A Role for Protein Phosphatase 2A–Like Activity, but Not Atypical Protein Kinase Cζ, in the Inhibition of Protein Kinase B/Akt and Glycogen Synthesis by Palmitate

Rosanna Cazzolli, Lee Carpenter, Trevor J. Biden, and Carsten Schmitz-Peiffer

We have shown previously that palmitate treatment of C2C12 skeletal muscle myotubes causes inhibition of the protein kinase B (PKB) pathway and hence reduces insulin-stimulated glycogen synthesis through the elevation of intracellular ceramide levels. Ceramide is known to activate both atypical protein kinase C (aPKC) ζ and protein phosphatase (PP) 2A, and each of these effectors has been reported to inhibit PKB. In the present study, palmitate pretreatment was found to elevate PP2A-like activity in myotubes and to prevent its inhibition by insulin. Incubation with the phosphatase inhibitor okadaic acid before insulin stimulation protected against the effect of the fatty acid on PKB phosphorylation. Palmitate was unable to inhibit PKB activity and glycogen synthesis in cells overexpressing the activated PKB mutant (T308D,S473D)-PKBα, which is unaffected by phosphatase. In contrast, PKB activity and glycogen synthesis were still inhibited by palmitate in cells overexpressing a membrane-targeted and, hence, activated PKB mutant that retains sensitivity to phosphatase. Although aPKC activity was also increased in palmitate-treated cells, overexpression of wild-type or kinase-dead aPKCζ did not alter the inhibitory effects of the lipid on either stimulation of PKB or glycogen synthesis by insulin. We conclude that palmitate disrupts insulin signaling in C2C12 myotubes by promoting PP2A-like activity and, therefore, the dephosphorylation of PKB, which in turn reduces the stimulation of glycogen synthesis. *Diabetes* 50:2210–2218, 2001

Insulin resistance is a major characteristic of type 2 diabetes, and it can be defined as a failure of target tissues to increase glucose disposal in response to insulin. The insulin resistance seen in skeletal muscle is of particular importance because this is the major site of insulin-stimulated glucose uptake and glycogen synthesis (1). Many studies have revealed an association between increased lipid availability and insulin resistance (2–4), suggesting that there is a causative link between the two, although the underlying mechanisms are not clear.

The regulation of glycogen synthesis by insulin is thought to be mediated primarily through the protein kinase B (PKB) pathway, downstream of the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and the stimulation of phosphatidylinositol (PI) 3-kinase activity. PKB is transiently recruited to the plasma membrane by the increase in PI (3,4,5)-trisphosphate (PI[3,4,5]P_3) levels, where it becomes phosphorylated on two residues crucial to its activation (5). Phosphorylation of Thr308 is mediated by PI(3,4,5)P_3-dependent kinase (PDK)-1, whereas the subsequent phosphorylation of Ser473 may be mediated by a hypothetical PDK-2 or by autophosphorylation (6). After activation in this manner, PKB is released from the membrane to phosphorylate substrates, including glycogen synthase kinase-3 (GSK-3), which is thus inactivated. GSK-3 is probably the major regulatory kinase of glycogen synthase. It phosphorylates the metabolic enzyme at three sites, causing marked inhibition, although phosphorylation by other kinases may also play a role. Therefore, inhibition of GSK-3, together with activation of protein phosphatase (PP) 1, may explain the increased synthesis of glycogen from glucose in response to insulin (7).

We have recently shown that pretreatment of C2C12 skeletal muscle cells with the saturated free fatty acid palmitate leads to inhibition of PKB phosphorylation in response to insulin, without effects on upstream signaling events, such as IRS-1 tyrosine phosphorylation and PI 3-kinase activation, and thus it leads to inhibition of insulin-stimulated glycogen synthesis (8). The elevation of ceramide levels in the muscle cells, which occurs specifically in response to palmitate but not to unsaturated free fatty acids, appears to be sufficient to account for these effects, because exogenously added ceramide was also able to inhibit PKB phosphorylation and glycogen synthesis (8). Ceramide has similar effects on PKB in a number of cell lines (9–12), and in each case, these are observed in the absence of alterations in PI 3-kinase activity.

Ceramide can be generated by de novo synthesis from palmitate (13), and also acutely upon the activation of sphingomylinases, such as in response to tumor necrosis

From the Garvan Institute of Medical Research, Sydney, Australia.

Address correspondence and reprint requests to Carsten Schmitz-Peiffer, Cell Signalling Group, Garvan Institute of Medical Research, 384, Victoria St., Darlinghurst, NSW 2010, Australia. E-mail: c.schmitz-peiffer@garvan.org.au.

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aPKC, atypical protein kinase C; aPKCζKD, kinase-dead aPKCζ; aPKCζwt, wild-type aPKCζ; BSA, bovine serum albumin; CAPP, ceramide-activated protein phosphatase; EMEM, minimum essential medium with Earles' salts; FCS, fetal calf serum; GFP, green fluorescence protein; GSK-3, glycogen synthase kinase-3; HA, hemagglutinin; IRS-1, insulin receptor substrate-1; m/p, myristoylated/palmitoylated; PBS, phosphate-buffered saline; PDK, phosphatidylinositol (3,4,5)P_3-dependent kinase; PI, phosphatidylinositol; PI(3,4,5)P_3, PI (3,4,5)-trisphosphate; PKB, protein kinase B; PMSF, phenylmethyl sulfoxide; PP, protein phosphatase; SF, serum-free.

factor-α stimulation. The lipid has been reported to activate several signaling proteins, including certain PKC isoforms (14–16) and a serine/threonine phosphatase of the PP2A family known as ceramide-activated protein phosphatase (CAPP) (17,18). Atypical protein kinase C (aPKC) ζ and PP2A are candidates for the inhibition of PKB phosphorylation after palmitate pretreatment, because they have been reported to be negative regulators of PKB activity (19–21).

We have determined the levels of both aPKCζ and PP2A-like activity in C2C12 myotubes pretreated with palmitate, as well as the effects of such activities on PKB phosphorylation and activation. Although both of these effectors appeared to be stimulated by palmitate in this system, we found evidence that phosphatase, but not aPKC activity, modulates PKB phosphorylation and activity and hence insulin-stimulated glycogen synthesis.

