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Activation of the ERK Pathway and Atypical Protein Kinase C Isoforms in Exercise- and Aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR)-stimulated Glucose Transport*

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Exercise increases glucose transport in muscle by activating 5′-AMP-activated protein kinase (AMPK), but subsequent events are unclear. Presently, we examined the possibility that AMPK increases glucose transport through atypical protein kinase Cs (aPKCs) by activating proline-rich tyrosine kinase-2 (PYK2), ERK pathway components, and phospholipase D (PLD). In mice, treadmill exercise rapidly activated ERK and aPKCs in mouse vastus lateralis muscles. In rat extensor digitorum longus (EDL) muscles, (*a*) AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR), activated PYK2, ERK and aPKCs; (*b*) effects of AICAR on ERK and aPKCs were blocked by tyrosine kinase inhibitor, genistein, and MEK1 inhibitor, PD98059; and (*c*) effects of AICAR on aPKCs and 2-deoxyglucose (2-DOG) uptake were inhibited by genistein, PD98059, and PLD-inhibitor, 1-butanol. Similarly, in L6 myotubes, (*a*) AICAR activated PYK2, ERK, PLD, and aPKCs; (*b*) effects of AICAR on ERK were inhibited by genistein, PD98059, and expression of dominant-negative PYK2; (*c*) effects of AICAR on PLD were inhibited by MEK1 inhibitor UO126; (*d*) effects of AICAR on aPKCs were inhibited by genistein, PD98059, 1-butanol, and expression of dominant-negative forms of PYK2, GRB2, SOS, RAS, RAF, and ERK; and (*e*) effects of AICAR on 2DOG uptake/GLUT4 translocation were inhibited by genistein, PD98059, UO126, 1-butanol, cell-permeable myristoylated PKC-ζ pseudosubstrate, and expression of kinase-inactive RAF, ERK, and PKC-ζ. AMPK activator dinitrophenol had effects on ERK, aPKCs, and 2-DOG uptake similar to those of AICAR. Our findings suggest that effects of exercise on glucose transport that are dependent on AMPK are mediated via PYK2, the ERK pathway, PLD, and aPKCs.

Exercise is an important adjunct for treatment of diabetes mellitus. Although there are a number of health benefits that accrue from exercise in diabetic subjects, a noteworthy effect of exercise is its ability to act like insulin and directly stimulate glucose transport in skeletal muscle. The mechanism for this insulin-like effect of exercise on glucose transport is only partly understood. Like insulin, exercise increases the translocation of Glut4 glucose transporters to the plasma membrane in skeletal muscle; however, these exercise-regulated glucose transporters may largely exist in functional pools different from those mobilized by insulin (1). Also, unlike insulin, exercise activates Glut4 translocation/glucose transport in skeletal muscle by a mechanism that does not involve the activation of phosphatidylinositol (PI)[1] 3-kinase (2). Instead, exercise stimulates Glut4 translocation/glucose transport by a mechanism thought to be at least partly dependent upon ATP breakdown, leading to increases in 5′-AMP and activation of 5′-AMP-activated protein kinase, AMPK (2). Accordingly, investigators have used chemical substances, most notably, 5-amino-imidazole-4-carboxamide-1-β-D-riboside (AICAR), which, after uptake and phosphorylation, acts like 5′-AMP to directly activate AMPK, or, to a lesser extent, dinitrophenol (DNP), which uncouples oxidative phosphorylation and thereby increases endogenous 5′-AMP levels to activate AMPK, as partial surrogates for exercise in stimulating Glut4 translocation/glucose transport in skeletal muscle preparations.

Despite the apparent involvement of AMPK during exercise, there is little insight into intracellular signaling factors that operate distally to AMPK during exercise-stimulated glucose transport. In the case of insulin-stimulated glucose transport, there is now considerable evidence that atypical protein kinase C (aPKC) isoforms (3–6) and possibly protein kinase B (PKB/Akt) (7–11), operating distally to PI 3-kinase, serve as more terminal effectors for activation of Glut4 translocation and glucose transport. Of these insulin-activated protein kinases, PKB has not been found to be activated by exercise (12). However, the role of aPKCs during exercise- or AICAR-stimulated glucose transport, to our knowledge, has not been examined.

Differently from insulin, we have recently reported that several agonists, including high levels of extracellular glucose (13) and sorbitol (14), stimulate Glut4 translocation and glucose transport in rat skeletal muscle preparations and cultured L6 myotubes by activating extracellular signal-regulated kinase (ERK), which in turn activates phospholipase D (PLD) to generate phosphatidic acid (PA), which directly activates aPKCs.

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[1] The abbreviations used are: PI, phosphatidylinositol; AMPK, 5′-AMP-activated protein kinase; AICAR, aminoimidazole-4-carboxamide-1-β-D-riboside; DNP, dinitrophenol; PKC, protein kinase C; aPKC, atypical PKC; PKB, protein kinase B; ERK, extracellular signal-regulated kinase; PLD, phospholipase D; PA, phosphatidic acid; PYK2, proline-rich tyrosine kinase-2; EDL, extensor digitorum longus; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; KRP, Krebs-Ringer phosphate; HA, hemagglutinin; KI, kinase-inactive; m.o.i., multiplicity of infection.

