10% (wt/vol) SDS and 25% (vol/vol) 2-mercaptoethanol, sonication for 2 × 10 s using a Branson Sonifier 250 and microtip at power setting 2 and 20% duty cycle, and heating to 95°C for 2 min. This extract was again centrifuged at 100,000*g* for 45 min and the supernatant retained as the cytoskeletal fraction. Material remaining in 100,000*g* pellets after this extraction under denaturing conditions was not subjected to SDS-PAGE.

PKC assays. Cytosolic and membrane fractions (400 µl) from red gastrocnemius muscle were applied at 4°C to 1-ml Macro-prep DEAE columns equilibrated in 20 mmol/l MOPS, pH 7.5, 20 mmol/l NaCl, 4% (vol/vol) glycerol, 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l dithiothreitol, 0.2 mmol/l PMSF, 20 µg/ml leupeptin, and 2 mmol/l benzamidine (equilibration buffer). This buffer was supplemented with 0.1% (wt/vol) decanoyl-*N*-methyl-glucamide for the membrane fractions. Columns were washed with 5 ml of equilibration buffer followed by 5 ml of equilibration buffer containing 100 mmol/l NaCl. PKC was eluted with equilibration buffer containing 300 mmol/l NaCl and immediately assayed as previously described (24) using 5 µmol/l PKC β–modified pseudosubstrate peptide (19–31; Ser25) as substrate. Assays were carried out under four conditions: in the presence of EGTA; calcium alone; EGTA, phosphatidylserine, and dioctanoylglycerol; and calcium, phosphatidylserine, and dioctanoylglycerol. Negligible amounts of PKC activity were detected in the column flowthrough, in 100 mmol/l NaCl washes, and in 500 mmol/l NaCl washes obtained after elution with 300 mmol/l NaCl.

Immunoblotting. After addition of a 133-µl sample buffer, cytosolic, membrane, and cytoskeletal fractions were heated to 95°C for 2 min, and 10 µl was subjected to SDS-PAGE using 10% gels, as described by Laemmli (25). Proteins separated on the gels were electroblotted onto nitrocellulose membrane (0.45 µm) in 25 mmol/l Tris, pH 8.3, 192 mmol/l glycine, at 500 mÅ for 2 h using a BioRad Transblot cell. Membranes were probed with 5 µg/ml Gibco rabbit anti-peptide antibody specific for PKC isozyme α, β, δ, ε, or ζ, or with 1 µg/ml Santa Cruz rabbit anti-peptide antibody specific for PKC isozyme θ for 2 h at room temperature, followed by horseradish peroxidase–linked donkey anti-rabbit antibody (1:10,000) for 1 h. PKC isozymes were visualized by incubation of the membrane with Amersham ECL reagents and exposure to X-ray film for between 30 s and 10 min as described in the manufacturer's instructions. Alternatively, 1 µg/ml Seikagaku mouse monoclonal antibody MC-2a, directed against purified rabbit brain PKC β, or 1 µg/ml Transduction Laboratories mouse monoclonal antibody P17720, directed against an expressed fragment of PKC β, was used, and binding was detected using goat anti-mouse antibody linked to horseradish peroxidase (1:4,000) with the ECL system. Densitometry of PKC bands was carried out using a Medical Dynamics Personal Densitometer SI and analyzed using IP Lab Gel software (Signal Analytics, Vienna, VA). The relationship between the amount of sample subjected to immunoblotting and the signal intensity observed was linear under the conditions described above.

Triglyceride and DAG measurements. Muscle triglyceride content was determined as described previously (26). Muscle DAG content was measured by a method adapted from Nakamura and Handa (27). Powdered tissue (50 mg) was extracted with 4 ml of chloroform:methanol (2:1, vol/vol). After mixing overnight, 2 ml of a 0.6% NaCl solution was added, and samples were centrifuged for 5 min at 2,000*g*. The resulting chloroform phase was collected and evaporated to dryness under N_2. The residue was reconstituted in 180 µl of chloroform and stored at –20°C until use. An aliquot (60 µl) was applied to a thin layer chromatography (TLC) plate together with a series of diolein standards, and TLC was carried out in petroleum ether:diethylether:acetic acid (82:18:4, vol/vol). Plates were stained with 0.03% Coomassie blue in 20% methanol for 30 min and destained for 3 min in 20% methanol. Dried plates were scanned with a Macintosh OneScanner, and data was analyzed with National Institutes of Health (NIH) Image software (NIH, Bethesda, MD).

Statistical analysis. All results are expressed as means ± SE. Analysis of group data was by analysis of variance (ANOVA), as described previously (20). Statistical calculations were performed using Statview SE + Graphics for Macintosh (Abacus Concepts, Berkeley, CA).