RESEARCH DESIGN AND METHODS

Materials. Bovine serum albumin (BSA), palmitic acid, and okadaic acid (sodium salt) were from Sigma Chemical (St. Louis, MO). Insulin was from Novo Nordisk (Copenhagen). aPKCζ antibodies were from Santa Cruz Biotechnology (sc-216; Santa Cruz, CA). Serine/threonine phosphatase assay system was from Promega (Madison, WI). Antibodies to PKB and phosphoSer473-PKB were from New England Biolabs (Beverly, MA). Protein A-Sepharose and goat–anti-mouse IgG-Sepharose were from Zymed (San Francisco, CA). Crosstide (GRPRTSSFAEG) and PKC-ε substrate peptide (PRKRQGSVRRRV) were from Biosource International (Camarillo, CA). 12CA5 mouse hybridoma cells secreting antibodies to hemagglutinin (HA) were a kind gift from W. Langdon, University of Western Australia. Other reagents were from Sigma or BDH (Merck).

Cell culture, palmitate preincubation, and glycogen assay. C2C12 myoblasts were maintained in minimum essential medium with Earles' salts (EMEM) containing 10% fetal calf serum (FCS) and differentiated into myotubes in 1% FCS-EMEM, as described previously (8). Palmitate-containing media were prepared by conjugation of the free fatty acid with BSA (8). Unless otherwise stated, myotubes in 6-well plates or 10-cm dishes were pretreated for 16 h in 2 ml/well or 10 ml/dish 1% FCS-EMEM containing 5% BSA with or without 0.75 mmol/l palmitate. They were then treated for 2 h in 1 ml/well or 5 ml/dish serum-free (SF)-EMEM, again containing 5% BSA with or without 0.75 mmol/l palmitate. For measurement of glycogen synthesis, lipid-pretreated myotubes in 6-well plates were incubated for 1 h in 1 ml/well SF-EMEM containing D-[U-^{14}C]glucose (4 μCi/ml) in the absence or presence of 100 nmol/l insulin and palmitate, and glycogen production was determined as previously described (8).

Generation and use of recombinant adenoviruses. Wild-type aPKCζ (aPKCζwt) cDNA (EMBL accession no. M94632) was a kind gift from H. Mischak, Laboratory of Genetics, National Cancer Institute, Bethesda, MD). Kinase-dead aPKCζ (aPKCζKD) was generated by polymerase chain reaction–based mutagenesis to give a K281W substitution (22,23) using 5′-TACGCCAT GTGGGTGGTAAAG-3′ as a primer. Adenoviruses containing these constructs were prepared by homologous recombination with the parental virus genome (pJM17) in HEK293 cells, as described by Graham and Prevec (24). Plaque assays were performed in HEK293 cells to determine the titer in plaque-forming units per milliliter of these viral stocks. A pJM17-derived adenovirus expressing green fluorescence protein (GFP) was used in control infections for aPKCζ constructs. The amount (in plaque-forming units per well) of this virus required for 80–100% infection of myotubes was determined by visualizing GFP expression in the myotubes using fluorescence microscopy, and this amount was also used for viruses expressing aPKCζ constructs (typically 5×10^7 pfu/well). HA epitope-tagged wild-type human PKBα (EMBL accession no. M63167), constitutively active (T308D,S473D)-HA-PKBα, and myristoylated/palmitoylated (m/p)-HA-PKBα were kind gifts from J. Tavaré, University of Bristol, Bristol, U.K., and have been characterized previously (5,25). The pAdEasy system was used to generate recombinant adenovirus for the expression of these PKB constructs, as described by He et al. (26), allowing co-expression of GFP and PKB. A pAdEasy-derived virus expressing GFP alone was used in control infections for PKB constructs. The amount of virus required to give 80–100% infection efficiency for each virus constructed using the pAdEasy system was individually determined by visualizing GFP expression. All viruses were used to infect C2C12 myotubes in 6-well plates at day 2 of differentiation, i.e., 3 days before experiments.

HA-PKBα immunoprecipitation and kinase assay. PKB activity was determined by a method adapted from Doornbos et al. (19). C2C12 myotubes in 6-well plates, infected with adenoviruses for expression of the HA-PKBα constructs described above, were treated as described in the figure legends and washed twice with ice-cold phosphate-buffered saline (PBS), and the cells were scraped into 500 μl PKB lysis buffer (20 mmol/l MOPS, pH 7.5, 1% [vol/vol] Triton X-100, 10 mmol/l sodium pyrophosphate, 10 mmol/l NaF, 150 mmol/l NaCl, 200 μg/ml leupeptin, 2 mmol/l phenylmethyl sulfoxide [PMSF], and 200 μg/ml aprotinin). After sonication (15 pulses using a Branson Sonifier 250 and microtip at power setting 2, 20% duty cycle), lysates were centrifuged at 20,000*g* for 10 min at 4°C. Supernatants were incubated with gentle rocking for 2 h at 4°C with 5 μl mouse monoclonal HA antibodies, prepared from 12CA5 cell culture supernatants by Protein A affinity chromatography. After the addition of 50 μl of a 1:1 suspension of goat–anti-mouse IgG-Sepharose beads in PKB lysis buffer and further rocking for 1 h, lysates were briefly centrifuged at 3,000*g*, and immunoprecipitates were washed three times with PKB lysis buffer. Beads were split into three aliquots and resuspended in 50 μl PKB kinase buffer (50 mmol/l Tris-HCl, pH 7.5, 1 mmol/l dithiothreitol, 30 μmol/l Crosstide peptide, and 1 μmol/l cAMP-dependent protein kinase inhibitor peptide, rabbit sequence). Reactions were started by the addition of 5 μl of 500 μmol/l [γ-^{32}P]ATP (0.25 mCi/ml) and 100 mmol/l $MgCl_2$ and then incubated for 10 min at 30°C. After the addition of 10 μl of 150 mmol/l ATP, 40-μl samples were spotted onto P81 paper squares (Whatman). Papers were washed 4×5 min in 8.5% (vol/vol) orthophosphoric acid and 1×5 min in ethanol, dried, and counted in water for Cerenkov radiation. Background activities observed in immunoprecipitates from myotubes overexpressing only GFP were used as assay blanks for each condition and subtracted from observed PKB activities.