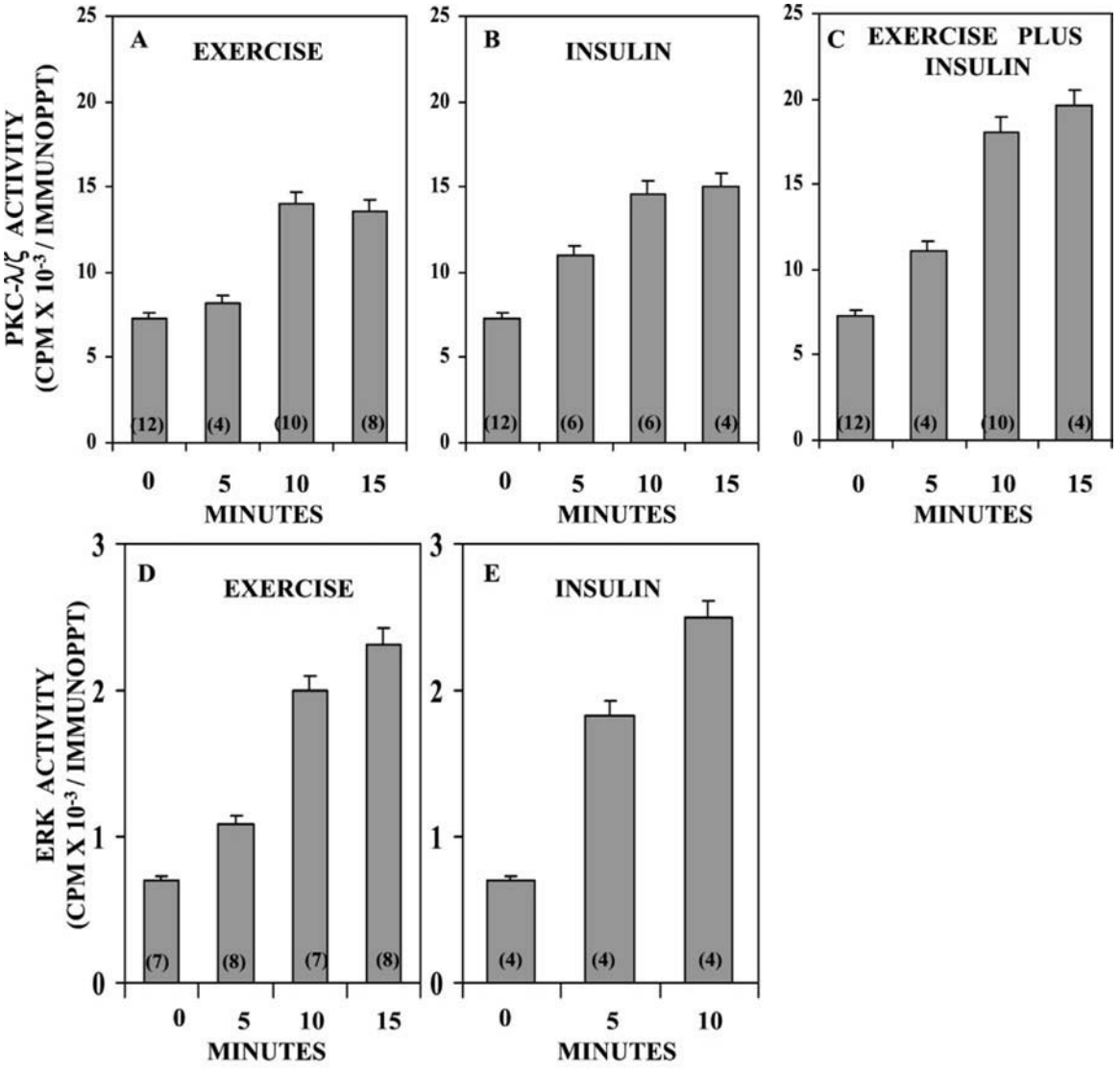

FIG. 1. **Effects of exercise and insulin on atypical PKC (ζ/λ) and ERK activity in mouse vastus lateralis muscle.** Mice were subjected to treadmill exercise or intraperitoneal insulin treatment (1 unit/kg of body wt) for indicated times, following which cell lysates were assayed for immunoprecipitable PKC-ζ/λ and ERK activity. Values are mean ± S.E. of the number of determinations shown in *parentheses*.

This activation of aPKCs via PLD and ERK, moreover, occurs in the absence of activation of PI 3-kinase and PKB, and this activation of ERK is dependent on the apparently sequential activation of the non-receptor tyrosine kinase, proline-rich tyrosine kinase-2 (PYK2), GRB2, SOS, RAS, RAF, and MEK1 (13, 14). Inasmuch as exercise is also known to activate the ERK pathway (12), we examined the possibility that the ERK/PLD/aPKC pathway may serve as a distal or terminal effector during exercise- and AICAR-stimulated glucose transport. Indeed, as reported herein, exercise, AICAR, and DNP were found to activate ERK and aPKCs in rodent skeletal muscles and/or cultured L6 myotubes, and AICAR- and DNP-stimulated glucose transport in these preparations was found to be dependent upon the activation of the ERK pathway, PLD, and aPKCs.

EXPERIMENTAL PROCEDURES

Exercise in Intact Mice—C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME), housed in a transgenic barrier facility (12 h light/dark cycles), and fed rodent chow (Ralston Purina, St. Louis, MO) *ad libitum*. The mice were subjected to treadmill exercise (0.2 m/s, Exer 4/8 Treadmill, Columbus Instruments, Columbus, OH) and/or treated with bovine insulin (1 unit/kg of body weight; Sigma), which was injected intraperitoneally. At indicated times of exercise and/or insulin treatment, the mice were sacrificed by decapitation, and vastus lateralis skeletal muscles were rapidly excised, frozen in liquid N_2, and stored at −70 °C for subsequent analyses. All procedures in mice were approved by the Committee on Animal Research of the University of California, San Francisco.

Rat Skeletal Muscle Incubations—Extensor digitorum longus (EDL) muscles were obtained from 200–225-g male Sprague-Dawley rats (Harlan Industries) and fed standard chow *ad libitum*. We chose to use the EDL rather than the soleus muscle, as AICAR acutely stimulates transport in the fast twitch EDL, but not in the slow twitch soleus muscle of the rat (15). As described previously (5, 13), muscles were ligated at both ends and stretched to maintain resting tension, slit lengthwise to enhance diffusion, and incubated under 95% O_2, 5% CO_2 in Krebs-Ringer bicarbonate medium containing 2.5 mM D-glucose and 1 mg/ml bovine serum albumin (BSA) for indicated times, with or without indicated additions. All procedures in rats were fully approved by the Institutional Animal Care and Use Committee of the University of South Florida College of Medicine and the James A. Haley Veterans Administration Medical Center Research and Development Committee.

L6 Myotube Incubations—L6 myotubes were cultured and differentiated as described previously (5, 14). On the day before experimental use, the culture medium was changed to Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml BSA. The cells were finally incubated in glucose-free Krebs-Ringer phosphate (KRP) medium containing 1 mg/ml BSA, first, for specified times with or without indicated concentrations of inhibitors (wortmannin (Sigma), genistein (Calbio-

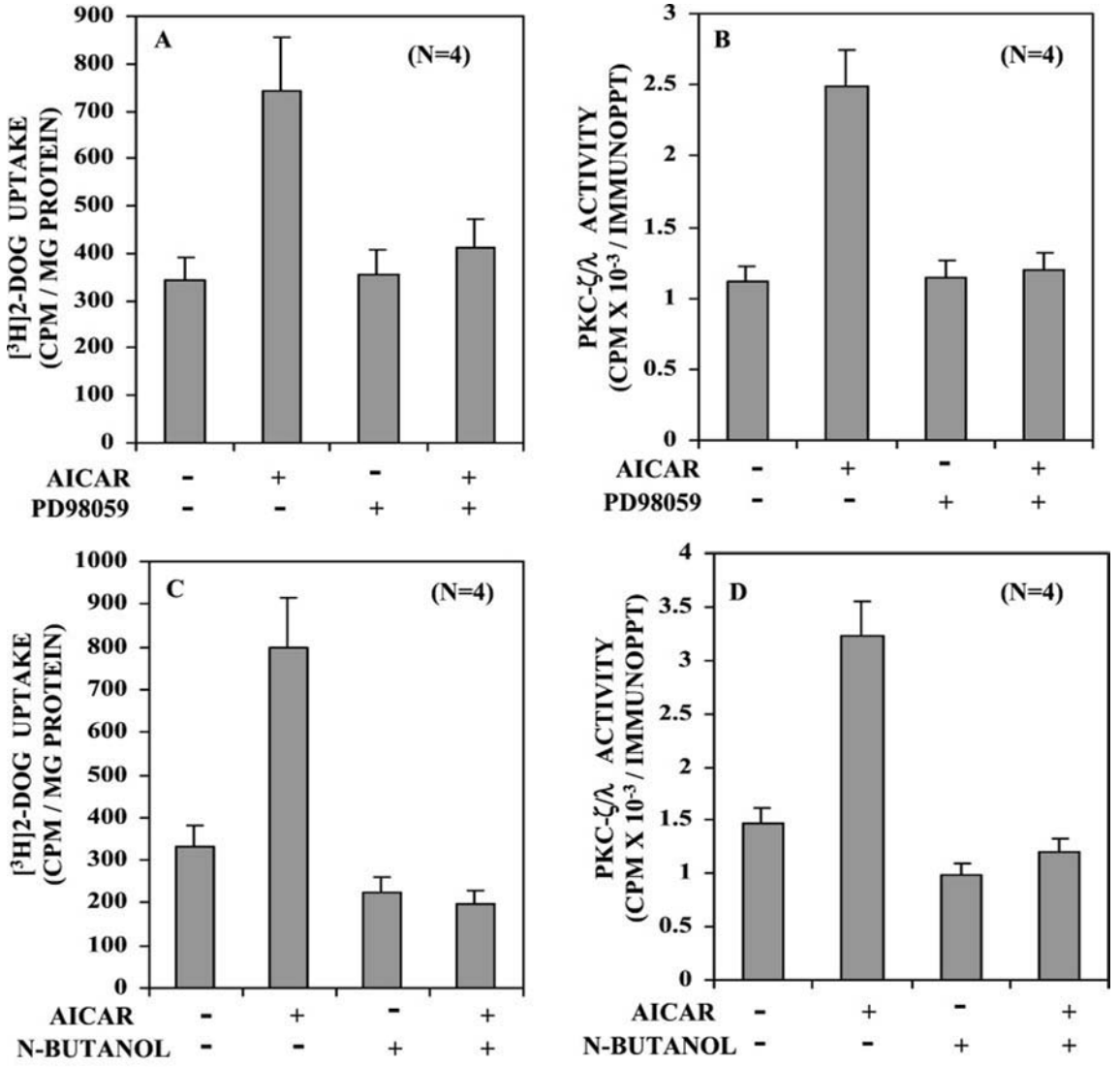

FIG. 2. **AICAR activates atypical PKCs (ζ/λ) and [^{3}H]2-DOG uptake in rat EDL muscles by a mechanism dependent on MEK1 and PLD.** EDL muscles were incubated for 30 min with MEK1 inhibitor PD98059 (50 μM) or PLD inhibitor 1-butanol (1.7%) and then for 40 min with 2 mM AICAR. [^{3}H]2-DOG uptake was determined during the last 10 min of incubation with AICAR. At the end of incubation, lysates were examined for immunoprecipitable PKC-ζ/λ activity. Values are mean ± S.E. of the number of determinations shown in *parentheses*.