RESULTS

Rats fed a high-fat diet for 3 weeks became insulin resistant relative to starch-fed controls, as previously reported (3). This was observed both as a reduced whole-body insulin sensitivity under euglycemic-hyperinsulinemic clamp conditions and as a decreased rate of insulin-stimulated glucose

TABLE 1
Plasma parameters and comparison of whole-body glucose turnover responses to diet during euglycemic-hyperinsulinemic clamp studies of starch and fat-fed rats

	Starch-fed	Fat-fed
Basal plasma glucose (mmol/l)	7.4 ± 0.2	7.7 ± 0.1
Basal plasma insulin (pmol/l)	275 ± 20	285 ± 25
Clamp plasma glucose (mmol/l)	6.9 ± 0.2	6.7 ± 0.1
Clamp plasma insulin (pmol/l)	590 ± 50	520 ± 20
Glucose infusion rate ($mg \cdot kg^{-1} \cdot min^{-1}$)	39.1 ± 2.4	12.2 ± 0.9*
Red quadriceps muscle glucose metabolic index ($\mu mol \cdot 100\ g^{-1} \cdot min^{-1}$)	46.5 ± 9.9	19.0 ± 1.8†

Data are means ± SE. Basal data are for 7-h fasted rats used for basal and clamp glucose turnover studies (n = 16–22); clamp data are for rats undergoing euglycemic-hyperinsulinemic clamps (n = 10–14). *P < 0.001; †P < 0.01.

uptake by skeletal muscle (Table 1). Thus, compared with control rats, a threefold lower glucose infusion rate is required to maintain plasma glucose concentrations when fat-fed rats are infused with insulin, and the glucose uptake by skeletal muscle under these conditions is also reduced, as indicated by the twofold reduction in glucose metabolic index of red quadriceps muscle (Table 1). However, this diet does not give rise to significant changes in basal plasma glucose or insulin (Table 1).

Our previous work has suggested that increased lipid availability plays a major role in the generation of skeletal muscle insulin resistance (4,26), and measurements of triglyceride levels in red gastrocnemius muscles from starch- and fat-fed rats (Fig. 1*A*) confirmed that an increased level of this lipid is associated with decreased insulin sensitivity. In addition, we now observed that the muscle DAG level is also raised (Fig. 1*B*). From Fig. 1*C*, it is clear that muscle triglyceride and DAG levels are closely related (simple regression, $P < 0.005$, $r^2 = 0.633$). Because DAG is an activator of most PKC isozymes, we investigated whether activity of these kinases, as well as total protein and cellular location, were also affected by the high-fat diet.

PKC activity in cytosolic and membrane fractions of red gastrocnemius muscle of starch- and fat-fed rats was measured. Total activity was comprised mainly of calcium-dependent PKC (Table 2), >90% of which was located in the cytosolic fraction, in agreement with an earlier study using soleus muscle (28). No effect of diet was observed on cPKC activity in either the cytosolic or membrane fractions. Calcium-independent (nPKC and aPKC) activity represented only 3% of the total PKC activity under the conditions used and was at the limit of detection in the membrane fractions from control rats (Table 2). Again, no significant effect of diet was seen on calcium-independent activity in the cytosolic fraction. However, in membrane fractions, fat-fed rats exhibited more calcium-independent PKC activity than starch-fed rats, although the difference did not reach significance (P < 0.09). Furthermore, a significant increase in the percentage of membrane-associated calcium-independent PKC, calculated individually for each muscle, was observed in fat-fed rats (P < 0.05).

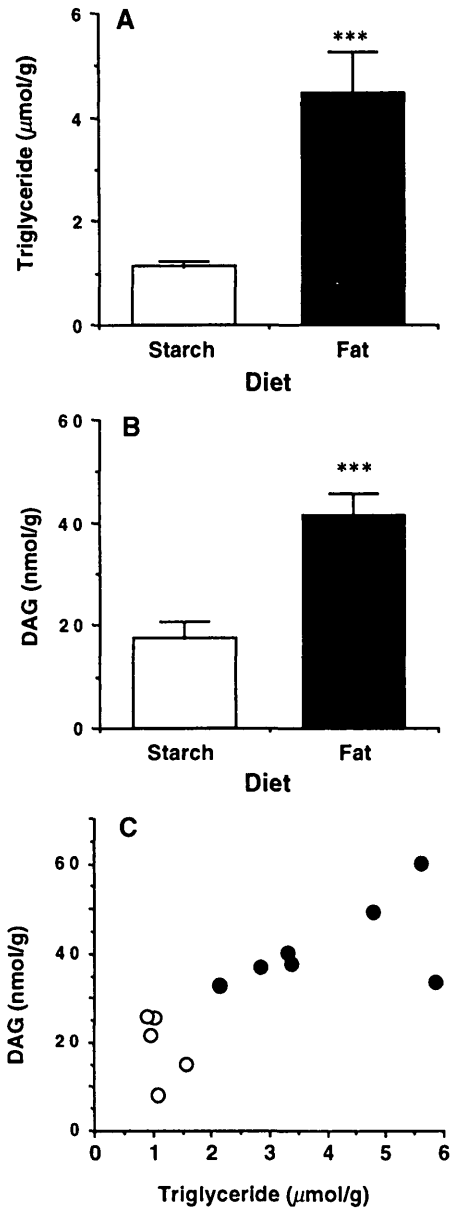


FIG. 1. Red gastrocnemius muscle basal triglyceride and DAG depots. Levels of triglyceride (*A*) and DAG (*B*) in samples from starch- and fat-fed rat muscle were determined as described in METHODS. Data are from at least five rats in each group; ***P < 0.005. *C*: relationship between triglyceride and DAG levels in red gastrocnemius muscle from starch- (○) and fat-fed (●) rats.