aPKC immunoprecipitation and kinase assay. Endogenous aPKC activity was determined by a method adapted from Bandyopadhyay et al. (27). C2C12 myotubes grown in 10-cm dishes were washed twice in ice-cold PBS and scraped into 500 μl aPKC lysis buffer (20 mmol/l MOPS, pH 7.5, 1% [vol/vol] Triton X-100, 0.5% [vol/vol] Nonidet P40, 250 mmol/l sucrose, 1.2 mmol/l EGTA, 1 mmol/l Na_3VO_4, 1 mmol/l sodium pyrophosphate, 1 mmol/l NaF, 150 mmol/l NaCl, 200 μg/ml leupeptin, 2 mmol/l PMSF, and 20 μg/ml aprotinin). Lysates were sonicated and centrifuged as described for PKB immunoprecipitation. After the addition of 5 μl aPKCζ antibodies, supernatants were gently rocked for 16 h at 4°C, followed by the addition of 50 μl of a 1:1 suspension of protein A-Sepharose beads in aPKC lysis buffer, and then further rocking for 1.5 h. Lysates were briefly centrifuged at 3,000*g*, and the beads were washed three times with lysis buffer. Immunoprecipitates were split into three aliquots after resuspension in 50 mmol/l Tris, pH 7.5, 100 μmol/l Na_3VO_4, 100 μmol/l sodium pyrophosphate, 1 mmol/l NaF, and 200 μmol/l PMSF; one of the aliquots was used for correction of aPKC protein recovery after immunoblotting, and two were assayed for kinase activity. Immunoprecipitates were resuspended in 50 μl of the same buffer, which also contained 80 μg/ml phosphatidylserine and 40 μmol/l PKC-ε peptide. Assays were started by the addition of 5 μl of 500 μmol/l [γ-^{32}P]ATP (0.25 mCi/ml) and 50 mmol/l $MgCH_3CO_2H$ and then incubated for 10 min at 30°C. Incorporation of phosphate into PKC-ε peptide was determined as described for PKB assays.

Protein phosphatase assays. Particulate fractions were prepared from C2C12 myotubes grown in 6-well plates that had been treated as stated in the figure legends. Cells were washed twice in ice-cold saline, scraped into 500 μl PP2A lysis buffer (50 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 1 mmol/l PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and sonicated as above. Lysates were centrifuged at 400,000*g* for 10 min at 4°C, and washed pellets were resuspended in 100 μl PP2A lysis buffer. Supernatants were passed through 10-ml Sepadex G-25 columns to eliminate ATP and free phosphate. The protein phosphatase activity in these fractions was determined by measuring the generation of free phosphate from the phosphopeptide RRA(pT)VA using the molybdate-malachite green-phosphate complex assay (Promega) as described by the manufacturer. This peptide substrate, suitable especially for type 2 phosphatases (28), was used in the absence of Ca^{2+} and Mg^{2+}, which are required for activity for protein phosphatases 2B and 2C (29). Assays were carried out using 20 μl cytosolic or particulate fraction in 50 μl of a Ca^{2+}- and Mg^{2+}-deficient reaction buffer (final concentrations 50 mmol/l imidazole [pH 7.2], 0.2 mmol/l EGTA, 0.02% 2-mercaptoethanol, 0.1 mg/ml BSA, and 100 μmol/l phosphopeptide substrate). After a 10-min incubation at room temperature, molybdate dye was added, and free phosphate was measured by optical density at 630 nm. A standard curve with free phosphate was used to determine the amount of free phosphate generated. Under these conditions, assays were linear with respect to the amount of cell fraction added (data not shown). Results were corrected for total protein in the cell fractions, measured by the method of Bradford (30).

Statistics. All results are expressed as the means ± SE and analyzed using Student's *t* test. Statistical calculations were performed using Statview SE + Graphics for Macintosh (Abacus Concepts, Berkeley, CA).

RESULTS

We have shown that pretreatment of C2C12 myotubes with palmitate reduces the level of phosphorylated PKB in response to insulin (8). To confirm that this was associated with a reduction in PKB activity, C2C12 myoblasts were infected with adenovirus during early differentiation to optimize expression of HA-tagged PKBα. We then assayed kinase activity in HA-immunoprecipitates prepared from basal and insulin-stimulated cells that had been preincubated in the absence or presence of palmitate. As expected, immunoprecipitates from control cells exhibited threefold stimulation of PKB activity in response to insulin, whereas kinase activity derived from palmitate-pretreated cells was greatly inhibited (Fig. 1*A*). Confirming previous results, palmitate also reduced the fraction of PKBα that was phosphorylated on Ser473, as determined by immunoblotting of cell lysates (Fig. 1*B*).

The elevation of ceramide levels induced by palmitate pretreatment appears to play an important role in the inhibition of insulin-stimulated PKB phosphorylation (8). Ceramide has been reported to stimulate a PP2A-like serine/threonine phosphatase activity (also known as CAPP) (17,18); because PP2A is capable of dephosphorylating and hence inactivating PKB (21), we investigated whether such a mechanism could explain the inhibition of PKB phosphorylation in palmitate-pretreated C2C12 cells. First, we measured phosphatase activity in extracts of myotubes under conditions selective for PP2A-like activity. Cytosolic fractions, which contained ~60% of the total activity, showed no significant change in response to either palmitate pretreatment or insulin stimulation (data not shown). In contrast, particulate fractions from insulin-stimulated cells exhibited a 50% reduction in PP2A-like activity, whereas palmitate elevated this activity in unstimulated cells by almost 50% and also totally prevented the inhibition by insulin (Fig. 2). This resulted in an almost threefold increase in membrane-associated PP2A-like activity in the presence of insulin in palmitate-treated cells compared with untreated cells.

Further evidence for the activation of protein phosphatase activity by palmitate was obtained using the phosphatase inhibitor okadaic acid. Although the use of 1 μmol/l okadaic acid, a concentration commonly used to inhibit both PP1 and PP2A in intact cells (31), caused detachment of myotubes within 10 min, this was prevented by reducing the length of lipid pretreatment to 4 h and the concentration of the inhibitor to 250 nmol/l. Under these conditions, the effect of palmitate on PKB phosphorylation was reduced, probably because of a lower accumulation of ceramide, but the cells did not become detached in the presence of the inhibitor. Incubation of myotubes with okadaic acid for 45 min was able to reverse the inhibitory effects of palmitate on subsequent stimulation of PKB phosphorylation by insulin (Fig. 3). The phosphatase inhibitor had little effect, however, on phosphorylation levels in insulin-stimulated cells in the absence of palmitate. Together with the observed increase in phosphatase activity, these results suggest that palmitate counteracts the