chem), PD98059 (Alexis), UO126 (Promega), dantrolene (Alexis), cell-permeable myristoylated PKC-ζ pseudosubstrate (BIOSOURCE), 1-butanol, and then for specified times with or without indicated concentrations of AICAR, insulin, or other agonists for studies of [^{3}H]2-deoxyglucose uptake or activation of PYK2, ERK, PLD, or PKC-ζ/λ, as described below.

Transfections in L6 Myotubes—For studies of epitope-tagged ERK activation, as described previously (5, 14), L6 myotubes in 100-mm plates were co-transfected in DMEM with LipofectAMINE and 3 μg of pCEP4-encoding HA-ERK2 or MYC-ERK2 (both kindly supplied by Dr. Melanie Cobb), along with 7 μg of plasmid encoding dominant-negative forms of PYK2 (in pRK5; kindly supplied by Dr. Ivan Dikic), GRB2 (in pCGN), SOS (in pSRα; kindly supplied by Dr. Masato Kasuga), RAS, cRAF-1 (in pEF; kindly supplied by Dr. Larry Kreutz), or MEK1 (in pCDNA3; kindly supplied by Dr. Melanie Cobb) (see Refs. 13, 14, 16, and 17 for further information on these constructs). After overnight incubation in DMEM/BSA medium to allow time for expression, cells were washed and incubated for indicated times in glucose-free KRP medium containing 1% BSA, with or without AICAR, DNP, insulin, or other substances, following which HA-ERK2 or MYC-ERK2 was precipitated with mouse monoclonal anti-HA (Covance) or anti-MYC (Upstate Biotechnologies Inc., Lake Placid, NY) antibodies and assayed as described below.

For studies of epitope-tagged PKC-ζ activation, L6 myotubes in 100-mm plates were co-transfected in DMEM with LipofectAMINE and 1 μg of pCMV5 encoding FLAG-PKC-ζ (kindly supplied by Dr. Alex Toker (18)) or pCDNA3 encoding HA-PKC-ζ (3, 4), along with 7 μg of plasmid encoding dominant-negative forms of PYK2, GRB2, SOS, RAS, cRAF-1, or MEK1 (see ERK transfection studies above). After overnight incubation in DMEM/BSA medium to allow time for expression, cells were washed and incubated for indicated times in glucose-free KRP medium containing 1% BSA, with or without AICAR, DNP, insulin, or other substances, following which HA-PKC-ζ or FLAG-PKC-ζ was precipitated with mouse monoclonal anti-HA (Covance) or anti-FLAG (Sigma) antibodies, respectively, and assayed as described below.

Adenoviral Gene Transfer Studies in L6 Myotubes—Adenoviruses encoding kinase-inactive (KI) forms of PKC-ζ, kinase-inactive c-RAF and kinase-inactive ERK1 were constructed using plasmid cDNA inserts described previously (3, 4, 13, 14, 16, 17) and Adeno-X Expression kits obtained from CLONTECH. L6 myotubes were infected with indicated concentrations (*i.e.* multiplicity of infection (m.o.i) or ratio of viral particles per cell) adenovirus alone or adenovirus encoding KI-PKC-ζ, KI-RAF, or KI-ERK1 as described previously (19). After 48-h incubation, cells were incubated in glucose-free KRP medium with or without AICAR, DNP, insulin, or other substances for studies of glucose transport.

Sample Preparations—Samples were homogenized as described previously (5, 13, 14, 19). For studies of PKC-ζ/λ activation, buffer contained 250 mM sucrose, 20 mM Tris/HCl (pH 7.5), 1.2 mM EGTA, 20 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM Na_3VO_4, 1 mM NaF, 1 mM $Na_4P_2O_7$, and 1 μM LR-microcystin. For studies of ERK activation, buffer con-

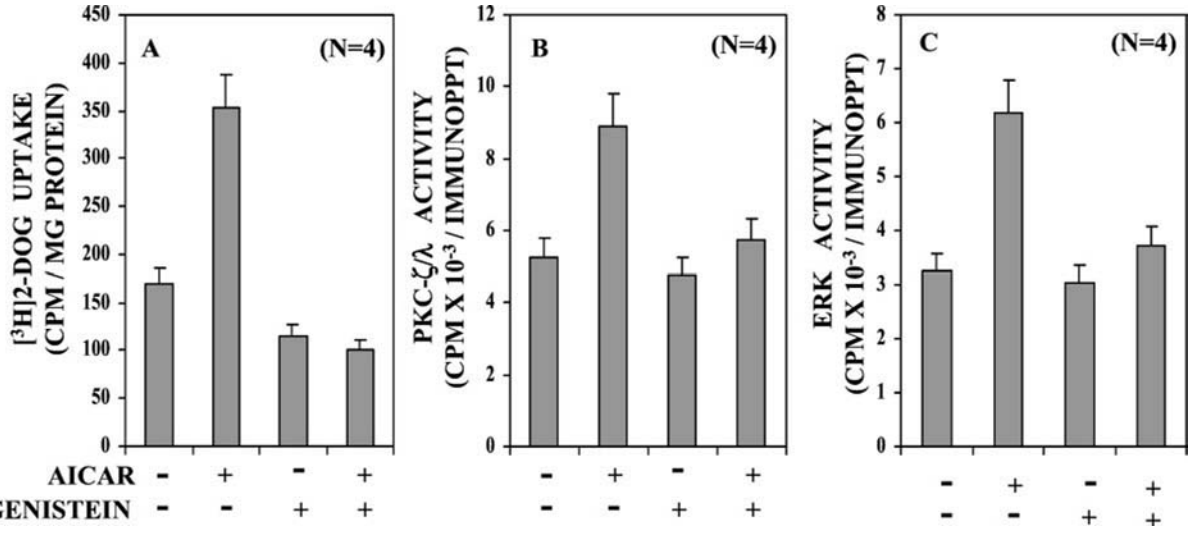

FIG. 3. **AICAR activates atypical PKCs (*ζ*/λ), ERK, and [^{3}H]2-DOG uptake in rat EDL muscles by a mechanism dependent on a tyrosine kinase.** EDL muscles were incubated for 30 min with tyrosine kinase inhibitor 100 μM genistein and then for 40 min with 2 mM AICAR. [^{3}H]2-DOG uptake was determined during the last 10 min of incubation with AICAR. At the end of incubation, lysates were examined for immunoprecipitable PKC-*ζ*/λ and ERK activity. Values are mean ± S.E. of the number of determinations shown in *parentheses*.

tained 40 mM β-glycerophosphate (pH 7.4), 1 mM EGTA, 0.5 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM Na_3VO_4, 0.5 mM NaF, 0.5 mM $Na_4P_2O_7$, and 1 μM LR-microcystin. Homogenates were centrifuged for 10 min at 700 × *g* to remove nuclei and cellular debris. Supernatants were then supplemented with (*a*) 0.15 M NaCl, 1% Triton X-100, and 0.5% Nonidet and used for immunoprecipitation of PKC−*ζ*/λ or (*b*) with 1% Triton X-100 for immunoprecipitation of ERK as described below.