Immunoblots were carried out on cytosolic and membrane fractions prepared from red and white skeletal muscles to determine which PKC isoforms contribute to the activities measured. An initial characterization using competing antigenic peptides was carried out to determine the specificity of the antibodies (Fig. 2). Several antibodies recognized more than one protein in the fractions from a mixed skeletal muscle extract, but in each case, PKC protein bands could be recognized by their specific abolition in blots when peptide was included. In this way, 82-kDa PKC α, 80-kDa PKC δ, 96-kDa PKC ε, and 72-kDa PKC ζ were detected. PKC θ was detected using a PKC θ–specific antibody to which no peptide was obtained; however, the expected 82-kDa band was the only protein detected, even after prolonged exposure. Only the 72-kDa PKC ζ signal and the 96-kDa PKC ε signal were analyzed

TABLE 2
PKC activity measured in cytosolic and membrane fractions from red gastrocnemius muscle of starch- and fat-fed rats

Diet	Cytosolic PKC activity		Membrane PKC activity		% membrane associated	
	Calcium-independent	Total	Calcium-independent	Total	Calcium-independent	Total
Starch	1,124 ± 136	35,877 ± 601	0.44 ± 41.8	3,062 ± 162	0.04 ± 3.6	8.4 ± 0.4
Fat	910 ± 96	33,391 ± 1,412	83.2 ± 20.9	3,217 ± 431	9.5 ± 2.2*	9.7 ± 1.2

Data are means ± SE. Total PKC activity was determined as that stimulated in the presence of phosphatidylserine and DAG in the presence of calcium; nPKC and aPKC activity was that stimulated by the lipids in the absence of calcium, as detailed in METHODS. Results are given as means from cytosolic and membrane fractions, each assayed in triplicate under the four conditions, from five starch-fed and six fat-fed rats. Data for cytosolic and membrane PKC activity are pmol of substrate peptide phosphorylated · min^{-1} · g $muscle^{-1}$. *$P < 0.05$.

in subsequent immunoblots using PKC ζ and PKC ε antibodies, since the other apparently specific signals of 82 kDa and 80 kDa represent conventional PKC protein (29,30) and PKC δ (C.S.-P., C.L.B., T.J.B., unpublished observations), respectively. Other PKC isozymes were not quantified: PKC β immunoreactivity was extremely low (not shown), using any of three PKC β-specific antibodies, making densitometry impracticable, while PKC γ, PKC η, and PKC ι are absent or only poorly expressed in skeletal muscle (24,31,32).

Studies using untreated red quadriceps muscle from starch-fed rats demonstrated that when equivalent amounts of cytosolic and membrane fractions were analyzed, all of the PKC isozymes were expressed predominantly in the cytosolic as opposed to the membrane fraction. This was especially pronounced for PKC α, PKC θ, and PKC ζ (92, 88, and >99% cytosolic, respectively), but also true for PKC δ (67%) and PKC ε (72%). Because of the prolonged exposures required to detect PKC ζ in the membrane fraction, the quantification of the level of the isozyme in this fraction was not carried out. When detergent-insoluble protein was further extracted with SDS and 2-mercaptoethanol and subjected to immunoblotting as above, only traces of PKC δ , PKC ε, and PKC θ were detected (not shown) and were not quantitated.

Next, the levels of the different muscle PKC isozymes were compared in control and fat-fed rats. Red quadriceps muscles from unstimulated rats and also from rats subjected to the euglycemic-hyperinsulinemic clamp were studied. In addition, red gastrocnemius muscles and the relatively insulin-insensitive white quadriceps muscle were investigated. After densitometry of immunoblots, the mean cytosolic PKC content of starch-fed rat muscle within each group was normalized to 100% and the cytosolic PKC from fat-fed rat muscle, as well as the membrane levels from both starch- and fat-fed rat muscle, were expressed relative to this. For each group, the relative cytosolic and membrane levels of a given PKC are displayed together, with the sum being a measure of the total expression of that PKC isozyme (Figs. 3, 4*A*, 5, 6*A*, and 7). Translocation of isozymes was assessed as changes in the mean ratio of membrane levels to cytosolic levels in individual muscles. For certain isozymes, these data are supplemented with a correlation between red gastrocnemius muscle measurements of PKC and lipid levels in individual rats (Fig. 4*B*, 6*B*, and 6*C*).

Total levels of PKC α exhibited no significant changes in response to fat-feeding in any of the red or white muscle groups studied (Fig. 3). Thus, the diet did not cause up- or downregulation of the total levels of this isozyme, nor did it affect the relative proportions in the cytosolic and membrane fractions from individual muscles. Furthermore, no differences in the amount or location of PKC α were observed between red quadriceps muscles from untreated and insulin-treated rats. However, there was a tendency ($P < 0.09$) for the proportion of the isozyme in the membrane fraction to be higher in red muscles (gastrocnemius and quadriceps) than in white quadriceps muscle. The importance of this last observation is not clear; it may reflect a greater basal activation of PKC α in oxidative muscle.

The total levels of PKC ε protein were similarly unaffected by the high-fat diet in either red or white muscle (Fig. 4*A*). However, a twofold increase was observed in the propor-