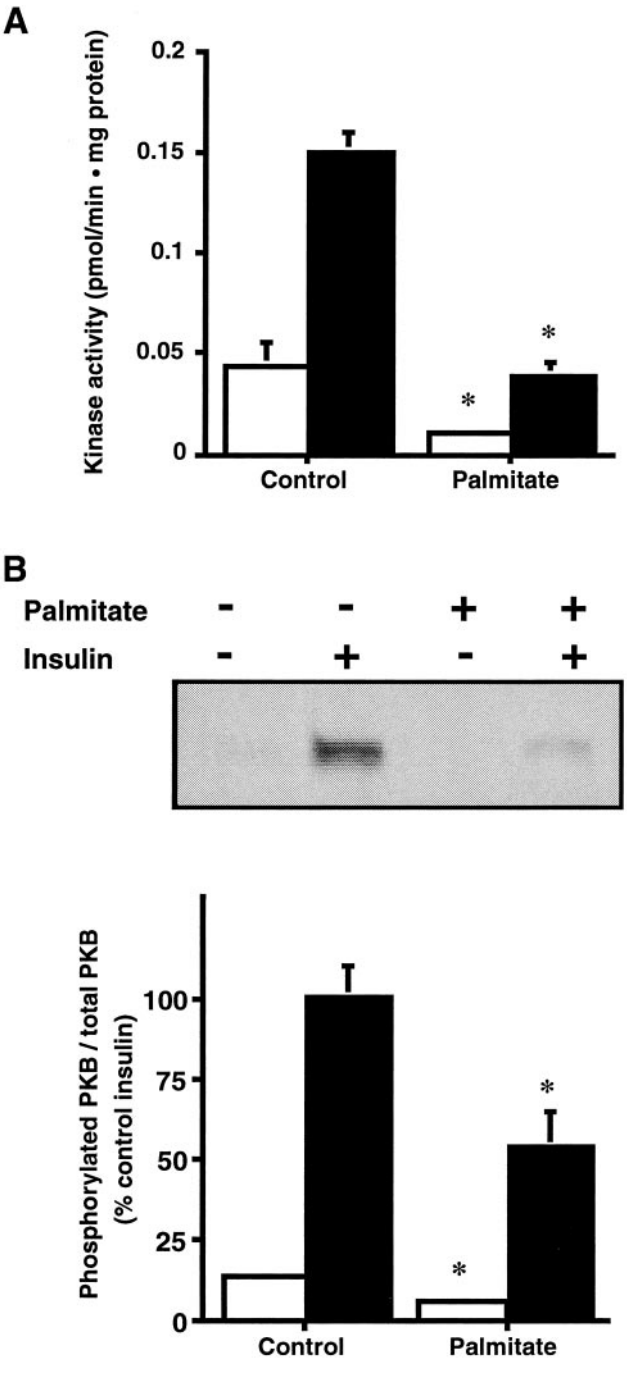

FIG. 1. Activation of PKB in C2C12 myotubes by insulin, and inhibition by palmitate pretreatment. C2C12 myotubes were infected with adenovirus to express wild-type HA-PKBα, and at 48 h postinfection, cells were incubated for 16 h in the absence or presence of 0.75 mmol/l palmitate in 1% FCS-EMEM. After a further 2 h incubation in SF-EMEM, again in the absence or presence of palmitate, myotubes were then incubated for 10 min with or without 100 nmol/l insulin. *A*: Kinase activity was determined in HA immunoprecipitates prepared from cell lysates containing 500 μg total protein. All procedures were carried out as detailed in RESEARCH DESIGN AND METHODS. *$P < 0.001$ for palmitate-treated versus appropriate untreated control. *B*: Cell lysates were also subjected to immunoblotting for phospho-Ser473 PKB, and data were corrected for total PKB protein levels, obtained from the same membranes by the stripping of antibodies and reprobing, as detailed in RESEARCH DESIGN AND METHODS. A representative immunoblot and the means from densitometry of three independent experiments are shown. *$P < 0.001$ for palmitate-treated versus appropriate untreated control. □, Basal; ■, insulin.

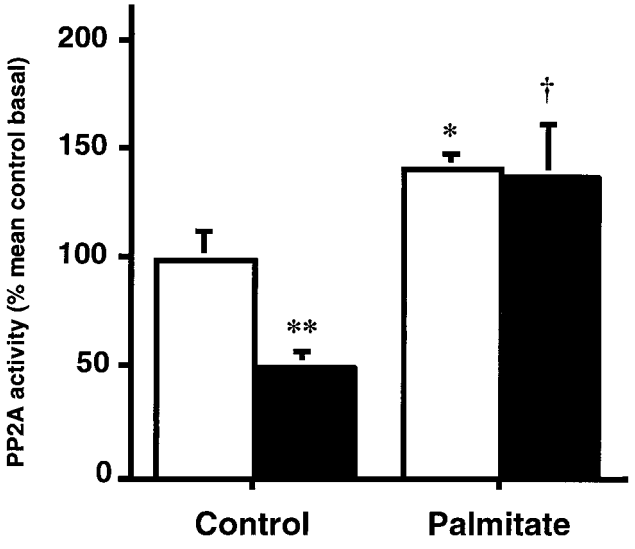

FIG. 2. Activation of PP2A in C2C12 myotubes by insulin and palmitate pretreatment. Cells were treated with palmitate and insulin as in Fig. 1, and a particulate fraction was prepared from cell lysates and assayed for PP2A activity as detailed in RESEARCH DESIGN AND METHODS. The results shown are the combined means from three independent experiments, each carried out in triplicate. *$P < 0.05$ for palmitate basal versus control basal; **$P < 0.005$ for control insulin versus control basal; †$P < 0.002$ for palmitate insulin versus control insulin. □, Basal; ■, insulin.