ERK Activation—Immunoprecipitable ERK activity was measured as described previously (13, 14, 16, 17).

Atypical PKC Activation—Atypical PKC-*ζ*/λ activity was measured as described previously (3–5, 13, 14, 19). In brief, atypical PKCs were immunoprecipitated from salt/detergent-treated cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies, Inc.) that recognizes the C termini of both PKC-*ζ* and PKC-λ for studies of total endogenous atypical PKC activity or with mouse monoclonal anti-FLAG (Sigma) or anti-HA (Covance) antibodies for studies of epitope-tagged PKC-*ζ* activity. Precipitates were collected on Sepharose-AG beads (Santa Cruz Biotechnologies) and incubated for 8 min at 30 °C in 100 μl of buffer containing 50 mM Tris/HCl (pH 7.5), 100 μM Na_3VO_4, 100 μM $Na_4P_2O_7$, 1 mM NaF, 100 μM phenylmethylsulfonyl fluoride, 4 μg of phosphatidylserine (Sigma), 50 μM [γ-^{32}P]ATP (PerkinElmer Life Sciences), 5 mM $MgCl_2$ and, as substrate, 40 μM serine analogue of the PKC-ϵ pseudosubstrate (BIOSOURCE), a preferred substrate for aPKCs. After incubation, ^{32}P-labeled substrate was trapped on P-81 filter paper and counted.

PYK2 Activation—PYK2 activation was assessed by immunoblotting for phosphorylation of Tyr^{402}, the autophosphorylation site, and Tyr^{881}, the GRB2-interacting site of PYK2 (20), using phosphopeptide-specific antisera obtained from BIOSOURCE, after resolution of 120-kDa PYK2 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (13, 14, 17).

PLD Activation—As described previously (13, 14, 21, 22), PLD was assayed by generation of ^{3}H-labeled phosphatidylethanol or phosphatidylbutanol (results were essentially the same) in L6 myotubes prelabeled by overnight incubation with [^{3}H]oleic acid (PerkinElmer Life Sciences) in DMEM, followed by washing and incubation for 15 min in glucose-free KRP medium containing 1.7% ethanol or 1-butanol. These primary alcohols substitute for H_2O during PLD-mediated hydrolysis of phosphatidylcholine and perhaps other phospholipids to yield phosphatidylethanol or phosphatidylbutanol, instead of PA.

Glucose Transport—Rat EDL muscles or L6 myotubes were incubated for 30 min with or without AICAR or insulin, following which uptake of [^{3}H]2-deoxyglucose was measured over 5 min, as described previously (5, 13, 14, 19).

GLUT4 Translocation—L6 myotubes were incubated for 30 min with or without AICAR, following which microsomes and plasma membranes were isolated by ultracentrifugation as described previously (5, 19).

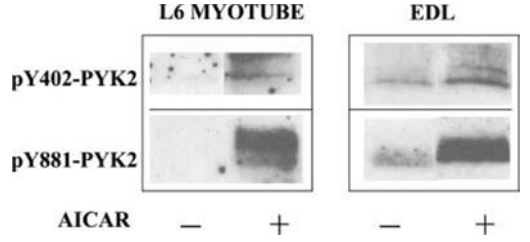

FIG. 4. **AICAR activates PYK2 in rat EDL muscles and L6 myotubes.** EDL muscles were incubated for 40 min with 2 mM AICAR. L6 myotubes were incubated for 45 min with 50 μM AICAR. At the end of incubation, lysates were examined for phosphorylation of tyrosine 402 (the autophosphorylation site) and tyrosine 881 (the GRB2-binding site) residues in PYK2. Shown here are representative blots.

RESULTS

Effects of Exercise on Signaling in Mouse Skeletal Muscles

Treadmill exercise in mice provoked rapid increases in aPKC activity in vastus lateralis skeletal muscles that were comparable, and, in part, additive, to those of maximally effective concentrations of insulin (Fig. 1). Similarly, treadmill exercise provoked rapid increases in ERK activity in mouse vastus lateralis muscles that were comparable in magnitude to those of insulin (Fig. 1).

Effects of AICAR in Rat EDL Muscles

The studies in mice suggested that exercise, like glucose (13) and sorbitol (14), might use the ERK and aPKC to activate glucose transport. To examine aspects of this signaling pathway in more detail, we turned to a well established model for studies of exercise that are mediated by AMPK, *viz*. the use of AMPK activator, AICAR, in rat EDL muscles (15). As seen in Fig. 2, AICAR provoked increases in both 2-deoxyglucose uptake and aPKC activity in rat EDL muscles. These effects of AICAR on aPKCs and 2-deoxyglucose uptake were similar in magnitude to, or only slightly less than, those elicited by insulin during comparable incubations of rat EDL muscles (data not shown).

The effects of AICAR on 2-deoxyglucose uptake and aPKC activity in EDL muscles were blocked by the MEK1 inhibitor, PD98059, and by 1-butanol, which as a primary alcohol, inhibits the production of PA by PLD (Fig. 2). These findings were

consistent the possibility that AICAR-induced increases in glucose transport and aPKC activity were mediated through ERK and PLD in rat EDL muscles.

Similarly, in keeping with the possibility that aPKCs operated downstream of ERK and PLD during AICAR action in rat EDL muscles, stimulatory effects of AICAR on aPKC activity, like effects on 2-deoxyglucose uptake, were inhibited by MEK1 inhibitor, PD98059, and PLD inhibitor, 1-butanol (Fig. 2).

Because a non-receptor tyrosine kinase, *viz*. PYK2, is required for effects of glucose (13) and sorbitol (14) on ERK/PLD/aPKCs and glucose transport, we hypothesized that PYK2 may be involved in AICAR-stimulated glucose transport in muscle cells. Indeed, in rat EDL muscles, we found that (*a*) the tyrosine kinase inhibitor, genistein, inhibited AICAR-induced increases in 2-deoxyglucose uptake, aPKC activity, and ERK activity (Fig. 3), and (*b*) the AICAR provoked increases in phosphorylation/activation of PYK2 (Fig. 4).

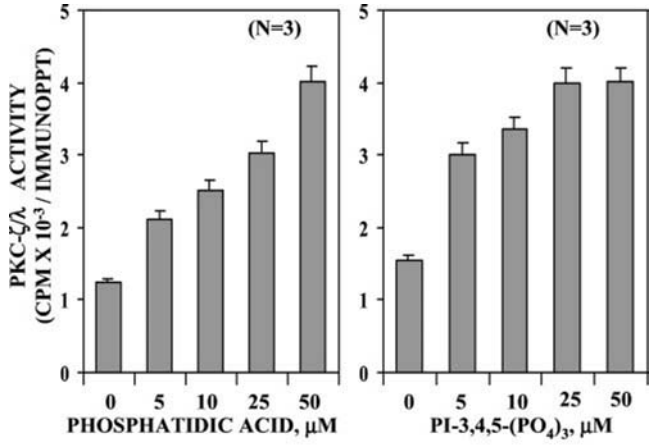

FIG. 5. **Activation of muscle PKC-ζ/λ by phosphatidic acid (*left*) and PI-3,4,5-$(PO_4)_3$ (*right*).** PKC-ζ/λ was immunoprecipitated from rat skeletal muscle and assayed in the presence of indicated concentrations of phosphatidic acid or PI-3,4,5-$(PO_4)_3$. Values are mean ± S.E. of the number of determinations shown in *parentheses*.

Taken together, these findings suggested that AICAR could activate glucose transport in rat EDL muscles through a mechanism that involved sequential activation of the non-receptor tyrosine kinase, PYK2, the ERK pathway, PLD, and aPKCs (Fig. 4). In keeping with this possibility in which PLD serves to activate aPKCs in skeletal muscle, we added PA, the acidic lipid product of PLD action, to incubations of aPKCs immunoprecipitated from rat muscle. As seen in Fig. 5, PA was nearly as effective as PI-3,4,5-$(PO_4)_3$, the acidic lipid product of PI 3-kinase, in activating immunoprecipitated aPKCs.