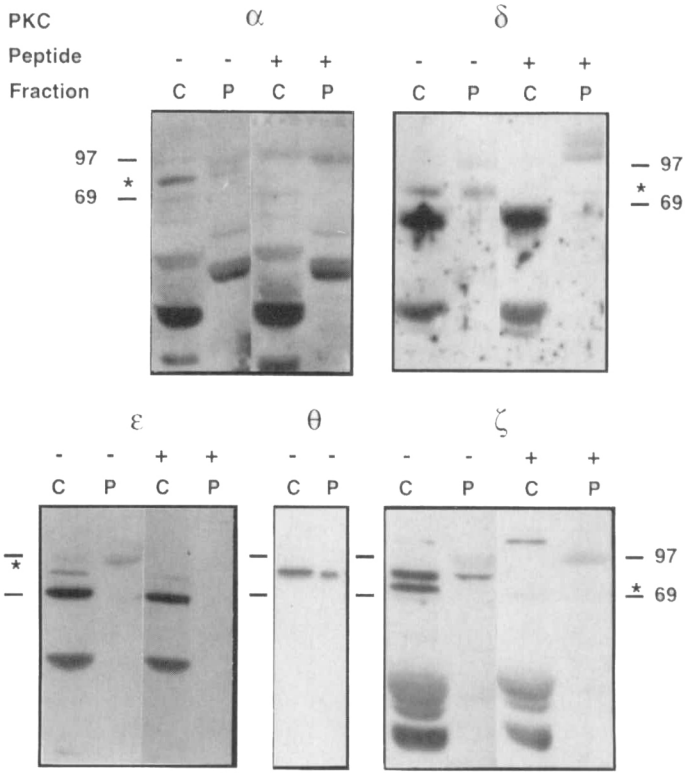


FIG. 2. Characterization of PKC-specific antibodies. Cytosolic (C) and particulate (P) fractions from mixed muscle extracts were prepared from control rats and subjected to SDS-PAGE and immunoblotting with the PKC-specific antibodies indicated, as detailed in METHODS. The specificities of the PKC α, δ, ε, and ζ antibodies were determined by carrying out incubations in the absence (–) and presence (+) of 10 μg/ml of the peptides against which they were raised. The migration of 97-kDa and 69-kDa molecular weight markers and of the PKC bands (*) are indicated.

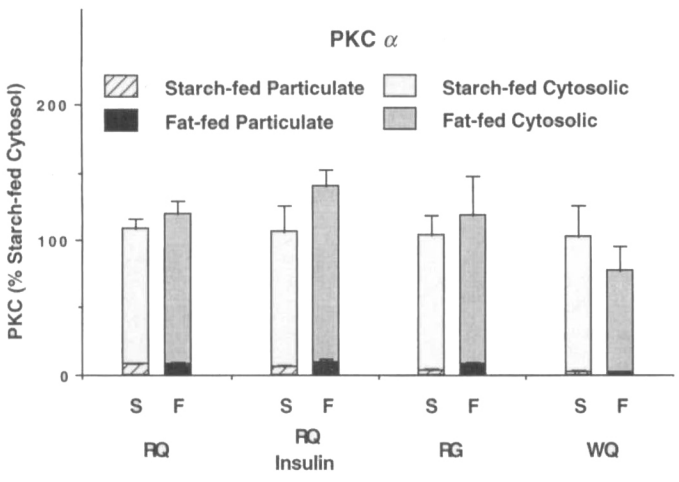


FIG. 3. Quantification of PKC α in cytosolic and membrane fractions of skeletal muscle from starch- and fat-fed rats. Samples from the four different groups of skeletal muscle indicated from both starch- (S) and fat-fed (F) rats were subjected to SDS-PAGE and immunoblotting with PKC α–specific antibodies, and results were analyzed by densitometry. RQ, red quadriceps; RG, red gastrocnemius; WQ, white quadriceps; insulin, muscle from rats subjected to euglycemic-hyperinsulinemic clamp. For further details, see METHODS. Within each muscle group, results are expressed relative to cytosolic PKC α from starch-fed rats, which was normalized to 100%. Mean membrane and cytosolic PKC α levels are summed and given as a single bar, with the proportion of PKC in each fraction indicated. Results are means of at least five rats per treatment. Results of statistical analyses are given in the text.

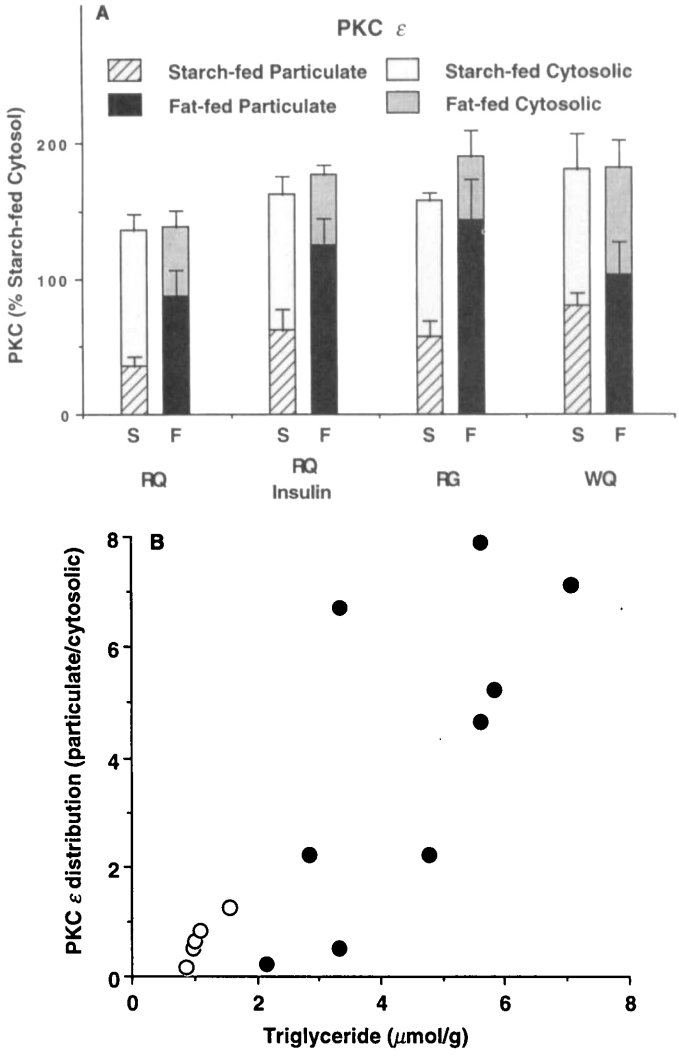


FIG. 4. Quantification of PKC ε in cytosolic and membrane fractions of skeletal muscle from starch- and fat-fed rats. *A*: data were generated as described in Fig. 3, except that PKC ε–specific antibodies were used in immunoblots. *B*: Relationship between PKC ε distribution and triglyceride levels in red gastrocnemius muscle from starch- (○) and fat-fed (●) rats. Results of statistical analyses are given in the text.