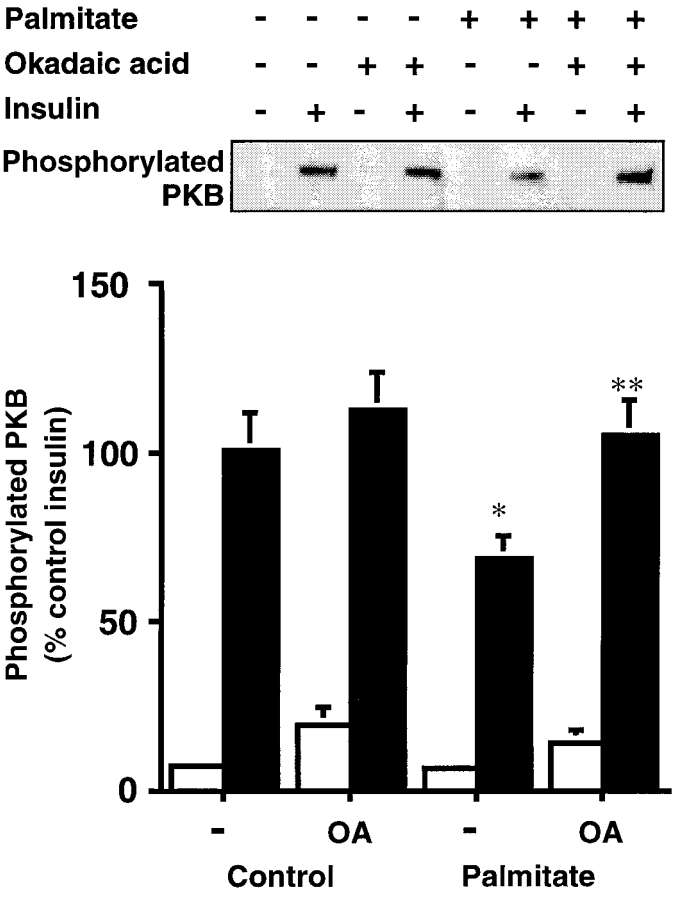

FIG. 3. Effect of the phosphatase inhibitor okadaic acid on the inhibition of PKB phosphorylation by palmitate. C2C12 myotubes were pretreated with palmitate for 4 h in SF-EMEM before the addition of 250 nmol/l okadaic acid. After a further 45 min, cells were incubated for 10 min in the absence and presence of 100 nmol/l insulin. Cell lysates were subjected to immunoblotting for phospho-Ser473 PKB, and data were corrected for total PKB protein levels. A representative immunoblot and the means from densitometry of three independent experiments are shown. *$P < 0.02$ for palmitate insulin versus control insulin; **$P < 0.001$ for palmitate okadaic acid versus palmitate alone. □, Basal; ■, insulin. OA, okadaic acid.

effect of insulin on PKB phosphorylation through the stimulation of a PP2A-like phosphatase.

To further address the role of increased phosphatase in the effect of palmitate, we assessed the sensitivity of wild-type and mutant HA-PKBα to inhibition by the lipid. The (T308D,S473D)-HA-PKBα mutant is constitutively active because aspartate residues mimic the effects of phosphorylation at Thr308 and Ser473 (5), and it is thus insensitive to downregulation by phosphatase activity. The (m/p)-HA-PKBα mutant is membrane-targeted, and although it is highly activated by phosphorylation even in unstimulated cells (25), it is still able to undergo dephosphorylation and inactivation. Because (T308D,S473D)-HA-PKBα is independent of phosphorylation, we did not compare the status of Ser473 phosphorylation in lysates from myotubes overexpressing the different PKB mutants, but instead determined the activity of these kinases (Fig. 4). As anticipated, the wild-type HA-PKBα was activated more than threefold by insulin in control cells, but it was greatly inhibited in cells that had been pretreated with palmitate. In contrast, (T308D,S473D)-HA-PKBα was not significantly affected by insulin, and most importantly, it did not undergo inhibition by palmitate. The (m/p)-HA-PKBα mutant was greatly activated even in the absence of insulin but showed similar sensitivity to the wild-type HA-PKBα in terms of downregulation by palmitate (Fig. 4). The differing sensitivities of the PKB mutants support the hypothesis that palmitate pretreatment inhibits PKB by promoting its dephosphorylation.

We also determined whether overexpression of the different HA-PKB constructs could modify the effect of palmitate treatment on insulin-stimulated glycogen synthesis, because this is regulated by PKB (7). Myotubes expressing only GFP as control exhibited a twofold increase in glycogen synthesis in response to insulin, which was abolished by pretreatment with palmitate (Fig. 5). Palmitate pretreatment also reduced glycogen synthesis in cells expressing wild-type HA-PKBα or (m/p)-HA-PKBα, but not in those overexpressing (T308D,S473D)-HA-PKBα, indicating that the effect of this mutant alone was insensitive to inhibition by the lipid. These results correlate with the sensitivities of the different PKB activities to inhibition by palmitate, further supporting a role for dephosphorylation of PKB in the effect of the lipid.

In addition to the activation of PP2A, ceramide has been reported to stimulate aPKCζ (15,32,33), which has been implicated in the inhibition of PKB in COS-1 cells (19) and in breast cancer cell lines (20). We therefore also investigated the activation of this enzyme in myotubes. As previously reported in L6 muscle cells (27), immunoprecipitates of aPKC from insulin-stimulated C2C12 myotubes exhibited a twofold increase in kinase activity compared with unstimulated cells (Fig. 6*A*). In the case of palmitate-pretreated cells, basal aPKC activity was elevated more than twofold, whereas insulin stimulation did not give rise to a further significant increase in kinase activity beyond that attributable to palmitate alone (Fig. 6*A*).

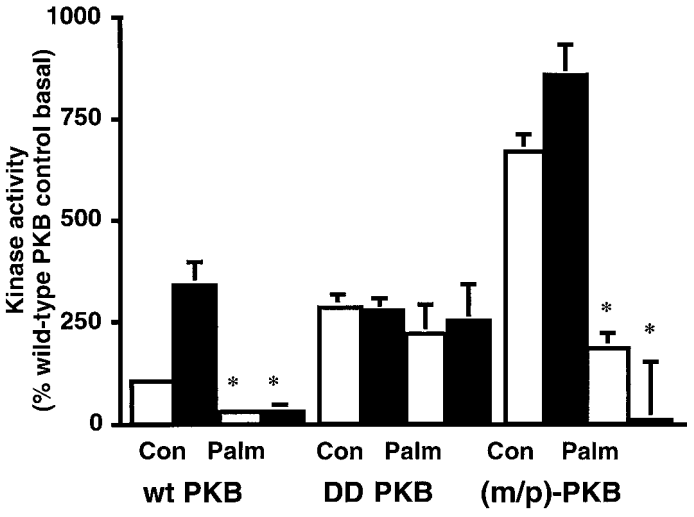

FIG. 4. Effect of palmitate pretreatment on the activity of wild-type and mutant PKB. C2C12 myotubes were infected with adenovirus to express either GFP as control, wild-type HA-PKBα, (T308D,S473D)-PKBα, or (m/p)-HA-PKBα as indicated. At 48 h postinfection, cells were pretreated with palmitate and stimulated with insulin as in Fig. 1. PKB activity was determined in HA immunoprecipitates prepared from cell lysates as detailed in RESEARCH DESIGN AND METHODS. Results shown are expressed relative to the basal activity from cells expressing wild-type HA-PKBα preincubated in the absence of palmitate and are the means from seven independent experiments. *P < 0.001 for palmitate-treated versus appropriate untreated control. □, Basal; ■, insulin. Con, control; DD, (T308D,S473D); Palm, palmitate; wt, wild-type.