Effects of AICAR in L6 Myotubes

Activation of Signaling Factors and Glucose Transport by AICAR—To further characterize signaling mechanisms used by AICAR for activating glucose transport, we used L6 myotubes, a cell line derived from fetal rat myocytes that is well suited for plasmid- and adenoviral-mediated expression studies. We first documented that AICAR activated 2-deoxyglucose uptake in L6 myotubes and observed maximal increases at 50 μM AICAR, above which concentration transport effects diminished (Fig. 6). Similarly, AICAR, at a concentration of 50 μM, provoked increases in activities of PYK2 (Fig. 4), ERK (Fig. 7), PLD (Fig. 7), and aPKCs (Figs. 6 and 8) in L6 myotubes. These AICAR-stimulated increases in 2-deoxyglucose uptake and activities of ERK, PLD, and aPKCs were comparable in magnitude with, or only slightly less than, those of insulin in L6 myotubes (see Fig. 9 and data not shown). Note, however, that insulin does not activate PYK2.

Requirements for AICAR Stimulation of Glucose Transport/GLUT4 Translocation—As in rat EDL muscles, the effects of AICAR on 2-deoxyglucose uptake in L6 myotubes were inhibited by the MEK1 inhibitor, PD98059 (and UO126; data not shown), the PLD inhibitor, 1-butanol, and the tyrosine kinase inhibitor, genistein (Fig. 6). Although not shown, inhibitors of p38 MAP kinase, which may also function downstream of PYK2, had only slight effects of AICAR-stimulated 2-deoxyglucose uptake. Effects of AICAR on 2-deoxyglucose uptake were also inhibited by the aPKC inhibitor, cell-permeable myristoylated PKC-ζ pseudosubstrate (Fig. 6), and adenoviral-mediated

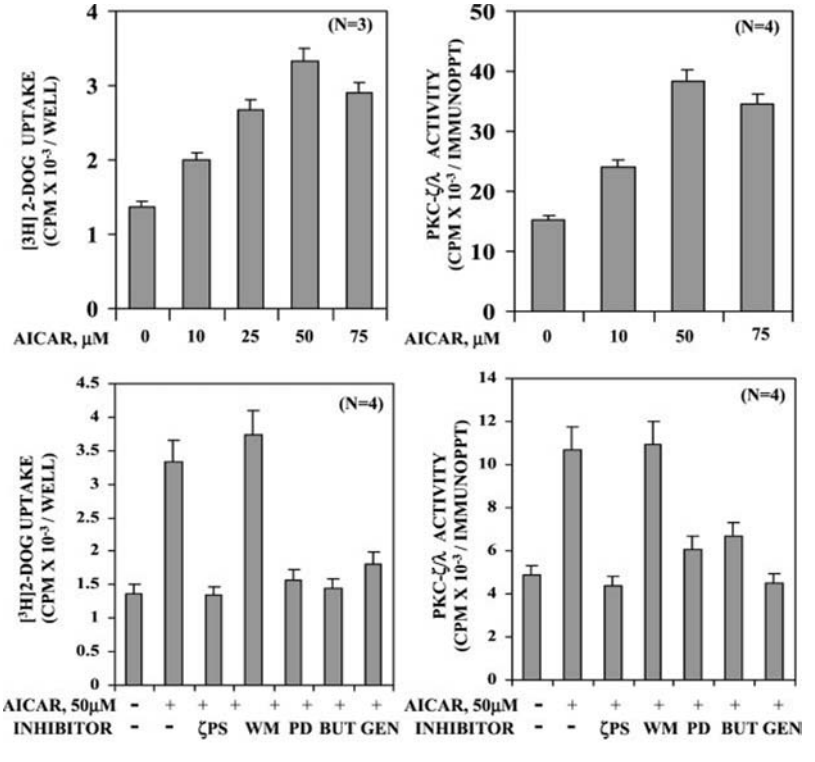

FIG. 6. **AICAR activates atypical PKCs (ζ/λ) and stimulates [^{3}H]2-DOG uptake in L6 myotubes by a mechanism independent of PI 3-kinase, but dependent on a tyrosine kinase, MEK1, PLD, and atypical PKCs.** L6 myotubes were incubated first, where indicated, for 30 min with PI 3-kinase inhibitor, 100 nM wortmannin (*WM*); tyrosine kinase inhibitor, 100 μM genistein (*GEN*); MEK1 inhibitor, 50 μM PD98050 (*PD*); PLD inhibitor, 1.7% 1-butanol (*BUT*); or PKC-ζ inhibitor, 100 μM cell-permeable myristoylated PKC-ζ-pseudosubstrate (*ζPS*) and then for 40 min with the indicated concentrations of AICAR. [^{3}H]2-DOG uptake was determined during the last 10 min of incubation with AICAR. At the end of incubation, lysates were examined for immunoprecipitable PKC-ζ/λ activity. Values are mean ± S.E. of the number of determinations shown in *parentheses*.

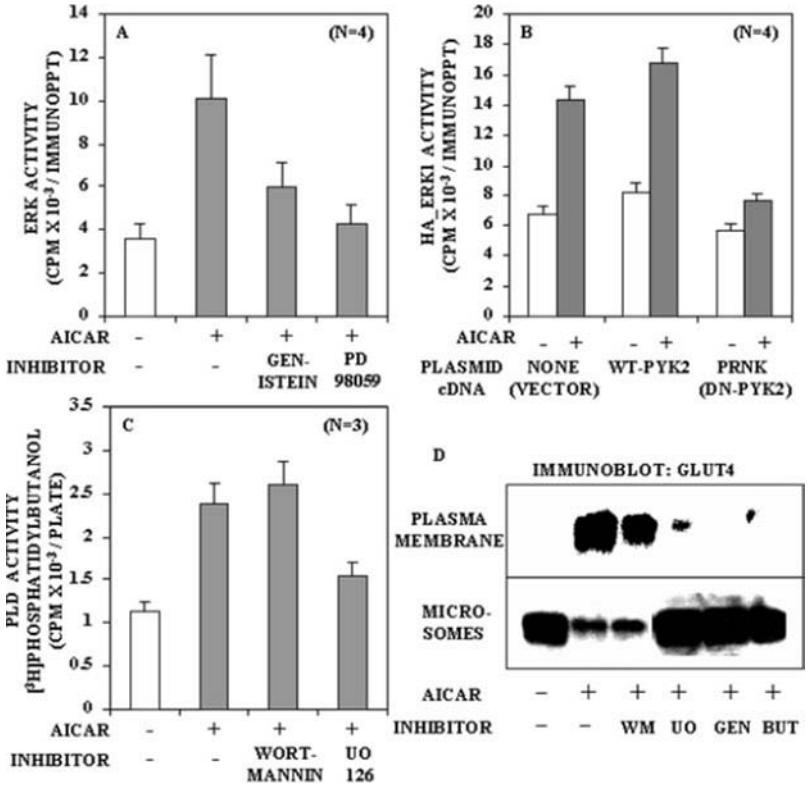

FIG. 7. **AICAR activates ERK through the tyrosine kinase PYK2 and MEK1 (*A* and *B*), PLD through the ERK pathway (*C*), and GLUT4 glucose transporter translocation to the plasma membrane through a tyrosine kinase, the ERK pathway and PLD (*D*) in L6 myotubes.** In *B*, myotubes were co-transfected with plasmids encoding HA-ERK1 along with plasmids alone (*VECTOR*) or plasmids encoding wild type (*WT*) PYK2 or the dominant-negative (*DN*) form of PYK2, PRNK, and then incubated for 24 h prior to the final incubation to allow time for expression. In *C*, myotubes were incubated for 24 h prior to the final incubation with [^{3}H]oleic acid to prelabel phospholipid pools, and 1.7% 1-butanol was present during the final incubation to trap phosphatidylbutanol released by PLD action. In *A*, *C*, and *D*, myotubes were preincubated, where indicated, for 30 min with PI 3-kinase inhibitor, 100 nM wortmannin (*WM*); tyrosine kinase inhibitor, 100 μM genistein (*GEN*); MEK1 inhibitors, 50 μM PD98050 (*PD*) or 25 μM UO126 (*UO*); and PLD inhibitor, 1.7% 1-butanol (*BUT*). In all panels, myotubes were finally incubated for 40 min with 50 μM AICAR. At the end of incubation, cell lysates were examined for immunoprecipitable ERK activity or extracted for determination of labeled phosphatidylbutanol or examined for microsomal or plasma membrane contents of GLUT4 glucose transporter. Values *A–C* are mean ± S.E. of the number of determinations shown in *parentheses*. Immunoblots shown in *D* are representative of two determinations.

expression of kinase-inactive PKC-ζ, kinase-inactive c-RAF and kinase-inactive ERK1 (Fig. 8), but not by the PI 3-kinase inhibitor, wortmannin (Fig. 6).