tion of this isozyme located in the membrane fraction of the red muscle groups (Fig. 4*A*). This was accompanied by a decrease in the cytosolic fraction (Fig. 4*A*), clearly indicating translocation of the kinase in response to fat-feeding ($P < 0.0001$ across the three red muscle groups). Distribution of PKC ε (assessed as the ratio of membrane to cytosolic PKC) in red gastrocnemius correlated significantly with triglyceride levels in this muscle (simple regression analysis, $P < 0.0005$, $r^2 = 0.675$; multiple regression analysis, triglyceride effect, $P < 0.01$, Fig. 4*B*), but less well with DAG muscle and not at all with basal plasma glucose (simple regression analysis, $P = 0.09$ and $P = 0.5$, respectively, data not shown). Interestingly, no significant effects of diet were seen on PKC ε in the white quadriceps, indicating that the translocation of this isozyme may be restricted to muscles showing large reductions in insulin sensitivity on fat-feeding.

In the case of PKC δ, an overall downregulation of the kinase in the cytosolic fractions from all muscle groups was observed (Fig. 5). When these results were analyzed by two-factor ANOVA, it was evident that muscle type had no effect on the cellular distribution of PKC δ, while fat-feeding had a significant effect on cytosolic ($P < 0.001$) but not membrane levels of the isozyme. Enzyme distribution was therefore also significantly affected by fat-feeding ($P < 0.05$). These results are in contrast with those obtained both for PKC α, where no effects of fat-feeding were observed on expression or translocation (Fig. 3), and for PKC ε, where the diet caused an increased proportion of the isozyme in the membrane fraction but no change in the total amount (Fig. 4*A*). Furthermore, PKC δ was the only isozyme found to undergo similar changes in both the red and white muscle groups in response to fat-feeding. Thus, the diet effect on PKC δ is not restricted to highly insulin-sensitive tissues, like red muscle, and therefore might not be involved specifically or selectively in the development of insulin resistance.

Fat-feeding caused a significant decrease in total PKC θ in red muscles only ($P < 0.005$ across the three groups), indicating downregulation. As with PKC δ, this effect was again confined to the cytosolic fraction ($P < 0.002$), while no decrease was seen in PKC θ from the membrane fractions of these muscles. This resulted in a highly significant change in the relative distribution of the kinase ($P < 0.005$). Moreover, PKC θ showed the greatest difference in cellular distribution between red and white muscle (Fig. 6*A*): a fivefold higher proportion of this isoform was found in the membrane fraction of white quadriceps from both starch- and fat-fed animals than in the same fraction of the red muscles ($P < 0.02$ for tissue effect, two-factor ANOVA). Thus, not only was the effect of the high-fat diet on PKC θ limited to the more insulin-sensitive red muscle groups, but the distribution of this isozyme was markedly different between white and red muscle in

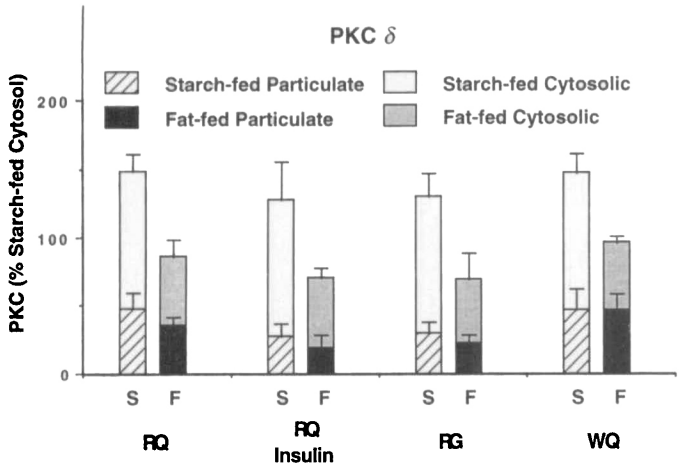


FIG. 5. Quantification of PKC δ in cytosolic and membrane fractions of skeletal muscle from starch- and fat-fed rats. Data were generated as described in Fig. 3, except that PKC δ–specific antibodies were used in immunoblots. Results of statistical analyses are given in the text.

starch-fed rats. PKC θ therefore responds differently both to PKC ε, which was translocated but not downregulated by fat-feeding, and to PKC δ, which showed no differences in cellular location between red and white muscle and was downregulated in all muscle groups studied.