It thus appeared that aPKCζ might also contribute to palmitate-induced inhibition of PKB. Even though aPKCζ is thought to play a positive role in insulin action, the existence of different pools of aPKC ativity that mediate distinct effects is possible. To further examine the involvement of aPKCζ, we assessed the effect of aPKCζKD overexpression on the rate of glycogen synthesis in C2C12 cells. This inactive mutant has previously been shown to behave in a dominant-negative fashion (22,34). Indeed, the same construct has been used to reverse the effect of endogenous aPKCζ on PKB (19). Insulin was able to stimulate glycogen synthesis ~1.5-fold in C2C12 cells overexpressing either GFP or aPKCζKD, suggesting that aPKCζ does not play an inhibitory role in the regulation of glycogen synthesis under these conditions. Furthermore, aPKCζKD overexpression was unable to overcome the inhibitory effect of palmitate pretreatment and in fact further increased the inhibitory effect of the lipid on insulin-stimulated glycogen synthesis (Fig. 6*B*). Finally, we determined the effect of aPKCζwt or aPKCζKD overexpression on endogenous PKB phosphorylation. Lysates from C2C12 cells overexpressing wild-type or mutant aPKCζ exhibited no inhibition of the insulin-stimulated increase in phosphorylation of PKB at Ser473 (Fig. 6*B*), in contrast to observations from COS-1 and BT-549 cells (19,20). Although it appeared that aPKCζwt in fact tends to increase Ser473 phosphorylation, this did not reach significance, and reciprocal effects were not seen with the dominant-negative mutant. In addition, overexpression of aPKCζwt did not inhibit PKB activity, and aPKCζKD did not protect PKB activity from the inhibitory effect of palmitate pretreatment (data not shown). Taken together, our results indicate that, although aPKC activity is elevated in palmitate-pretreated myotubes, this is unlikely to play a role in the inhibition of PKB and insulin-stimulated glycogen synthesis.

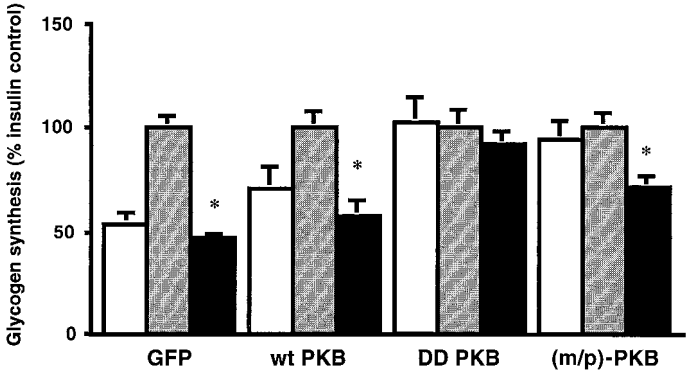

FIG. 5. Effect of wild-type and mutant PKB on insulin-stimulated glycogen synthesis in palmitate-pretreated C2C12 myotubes. C2C12 myotubes were infected with adenovirus and pretreated with palmitate as in Fig. 4, and they were then stimulated with insulin for 1 h in the presence of ^{14}C-glucose for the determination of glycogen synthesis, as detailed in RESEARCH DESIGN AND METHODS. The results shown are the combined means from three independent experiments, each carried out in triplicate, and are presented as a percentage of the mean insulin-stimulated rate of glycogen synthesis for each virus: GFP 1.58, wild-type PKB 1.78, DD PKB 1.09, and (m/p)-PKB 1.33 nmol glucose units/mg protein · h. *P < 0.01 for palmitate-treated and insulin-stimulated versus insulin-stimulated alone. □, Basal; ▨, insulin; ■, insulin + palmitate.

DISCUSSION

The stimulation of the PKB pathway by insulin is an important step in the regulation of glycogen synthesis in skeletal muscle, and we have recently shown that palmitate inhibits this process through the elevation of ceramide, resulting in a reduction in PKB phosphorylation (8). In the present study, we have further addressed this mechanism and present evidence for the involvement of a PP2A/CAPP-like phosphatase. We demonstrate that pretreatment of myotubes with palmitate increases particulate PP2A-like activity, preventing the expected decrease in insulin-stimulated cells. We were able to reverse the effect of palmitate on PKB phosphorylation by incubation of lipid-treated cells with okadaic acid before stimulation with insulin. In addition, the constitutively active (T308D,S473D)-PKBα mutant, which is independent of phosphorylation for activity, was insensitive to palmitate pretreatment, whereas the activity of wild-type PKB and the (m/p)-PKB mutant were greatly reduced by the lipid. PP2A-like activity, probably corresponding to CAPP, may therefore be responsible at least in part for the reduction in PKB phosphorylation and activity observed in palmitate-pretreated myotubes.

Although a role for phosphatases other than PP2A in the action of palmitate may not be totally excluded by the use of the in vitro phosphatase assay and the inhibitor okadaic acid, several of the more recently described protein serine/threonine phosphatases are probably not involved. Unlike PP2A, PP4/PPX is expressed at very low levels in skeletal muscle (35,36), whereas PP7 appears to be limited to retinal cells and is dependent on magnesium (37). Although PP5 is ubiquitous and stimulated by polyunsaturated fatty acids, neither saturated fatty acids, including palmitate, nor ceramide are able to activate this enzyme (38,39). Although ceramide can stimulate PP1 activity (40),