In addition to increasing 2-deoxyglucose uptake, AICAR increased the translocation of GLUT4 from the microsomal to the plasma membrane fraction (Fig. 8). As with 2-deoxyglucose uptake, effects of AICAR on GLUT4 translocation were blocked by UO126, 1-butanol, genistein, and myristoylated PKC-ζ pseudosubstrate, but not by wortmannin (Fig. 8). Taken together, these findings suggested that AICAR-stimulated GLUT4 translocation/glucose transport requires the activation of a tyrosine kinase, the ERK pathway, PLD, and aPKCs, but not PI 3-kinase.

In contrast to AICAR, effects of insulin on 2-deoxyglucose uptake were inhibited by wortmannin, but not by PD98059 (Fig. 9) or expression of kinase-inactive c-RAF or kinase-inactive ERK1 (Fig. 9). On the other hand, as with AICAR, effects of insulin on 2-deoxyglucose uptake were inhibited by genistein, 1-butanol, myristoylated PKC-ζ pseudosubstrate, and kinase-inactive PKC-ζ (Fig. 9). These findings suggested that insulin-stimulated glucose transport requires the activation of a tyrosine kinase (presumably the insulin receptor), PI 3-kinase, aPKCs, and PLD, but not the ERK pathway. In this regard, as discussed further below, note that, differently from AICAR, (*a*) the activation of aPKCs and PLD by insulin was dependent on PI 3-kinase, but not on the ERK pathway (Fig. 9), and (*b*) activation of aPKCs by insulin was dependent on PI 3-kinase, but not on the ERK pathway or PLD (Fig. 9). It may therefore be surmised that AICAR and insulin activate PLD by decidedly different mechanisms, and the requirement for PLD during insulin-stimulated glucose transport is unrelated to aPKC activation.

Requirement for Tyrosine Kinase in AICAR Stimulation of Glucose Transport and ERK—As in rat EDL muscles, AICAR-induced increases in ERK activity in L6 myotubes were inhibited by the tyrosine kinase inhibitor, genistein (Fig. 7). In keeping with the possibility that this tyrosine kinase may be PYK2, (*a*) AICAR increased the phosphorylation of PYK2 in rat EDL muscles (Fig. 4), and (*b*) AICAR-induced activation of HA-epitope-tagged ERK was inhibited by expression of dominant-negative, but not wild type, forms of PYK2 (Fig. 7).

Requirements for AICAR Stimulation of aPKCs—As in rat EDL muscles, AICAR-induced increases in aPKC activity were inhibited by MEK1 inhibitor, PD98059, tyrosine kinase inhibitor, genistein, and PLD inhibitor, 1-butanol, but not by PI 3-kinase inhibitor, wortmannin (Fig. 6). Thus, as with AICAR-stimulated glucose transport, the activation of aPKCs by AICAR appeared to be dependent on a tyrosine kinase, the ERK pathway, and PLD-generated PA, rather than PI 3-kinase. Further support for dependence of AICAR-induced increases in aPKC activity on the non-receptor tyrosine kinase, PYK2, and the ERK pathway was provided by finding that expression of dominant-negative forms of PYK2, GRB2, SOS, RAS, c-RAF, and ERK2 inhibited the activation of epitope-tagged PKC-ζ (Fig. 8).

In contrast to AICAR, effects of insulin on aPKC activity

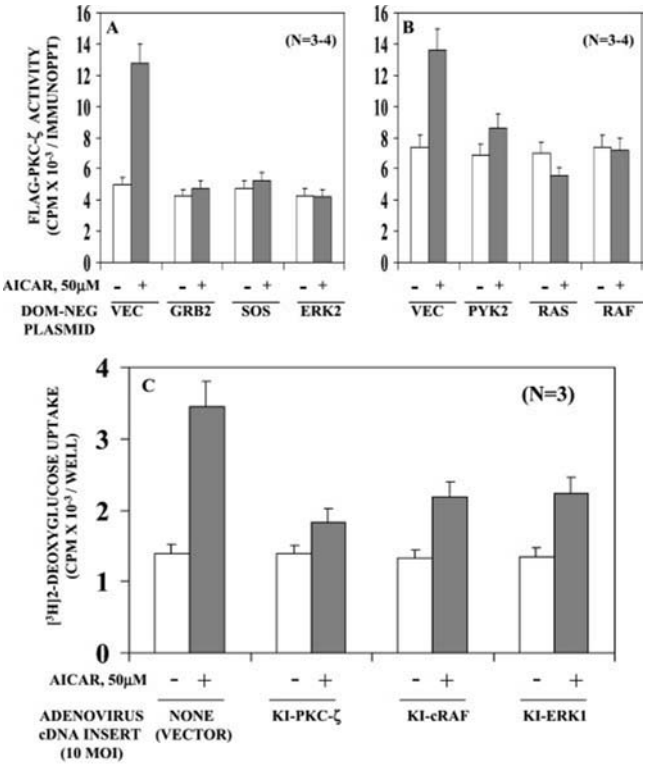

FIG. 8. **AICAR activates PKC-ζ through PYK2 and the ERK pathway (*A* and *B*) and [³H]2-deoxyglucose uptake through the ERK pathway and atypical PKCs (*C*) in L6 myotubes.** In *A* and *B*, myotubes were co-transfected with plasmids encoding FLAG-PKC-ζ along with plasmids alone (*VEC*) or plasmids encoding dominant-negative (*DOM-NEG*) forms of PYK2 (PRNK), GRB2, SOS, RAS, cRAF, or ERK2 and then incubated for 24 h prior to the final incubation to allow time for expression. In *C*, myotubes were infected with 10 m.o.i. adenovirus alone (*VECTOR*) or adenovirus encoding kinase-inactive PKC-ζ, KI-cRAF, or KI-ERK1 and then incubated for 48 h prior to the final incubation to allow time for expression. In all panels, myotubes were finally incubated for 40 min with 50 μM AICAR. [³H]2-DOG uptake was determined during the last 10 min of incubation with AICAR. At the end of incubation, lysates were examined for immunoprecipitable PKC-ζ. Values are mean ± S.E. of the number of determinations shown in *parentheses*.

were inhibited by wortmannin, but not by PD98059 or 1-butanol (Fig. 9). As with AICAR, effects of insulin on aPKC activity were inhibited by genistein and myristoylated PKC-ζ pseudosubstrate (Fig. 9). These findings suggested that insulin stimulation of aPKCs requires the activation of a tyrosine kinase and PI 3-kinase, but not the ERK pathway or PLD.