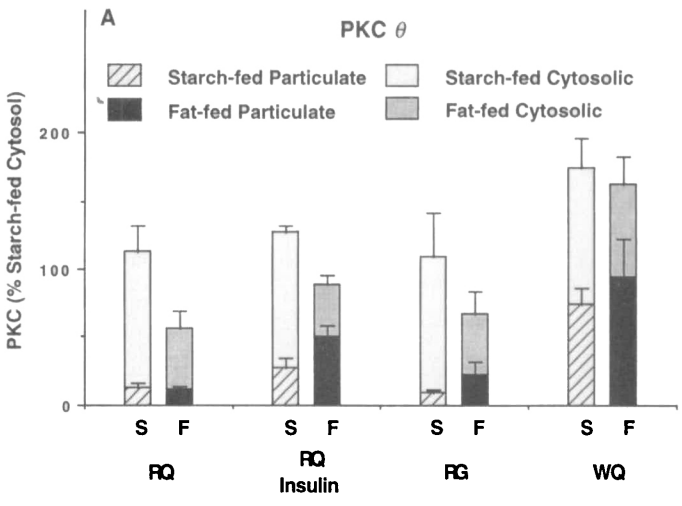
PKC θ was also the only isozyme in this study to be affected during physiological hyperinsulinemia. Thus, a greater proportion of PKC θ was observed in the membrane fraction of red muscle from rats that had undergone a euglycemic-hyperinsulinemic clamp than in this fraction of muscle from untreated rats ($P < 0.05$ for insulin effect, two-factor ANOVA). This effect was further enhanced in the fat-fed animals, indicating a combined action of insulin and diet ($P < 0.05$, two-factor ANOVA). These results indicate that while muscle from fat-fed rats is insulin resistant in terms of glucose transport, other effects of the hormone (in this case, the translocation of PKC θ) are in fact augmented by the fat diet.

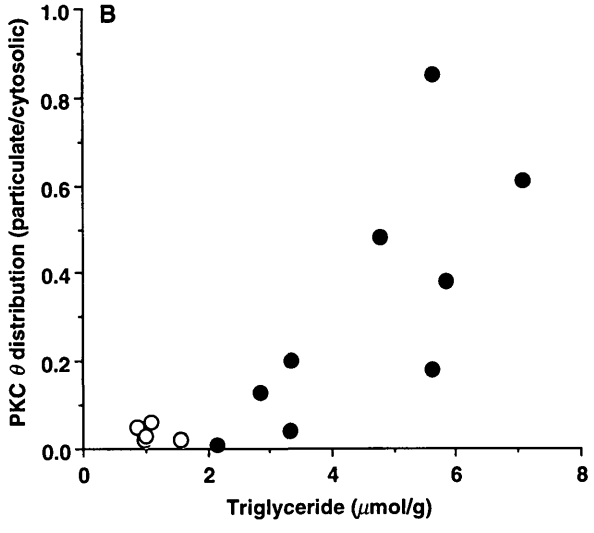
When PKC θ levels in red gastrocnemius from individual rats were compared with lipid measurements, it was found that distribution of the isozyme correlated well with muscle triglyceride (simple regression analysis, $P < 0.001$, $r^2 = 0.654$; multiple regression analysis, triglyceride effect, $P < 0.005$, Fig. 6*B*). Furthermore, PKC θ levels also correlated with muscle DAG (simple regression analysis, $P < 0.005$, $r^2 = 0.612$; multiple regression analysis, triglyceride effect, $P < 0.02$, Fig. 6*C*). Basal plasma glucose levels were not related to the translocation of PKC θ (simple regression analysis, $P = 0.98$, data not shown). As in the case of PKC ε, these findings support a role for increased muscle lipid storage, as opposed to plasma glucose in the alteration of kinase levels.

Finally, the cytosolic levels of PKC ζ in the red and white muscle groups were not significantly affected by fat-feeding (Fig. 7). Thus, although PKC α and PKC ζ exhibit markedly different properties in vitro, not least in their sensitivity to activators, they were the only isozymes in this study that did not exhibit changes in their cytosolic levels in response to fat-feeding.

DISCUSSION

In this study, we have examined the potential role of PKC isozymes in the modulation of insulin action in skeletal mus-





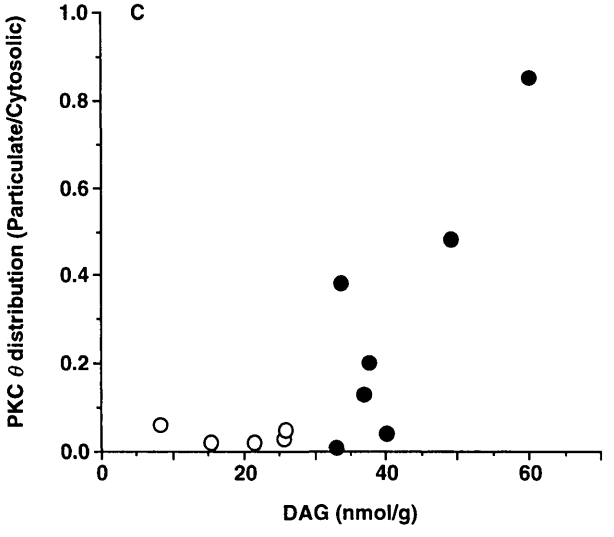


FIG. 6. Quantification of PKC θ in cytosolic and membrane fractions of skeletal muscle from starch- and fat-fed rats. *A*: data were generated as described in Fig. 3, except that PKC θ–specific antibodies were used in immunoblots. *B*: relationship between PKC θ distribution and triglyceride levels in red gastrocnemius muscle from starch- (○) and fat-fed (●) rats. *C*: relationship between PKC θ distribution and DAG levels in red gastrocnemius muscle from starch- (○) and fat-fed (●) rats. Results of statistical analyses are given in the text.