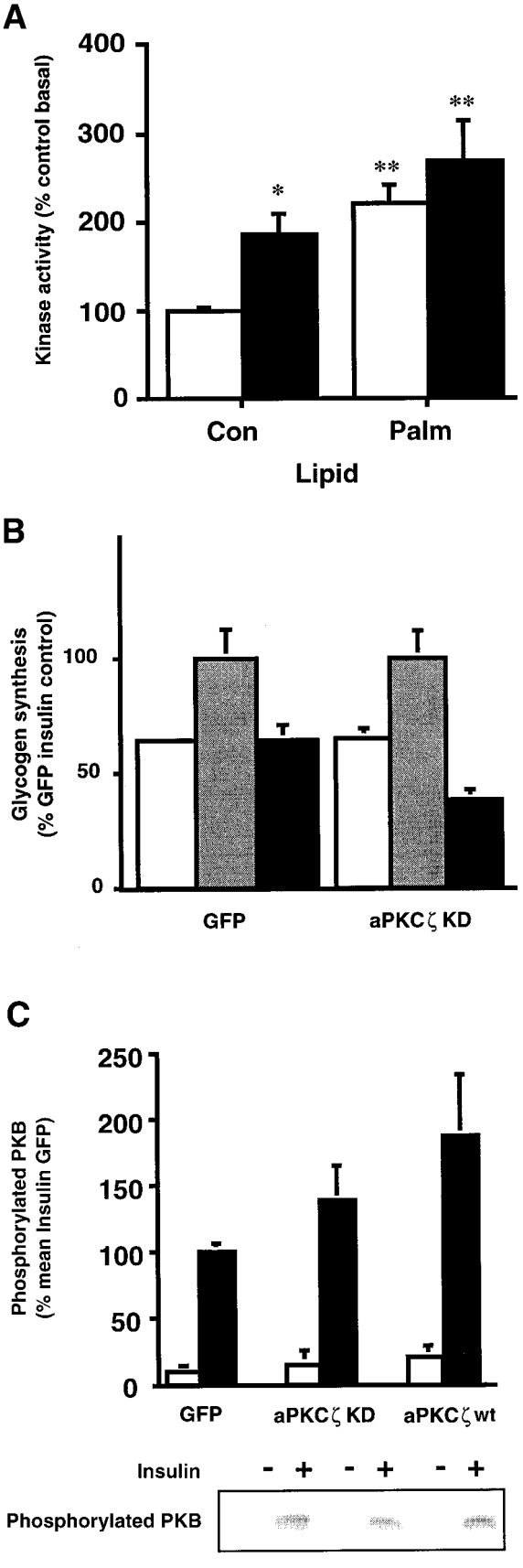

FIG. 6. The role of aPKCζ in the inhibition of PKB and glycogen synthesis. *A*: Measurement of aPKC activity in C2C12 myotubes. Cells were pretreated with palmitate and stimulated with insulin as described for Fig. 1. Kinase activity was determined in aPKC immunoprecipitates prepared from cell lysates. All procedures were carried out as detailed in RESEARCH DESIGN AND METHODS. Activities were corrected for recovery of aPKC protein, as determined by immunoblotting and densitometry of aliquots from each immunoprecipitate. Results shown are combined means from three experiments, each carried out in duplicate. □, Basal; ■, insulin. *$P < 0.005$ for insulin-stimulated versus basal; **$P < 0.005$ for lipid-pretreated basal or insulin-stimulated versus untreated basal. *B*: Effect of aPKCζKD on glycogen synthesis. C2C12 cells were infected with adenovirus to express either GFP or aPKCζKD, as indicated, and then they were pretreated with palmitate as in Fig. 1. Glycogen synthesis was determined after incubation with insulin for 1 h in the presence of ^{14}C-glucose, as detailed in RESEARCH DESIGN AND METHODS. Results shown are the combined means from three experiments, each carried out in triplicate, and are presented as a percentage of the mean insulin-stimulated rate of glycogen synthesis for each virus (GFP 1.66 and aPKCζKD 1.74 nmol glucose units/mg protein · h). □, Basal; ▨, insulin; ■, insulin + palmitate. *C*: Determination of phosphorylated PKB in aPKCζ-overexpressing C2C12 myotubes. Cells were infected with adenovirus to express either GFP, aPKCζKD, or aPKCζwt, and they were then treated with palmitate and insulin as in Fig. 1. Cell lysates were subjected to immunoblotting for phospho-Ser473 PKB and total PKB. All procedures were carried out as detailed in RESEARCH DESIGN AND METHODS. Results shown are a representative immunoblot for phospho-Ser473 PKB, and the combined means of densitometry from three experiments, each carried out in triplicate and corrected for total PKB immunoreactivity. □, Basal; ■, insulin.

the peptide substrate used here is poorly dephosphorylated by PP1, and this phosphatase is in fact activated by insulin in cultured myotubes (41) and skeletal muscle in vivo (42). In addition, the low concentration of okadaic acid used here is likely to have a greater effect on PP2A than PP1 (30). When our data are taken together with previous reports that PP2A but not PP1 can dephosphorylate and inactivate PKB (21), and that ceramide activates a phosphatase of the PP2A family (17,18), it is most likely that palmitate acts through PP2A. However, because the biochemical properties of PP6 have not been reported, and because PP3 shares some characteristics with PP2A (43), these phosphatases cannot be excluded. Positive identification of the lipid-stimulated phosphatase present in the particulate fraction as PP2A was not carried out, and it is referred to here as a PP2A-like activity. Identification (e.g., through immunoprecipitation or purification) is complicated by the noncovalent nature of the activation: the use of detergent, necessary for such procedures, will remove associated ceramide so that activation is not preserved. Indeed, we were unable to observe differences between activities from control and palmitate-treated myotubes when fractions were prepared in the presence of detergent (not shown).

The regulation of PP2A activity is complex, because several signals converge on the holoenzyme, which displays different characteristics, in terms of activity and cellular localization, depending on the constituent regulatory subunits (rev. in 44). Our data are consistent with reports that insulin can inhibit PP2A in skeletal muscle (45,46), which may be mediated through phosphorylation of the catalytic subunit (47–49). Such inhibition is not surprising, because PP2A counter-regulates several phosphorylation events stimulated by insulin, including the phosphorylation of PKB by PDK1 (21,50) and that of phosphodiesterase 3B by PKB itself (51).