Requirements for AICAR Stimulation of PLD—In keeping with the possibility that PLD operated downstream of ERK, rather than PI 3-kinase, during AICAR action, effects of AICAR on PLD were blocked by the MEK1 inhibitor, UO126, but not by wortmannin (Fig. 7). As would be expected, the activation of ERK by AICAR was fully blocked by the MEK1 inhibitor, PD98059 (Fig. 7). Unlike AICAR, insulin, which was previously reported to activate PLD in L6 myotubes (20), was presently found to activate PLD independently of the ERK pathway (*i.e.* insensitive to MEK1 inhibitors), but, as in rat adipocytes (19), dependent upon PI 3-kinase (*i.e.* inhibited by wortmannin) (Fig. 9).

Effects of DNP in L6 Myotubes

Similar to AICAR, DNP activated ERK and PKC-ζ/λ and stimulated 2-deoxyglucose uptake in L6 myotubes (Fig. 10). The activation of PKC-ζ/λ and the stimulation of 2-deoxyglucose uptake, moreover, were insensitive to the PI 3-kinase inhibitor, wortmannin, and inhibited by MEK1 inhibitors, PD98059 and UO126, and PLD inhibitor, D609 (Fig. 10). Of further note, effects of DNP on 2-deoxyglucose uptake were inhibited by the cell-permeable myristoylated PKC-ζ pseudosubstrate and adenoviral-mediated expression of kinase-inactive PKC-ζ (Fig. 10). These findings were therefore similar in all respects to those seen with AICAR treatment, and this similarity suggested that, regardless of whether AMPK is directly activated by AICAR or indirectly by DNP-induced increases in 5′-AMP, there is activation of a common distal signaling mechanism via ERK, PLD, and aPKCs that eventuate in increases in glucose transport.

DISCUSSION

Although exercise stimulates glucose transport in skeletal muscle through signaling pathways involving AMPK (15, 24), downstream aspects of this signaling are poorly understood. In this study, using several different types of muscle cells, we found that AICAR activates the non-receptor tyrosine kinase, PYK2, which apparently leads to sequential activation of the ERK pathway, PLD, aPKCs, and glucose transport. Our studies help to define signaling events in what we believe is a more primordial pathway that is distinct from that used by insulin.

Our findings showed that aPKCs are potently activated by AICAR in rat EDL skeletal muscles and cultured L6 myotubes. Moreover, in view of the presently observed inhibitory effects of two independent specific aPKC inhibitors, *i.e.* the PKC-ζ pseudosubstrate and expression of kinase-inactive PKC-ζ, on AICAR-stimulated glucose transport, it may be surmised that aPKCs are required for effects of AICAR on glucose transport. This dependence of glucose transport effects of AICAR on aPKCs is like that of insulin, which activates aPKCs largely via PI 3-kinase and PDK-1 (23), and like that of several other agonists, including high levels of glucose (13) and sorbitol (14), which activate aPKCs largely via PYK2, ERK, and PLD. These similarities raise the possibility that aPKCs may serve as common terminal activators of the glucose transport system for a number of agonists, regardless of the diverse initial signaling pathways that are used by these agonists to activate aPKCs.

Taken together, our findings with inhibitors and dominant-negative signaling factors suggested that all components of the PYK2/GRB2/SOS/RAF/RAF/MEK1/ERK pathway are required for aPKC activation during AICAR action in L6 myotubes. Moreover, since AICAR-stimulated aPKC activation was 1-butanol-sensitive and therefore appeared to be dependent upon PLD, and since AICAR effects on PLD were blocked by MEK1 inhibitors, it seems likely that (*a*) the PYK2/GRB2/SOS/RAF/RAF/MEK1/ERK pathway functions upstream of both PLD and aPKCs, and (*b*) PLD functions between ERK and aPKCs during AICAR action. On the other hand, although PLD appeared to operate downstream of PI 3-kinase, rather than ERK, during insulin action, this activation of PLD was not essential for aPKC activation by insulin. The reason for differing requirements for PLD during aPKC activation by AICAR and insulin is presently unclear. Moreover, since PLD was not required for aPKC activation during insulin-stimulated glucose transport, it may be surmised that PLD subserves another essential function that is required for glucose transport, perhaps for GLUT4 vesicle budding or fusion of GLUT4 vesicles to the plasma membrane during insulin action. Accordingly, during AICAR action, PLD may be required for both aPKC activation and GLUT4 vesicle function.

Although we did not directly show that AICAR activated the PYK2/GRB2/SOS/RAS/RAF/MEK1/ERK pathway via AMPK, the fact that DNP, which activates AMPK and glucose transport by uncoupling oxidative phosphorylation and increasing 5′-AMP, had effects similar to those of AICAR on ERK, aPKCs, and glucose transport lends strong support to the postulation

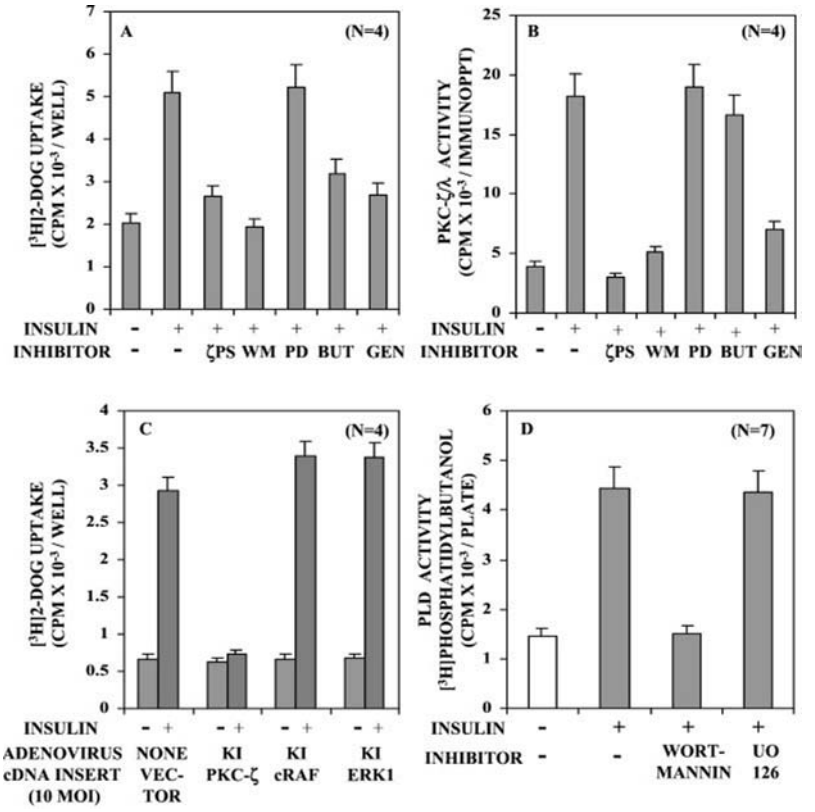

FIG. 9. **Requirements for effects of insulin on 2-deoxyglucose uptake (*A* and *C*) and activation of aPKCs (*B*) and PLD (*D*) in L6 myotubes.** In *C*, myotubes were infected with 10 m.o.i. adenovirus alone (*VECTOR*) or adenovirus encoding kinase-inactive (*KI*) PKC-ζ, KI-cRAF, or KI-ERK1 and then incubated for 48 h prior to the final incubation to allow time for expression. In *D*, myotubes were incubated for 24 h prior to the final incubation with [^{3}H]oleic acid to prelabel phospholipid pools, and 1.7% 1-butanol was present during the final incubation to trap phosphatidylbutanol released by PLD action. Where indicated, L6 myotubes were incubated first for 30 min with PI 3-kinase inhibitor, 100 nM wortmannin (*WM*); tyrosine kinase inhibitor, 100 μM genistein (*GEN*); MEK1 inhibitor, 50 μM PD98050 (*PD*) or 25 μM UO126 (*UO*); PLD inhibitor, 1.7% 1-butanol (*BUT*); or PKC-ζ inhibitor, 100 μM cell-permeable myristoylated PKC-ζ-pseudosubstrate (*ζPS*); and then, in all cases, for 15 min with 100 nM insulin. [^{3}H]2-DOG uptake was determined during the last 10 min of incubation with AICAR. At the end of incubation, lysates were examined for immunoprecipitable PKC-ζ/λ activity or generation of [^{3}H]phosphatidylbutanol. Values are mean ± S.E. of the number of determinations shown in *parentheses*.