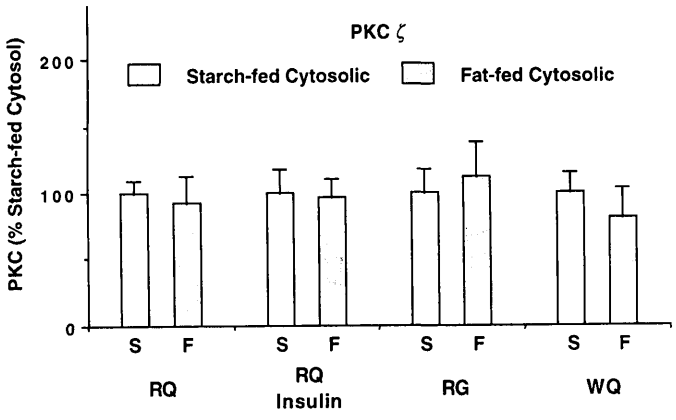


FIG. 7. Quantification of PKC ζ in cytosolic and membrane fractions of skeletal muscle from starch- and fat-fed rats. Data were generated as described in Fig. 3, except that PKC ζ-specific antibodies were used in immunoblots. Membrane levels of this isozyme were not quantitated. Results of statistical analyses are given in the text.

cle. By using the high-fat–fed rat model of insulin resistance, we have been able to correlate the expression and cellular distribution of PKC isozymes with several aspects of insulin sensitivity: with the different inherent insulin sensitivities of red and white skeletal muscles, with diet-induced muscle insulin resistance, and with acute changes in insulin-stimulated glucose metabolism. Our results, summarized in Table 3, are consistent with the hypothesis that aberrant PKC activation is involved in the generation of insulin resistance.

Previous studies have identified several PKC isozymes in muscle. PKC α and PKC βI and βII have been detected on muscle fiber surface membranes and within muscle fibers by immunohistochemical analysis of human skeletal muscle (33). In another such study, PKC β was not found in rat skeletal muscle cells but detected in motoneurons and myoneural junctions (34). The intracellular localization of PKC α and PKC θ has been studied by immunocytochemistry, the most intense PKC α immunoreactivity appearing in the cytosol, while PKC θ was associated with the sarcolemma, especially at the neuromuscular junction (35). PKC α, PKC β, PKC ε, and PKC θ were detected in soleus by immunoblotting in one recent study (36) and PKC α, PKC δ, and PKC ζ in rat skeletal muscle in another (37), although there have been no reports of the intramuscular location of PKC δ, PKC ε, or PKC ζ. In two studies involving Northern analyses, mRNA for PKC β was poorly detected in skeletal muscle extracts: the major isozymes observed were PKC α and PKC θ (39) and PKC α, PKC ε, and PKC θ (36). These results are consistent with our findings that PKC α is the most abundant cPKC activity in muscle cytosol, while PKC β activity is relatively scarce, and that the calcium-independent isozymes PKC δ, PKC ε, PKC θ, and PKC ζ are also present.

A recent study (36) found that PKC α, PKC β, and PKC ε are present in soleus with approximate ratios of total cytosolic immunoreactive PKC to total membrane immunoreactive PKC of 3–4:1 (although, as in our study, PKC β was harder to detect), while a ratio of 1:1 was reported for PKC θ. Others have found approximately equal amounts of cPKC and PKC θ immunoreactive protein in cytosol and membranes from white skeletal muscle (38). The lower amounts of certain PKC isozymes observed in the membrane fraction in our study is unlikely to be due to differences in extraction buffers or centrifugation, which are quite similar. However, we have included a wash step before solubilization of particulate PKC, which reduces contamination of the large 100,000*g* pellets with cytosolic PKC and, hence, will lower the percentage of PKC observed in the membrane fraction. Furthermore, some differences may be attributed to muscle type, as we also saw a larger proportion of membrane-associated PKC θ in white quadriceps muscle compared with red quadriceps or red gastrocnemius muscle (Fig. 6*A*). It has been suggested that this difference might underlie the reduced insulin sensitivity of white muscle relative to red (38). Although a high proportion of PKC θ has been found in cytoskeletal fractions when expressed in Cos-1 cells (39,40), we found little evidence of the isozyme in this fraction of skeletal muscle.

PKC activity assays gave an indication that the insulin resistance induced by a high-fat diet is accompanied by changes in PKC activity. However, the results are difficult to interpret, since although calcium-dependent and -independent activities can be differentiated, these are still the products of several isozymes, only some of which may be altered by fat-feeding. In addition, the assay conditions are not optimal for all isozymes, as suggested by the low levels of calcium-independent activity detected, even though a substrate peptide was chosen that is phosphorylated by PKC isoforms α, β, γ, δ, ε, θ, η, and ζ (41,42). Finally, despite partial purification of PKC by ion exchange chromatography, some endogenous lipid activators from muscle may still be present during assay that will reduce the activity stimulated in the presence of added phosphatidylserine and DAG (43). Therefore, we consider that our results from immunoblotting with isozyme-specific antibodies are more informative. Even so, both approaches are broadly consistent in that they suggest a role

TABLE 3
Summary of changes in PKC levels and distribution in red skeletal muscle of fat-fed rats relative to starch-fed control rats

PKC isozyme	Total	Membrane	Cytosolic	Distribution	Insulin effects on distribution
α	NS	NS	NS	NS	NS
ε	NS	262	49	576	NS
θ	56	NS	45	390	867
δ	64	NS	54	193	NS
ζ	ND	ND	NS	ND	ND

Data are %. Values were calculated from the combined results from red quadriceps and red gastrocnemius muscles and indicate the amount or distribution (membrane/cytosolic levels) of PKC observed in red skeletal muscle fractions of the fat-fed rat, expressed relative to the corresponding fraction in starch-fed rats. ND, not determined.

for novel, as opposed to conventional, PKC isozymes in the development of muscular insulin resistance.