In the present study, the inhibitory effects of insulin on phosphatase activity were observed only in particulate fractions from C2C12 myotubes, whereas cytosolic activity was decreased in a previous study using L6 myotubes (45). The reason for this discrepancy is not clear, but it may be caused by differences in cell lines, fractionation, or phosphatase assay. The effects of palmitate on phospha-

tase activity were also seen here only in the particulate fraction. The previously reported activation of PP2A by ceramide (52,53) appears to involve a direct binding of the lipid to the catalytic subunit of the enzyme (40), and it is likely that ceramide derived from palmitate in C2C12 cells will be associated with phospholipid membranes in the particulate fraction prepared in the absence of detergent, as in the present study. Because palmitate prevented the inhibition caused by insulin, it appears that the lipid either prevents the inhibitory phosphorylation of PP2A or overcomes its effect. Although these possibilities remain to be investigated, the equilibrium between PKB phosphorylation by PDK1 and dephosphorylation by PP2A appears to be sufficiently affected to account for the decrease in insulin-stimulated PKB activity. If the lipid activates a subset of phosphatase colocalized with PKB during its activation at the membrane, such as in a scaffolding complex similar to those described for PP2A with other kinases (54), then the changes we have observed in PP2A-like activity may in fact be underestimations of the effect of palmitate. Similarly, whereas the threefold change in phosphatase activity was accompanied by a 30–50% decrease in PKB phosphorylation observed in total cell lysates, greater effects on PKB may occur in a specific pool of the kinase colocalizing with PP2A, which might be more specifically involved in the regulation of glycogen synthesis.

An activation of PP2A similar to that seen here has been reported in mitochondrial membrane fractions by treatment of HL60 cells with ceramide (55), whereas in rat adipocytes, palmitate had specific effects on PP2A activity in different membrane fractions, activating the enzyme in a high-density microsomal fraction but inhibiting it in a plasma membrane fraction (56). Interestingly, a failure of insulin-mediated inhibition of PP2A activity has been reported in type 2 diabetic Goto-Kakizaki rats (57), and it is possible that inhibition of PKB by PP2A may be a common mechanism induced by insulin-desensitizing factors to oppose the effects of the hormone.

Our results are consistent with recent findings that the elevation of PP2A activity by ceramide is responsible for the inhibition of PKB activity in PC12 cells (11) and TF-1 cells (12). In these studies, PKB inhibition occurred in the absence of an effect on PI 3-kinase (11,12), similar to our findings with palmitate-treated C2C12 cells (8). In the case of PC12 cells, inhibition of PDK1 activity was also excluded (11), and inhibition of this kinase has also been ruled out in the dephosphorylation and inactivation of PKB by osmotic stress (50) and upon disruption of PKB complexes with the heat-shock protein Hsp90 (58). Furthermore, ceramide does not inhibit PDK1 in vitro or in CHO/IR cells (59). Thus, it appears that PP2A activated by ceramide can directly dephosphorylate PKB rather than act upstream of the kinase. Recent work has shown that PKB translocation to the plasma membrane is inhibited in cells treated with ceramide (60,61), preventing its phosphorylation and activation. Because this effect is insensitive to okadaic acid (61), in contrast to the effect of palmitate seen here, it appears that the elevation of ceramide by synthesis from the fatty acid does not act in the same way, and that phosphatase activation is of greater importance. The use of different cell lines may also contribute to variations in the observed effects of ceramide, which are known to be cell specific.

Comparison of the effects of palmitate on PKB activation and phosphorylation (Fig. 1) and on glycogen synthesis (Fig. 5) indicate that there is not always a simple relationship between these parameters. First, whereas PKB activity from both basal and stimulated cells is markedly reduced, inhibition of PKB phosphorylation does not correspond precisely, and second, whereas in each case an effect of insulin is still observed, insulin-stimulated glycogen synthesis is abolished by palmitate. Although this may in part reflect differences in the sensitivities of the assays for glycogen synthesis and PKB activity and phosphorylation, it is also possible that a threshold level of PKB activity is required to promote glycogen synthase activation by insulin, which results from the net dephosphorylation of the enzyme upon inhibition of GSK-3 by PKB and activation of PP1. In addition, overexpression of wild-type PKB did not cause a significant increase in glycogen synthesis upon insulin stimulation compared with control cells. This suggests that other factors, such as the amount of glycogen synthase or its dephosphorylation by PP1, become rate-limiting in the presence of excess PKB activity. Furthermore, overexpression of (T308D,S473D)-HA-PKBα or (m/p)-HA-PKBα caused lower rates of glycogen synthesis compared with insulin-stimulated control cells. In the case of the (m/p)-HA-PKBα, this may be due in part to the permanent membrane localization of the protein, away from potential substrates such as glycogen synthase kinase-3. In addition, persistent activation of the PKB pathway may result in desensitization, which has previously been reported in 3T3-L1 adipocytes overexpressing either a myristoylated PKB mutant (62) or constitutively active PI 3-kinase (63). Importantly, however, glycogen synthesis in cells overexpressing (T308D,S473D)-HA-PKBα in the present study was not sensitive to inhibition by palmitate pretreatment, in contrast to that seen in cells overexpressing either wild-type or (m/p)-HA-PKBα. This finding is consistent with inhibition of the PKB pathway by palmitate through dephosphorylation of PKB itself.

Other ceramide-activated enzymes that were candidates for a role in the action of palmitate included MAP kinase (64) and aPKCζ (15,32,33). Although we have previously shown that palmitate pretreatment of C2C12 cells also led to stimulation of MAP kinase, this does not account for inhibition of PKB (8). Similarly, although we observed an increase in aPKC activity in palmitate-pretreated C2C12 myotubes, overexpression of aPKCζKD did not increase glycogen synthesis either with or without palmitate pretreatment. In addition, we were unable to demonstrate alterations in PKB phosphorylation or activity in C2C12 cells that overexpressed aPKCζwt or aPKCζKD, in contrast to previous observations made in COS-1 cells (19) and breast cancer cell lines (20). Taken together, these results do not support a role for aPKCζ in the inhibitory action of palmitate in myotubes. Interestingly, we have observed inhibition of PKB activity in cells overexpressing a constitutively active mutant of aPKCζ (data not shown), indicating that aPKCζ is sufficient to regulate PKB in C2C12 cells, although our results indicate that it is not necessary to explain the effects of palmitate.

In summary, we have investigated possible mechanisms by which palmitate pretreatment inhibits insulin-stimulated glycogen synthesis. Although we have demonstrated that both insulin and palmitate activate aPKC activity in C2C12 myotubes, this is unlikely to account for the lipid effects on PKB activity and glycogen synthesis, because these were unaffected by overexpression of either aPKCζwt or dominant-negative aPKCζ. We have also shown that palmitate pretreatment increases basal PP2A-like activity and prevents its inhibition by insulin, and that the protein phosphatase inhibitor okadaic acid can reverse the effects of the lipid on PKB phosphorylation. Using different PKB mutants, we provide further evidence that the palmitate may promote inactivation of PKB through dephosphorylation and hence lead to a reduced level of glycogen synthesis. This mechanism may therefore contribute to the insulin resistance of skeletal muscle associated with increased lipid availability.

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