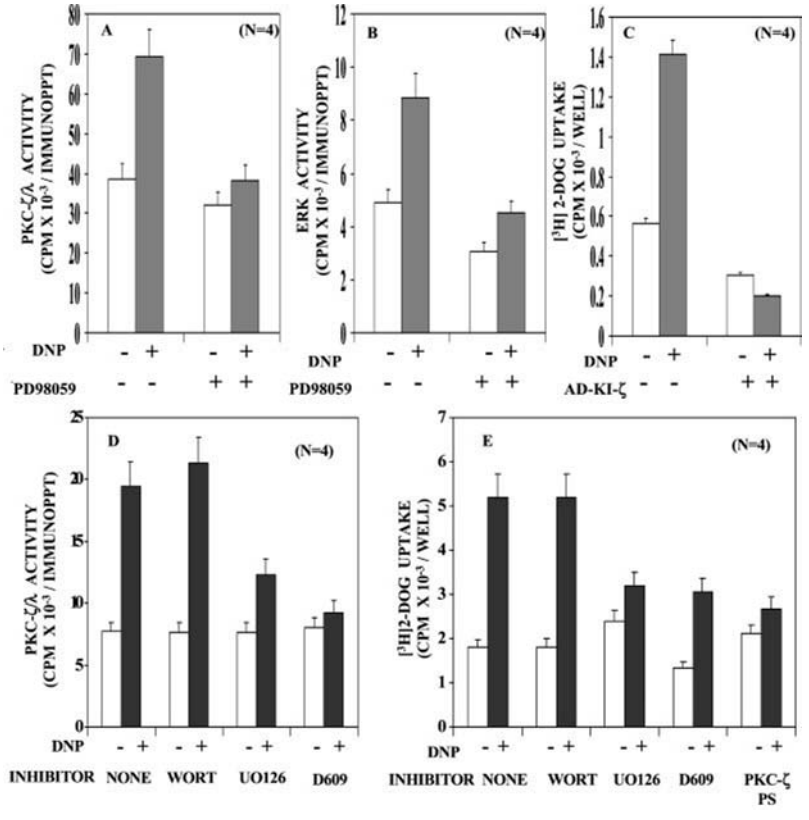

FIG. 10. **DNP activates ERK through MEK1 (*B*), PKC-ζ through ERK pathway and PLD, independently of PI 3-kinase (*A* and *D*), and [^{3}H]2-deoxyglucose uptake through the ERK pathway, PLD, and atypical PKCs (*C* and *E*) in L6 myotubes.** In *A*, *B*, *D*, and *E*, myotubes were preincubated, where indicated, for 30 min with PI 3-kinase inhibitor, 100 nM wortmannin (*WORT*); PLD inhibitor, 300 μM D609; MEK1 inhibitor, 25 μM PD98050; MEK1 inhibitor, 10 μM UO126; or cell-permeable PKC-ζ inhibitor, 100 μM myristoylated PKC-ζ-pseudosubstrate (*PKC-ζ PS*). In *C*, myotubes were infected with 10 m.o.i. adenovirus alone (−) or adenovirus encoding kinase-inactive PKC-ζ (*AD-KI-ζ*) (+) and then incubated for 48 h prior to the final incubation to allow time for expression. In all panels, myotubes were finally incubated for 60 min with 1 mM DNP. [^{3}H]2-DOG uptake was determined during the last 10 min of incubation with DNP. At the end of incubation, lysates were examined for immunoprecipitable PKC-ζ. Values are mean ± S.E. of the number of determinations shown in *parentheses*.

that both AICAR and DNP use AMPK to activate the same downstream signaling factors that are operative in stimulating glucose transport. There presently is, however, little insight into mechanisms that AMPK may use to activate PYK2 and the ERK pathway. In this regard, PYK2 is known to be activated by a mechanism requiring a dantrolene-sensitive internal Ca^{2+} pool (20), and, indeed, we have found[2] that AICAR effects, like those of glucose (13) and sorbitol (14), are dantrolene-sensitive. However, PYK2 can also be activated by other non-receptor

[2] H. C. Chen, G. Bandyopadhyay, M. P. Sajan, Y. Kanoh, M. Standaert, R. V. Farese, Jr., and R. V. Farese, unpublished data.

tyrosine kinases, *e.g.* Src family members (20), and further studies are needed to evaluate these and other possibilities.

Like AICAR, exercise potently activated aPKCs, as well as ERK, in mouse vastus lateralis muscle (similar activation of aPKCs and ERK is seen in the vastus lateralis muscle following exercise in humans).[3] Due to methodologic limitations, it is presently not possible to directly study signaling requirements for activation of ERK, aPKCs, and glucose transport during exercise in intact animals. Accordingly, we relied upon studies of signaling requirements during AICAR action in isolated EDL muscle strips and cultured L6 myotubes. However, in correlating the effects of exercise and AMPK activators, such as AICAR, note that studies of transgenic mice expressing dominant-negative AMPK have suggested that exercise/contraction-stimulated glucose transport in muscle is partly dependent on, and partly independent of, AMPK (24). Thus, AICAR most likely serves as a valid chemical surrogate for only a portion of the effects of exercise in skeletal muscles. Also note that AICAR stimulates glucose transport primarily in fast twitch muscle fibers (15), and the ERK pathway does not appear to be required for contraction-stimulated glucose transport in soleus muscles of the rat, which contain largely slow twitch fibers (25, 26). It is therefore tempting to speculate that exercise may use AMPK and the ERK/PLD/aPKC signaling pathway to activate glucose transport in skeletal muscles that primarily contain fast-twitch fibers, such as the EDL, whereas in slow twitch fibers, exercise may use an AMPK- and ERK-independent pathway to activate glucose transport. Accordingly, it will be of great interest to see whether aPKCs are activated by exercise in slow twitch muscle fibers, and, if so, by what mechanism and of what importance for the activation of glucose transport in slow twitch fibers.

Our hypothesis that ERK activation by exercise or AICAR led to activation of glucose transport may appear to be inconsistent with the fact that insulin-induced activation of ERK is not required for insulin-stimulated glucose transport. However, as alluded to above, insulin activates aPKCs primarily via PI-3,4,5-$(PO_4)_3$ generated by PI 3-kinase action, whereas AICAR apparently activates aPKCs largely via PA generated by PLD. This may in part be due to the fact that PLD operates (*a*) downstream of PI 3-kinase, rather than ERK, during insulin action and (*b*) downstream of ERK, rather than PI 3-kinase, during AICAR action. Accordingly, the differences in mechanisms that are used by insulin and AICAR to activate aPKCs may account for non-involvement of ERK in insulin-stimulated glucose transport, and, conversely, for involvement of ERK in AICAR-stimulated glucose transport. Nevertheless, in this scenario, it remains enigmatic as to why activation of the ERK pathway would result in PLD and aPKC activation during AICAR action, but not during insulin action. Possibly relevant to this question, both PI 3-kinase and aPKCs are required for insulin-induced activation of ERK, not only as reported (15) in rat adipocytes, but also in both rat EDL muscles and L6 myotubes.[2] It may therefore be conjectured that the initial activation of aPKCs and/or PLD by PI 3-kinase may down-regulate aPKC and/or PLD activation by ERK during insulin action.

To summarize, both exercise and AMPK activators, AICAR and DNP, were found to activate ERK and aPKCs in rodent skeletal muscle preparations. Studies of AICAR action further suggested that glucose transport was activated by sequential activation of PYK2, GRB2, SOS, RAS, RAF, MEK1, ERK, PLD, and aPKCs. Further studies are needed to elucidate the mechanisms used by exercise and AMPK to activate this signaling pathway.

[3] M. Beeson, M. P. Sajan, G. Bandyopadhyay, M. L. Standaert, and R. V. Farese, Sr., unpublished observations.

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