From studies showing that in vitro activation of PKC can inhibit insulin signaling, we anticipated that insulin-resistant skeletal muscle from the high-fat–fed rat would exhibit upregulation or greater activation of PKC isozymes. In fact, our results demonstrated that none of the investigated PKC isozymes increased in total amount, suggesting that fat-feeding does not lead to higher expression of any PKC. On the other hand, PKC ε did exhibit translocation in response to the diet (Fig. 5*A*). A number of studies in several cell types have demonstrated translocation of nPKC isozymes in response both to phorbol esters and physiological stimuli, and such translocation has been associated with increased DAG (44,45) or free fatty acid levels (46) and phosphorylation of known PKC substrates in the intact cells, such as the 80-kDa MARCKS protein (47) and pleckstrin (46,48). This suggests that activation of nPKC, as of cPKC, also involves translocation. While there have been no reports to our knowledge about the effects of PKC ε activation in muscle, overexpression of this isozyme in other cell types has been associated with growth control disorders (49,50). Thus, our results suggest a chronic activation of PKC ε without ensuing downregulation, which may have effects on muscle cell signaling and protein expression. We suggest that the kinase might interfere with components of the insulin signal transduction cascade, activate tyrosine phosphatases by direct phosphorylation, or alternatively affect the expression of particular components of the signaling pathway.

Downregulation of nPKCs has also been reported after prolonged activation, for example, by phorbol esters (51), nerve growth factor (52), insulin (47), and thyrotropin-releasing hormone (53). Frequently, PKC ε has been found to be more resistant than other PKC isozymes to such downregulation (47,51,54,55), which is in agreement with our findings concerning PKC δ, PKC ε, and PKC θ. The downregulation of cytosolic PKC θ, giving an increased proportion of membrane-associated enzyme, was found to be restricted to red skeletal muscle in the present study. Thus, we consider an increase in the relative amount of membrane-associated PKC θ to be more likely to relate to the development of insulin resistance than that observed for PKC δ.

Another important finding is the highly selective effect of insulin on the PKC isozymes, again increasing the proportion of PKC θ in the membrane fraction (Fig. 6*A*). Despite the loss of cytosolic PKC θ caused by the diet alone, there was a synergistic effect of insulin and fat-feeding on the translocation. Because translocation is enhanced while glucose disposal is diminished by fat-feeding, it is possible that PKC θ plays an inhibitory role in insulin signaling and contributes to insulin resistance in an acute fashion rather than in the chronic manner suggested for PKC ε. However, further work is needed to establish definitively whether a causative link exists between these observed changes in PKC regulation and the development of insulin resistance.

While it has been suggested that PKC activation arises from increased glucose incorporation into DAG on hyperglycemia (56–58), our results suggest that additional or alternative mechanisms might apply in the chronic regulation of PKC in insulin target tissues. Basal plasma glucose does not differ between starch- and fat-fed rats (Table 1) nor does the postabsorptive flux of glucose into skeletal muscles (20). Consequently, effects of increased muscle lipid species on PKC isoforms are unlikely to be secondary to hyperglycemia after the generation of insulin resistance. This view is supported by the lack of correlation in individual rats between plasma glucose and the changes in PKC reported here. Other studies involving models of insulin resistance where glucose levels are not elevated have also found increases in membrane-associated PKC activity or protein, such as in the liver of diabetic Zucker rats treated with phlorizin or insulin (59), in denervated rat soleus muscle (28), and in white skeletal muscle of fructose-fed rats (38).

On the other hand, our data strongly suggest a relationship among increased lipid availability, alteration of PKC activity, and insulin resistance. Changes in PKC ε and PKC θ distribution correlated well with muscle triglyceride levels (Figs. 4*B* and 6*B*). While it has not been reported that triglyceride has a direct effect on these enzymes, its accumulation might be indicative of a rise in other lipid species within the cell, which themselves could activate PKC. Thus, the levels of DAG, a well-established activator of PKC, increased along with triglyceride in skeletal muscle (Fig. 1*C*) and also correlated with PKC θ distribution (Fig. 6*C*). In addition to DAG, other lipids are known to affect PKC activity in vitro, such as unsaturated fatty acids (60,61) and fatty acyl CoAs (62–64). These could also be responsible for the changes observed in specific PKC isozymes.

In summary, we have observed selective changes in the expression and cellular localization of PKC isozymes in association with insulin resistance in the high-fat–fed rat. The chronic translocation, and hence possible activation, of PKC ε makes this isozyme a candidate for a causative link between PKC activity and insulin resistance. We have also shown that PKC θ is translocated during hyperinsulinemia and that fat-feeding potentiates this effect, consistent with an inhibitory role of this isozyme in the action of the hormone. The observed changes in PKC do not appear to be secondary to hyperglycemia. Instead, our results provide a conceptual explanation for the previously reported correlation between muscle lipid availability and insulin resistance.

Note added in proof: A recent study of skeletal muscle from diabetic G-K rats has reported further changes in expression and distribution of PKC isozymes in addition to those described here (Avignon et al., *Diabetes* 45:1396–1404, 1996). These changes were interpreted as being secondary to hyperinsulinemia. However, hyperinsulinemia is not present in the high-fat–fed rat and therefore cannot explain the more limited alterations in nPKCs reported here, which we suggest are due to increased muscle lipid availability.

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