AICAR and metformin, but not exercise, increase muscle glucose transport through AMPK- , ERK- and PDK1-dependent activation of atypical PKC.


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Running Head: Atypical PKC in AICAR, Metformin and Exercise Actions

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Abstract

Activators of 5'-AMP-activated protein kinase (AMPK), 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR), metformin and exercise activate atypical protein kinase C (aPKC) and ERK, and stimulate glucose transport in muscle by uncertain mechanisms. Here, in cultured L6-myotubes: AICAR- and metformin-induced activation of AMPK was required for activation of aPKC and ERK; aPKC activation involved and required phosphoinositide kinase-dependent-1 (PDK1) phosphorylation of thr-410-PKC-ζ; aPKC thr-410 phosphorylation and activation also required MEK1-dependent ERK; and glucose transport effects of AICAR and metformin were inhibited by expression of dominant-negative AMPK, kinase-inactive PDK1, MEK1 inhibitors, kinase-inactive PKC-ζ and RNAi-mediated knockdown of PKC-ζ. In mice, muscle-specific aPKC (PKC-λ) depletion by conditional gene targeting impaired: AICAR-stimulated glucose disposal and stimulatory effects of both AICAR and metformin on 2-deoxyglucose/glucose uptake in muscle in vivo; and AICAR stimulation of [3H]2-deoxyglucose uptake in isolated extensor digitorum longus muscle; however, AMPK activation was unimpaired. In marked contrast to AICAR and metformin, treadmill exercise-induced stimulation of 2-deoxyglucose/glucose uptake was not inhibited in aPKC knockout mice. Finally, in intact rodents, AICAR and metformin activated aPKC in muscle, but not in liver, despite activating AMPK in both tissues. The findings demonstrate that, in muscle: AICAR and metformin activate aPKC via sequential activation of AMPK, ERK and PDK1; and AMPK/ERK/PDK1/aPKC pathway is required for metformin- and AICAR-stimulated increases in glucose transport. On the other hand, although aPKC is activated by treadmill exercise, this activation is not required for exercise-induced increases in glucose transport, and therefore may be a redundant mechanism.

Key Words/Phrases

Tissue-specific atypical PKC activation by AMPK
Primacy of AMPK in muscle atypical PKC activation
Redundant activation of atypical PKC during exercise

Introduction

5'-AMP-dependent protein kinase (AMPK) senses 5'-AMP levels and regulates ATP supply (12). AMPK is activated physiologically by hypoxia and exercise, which increase cellular levels of 5'-AMP at the expense of ATP, and certain hormones, such as, leptin and adiponectin (12). AMPK can also be activated by chemical agents that either increase 5'-AMP, e.g., by uncoupling mitochondrial oxidative phosphorylation, or mimic 5'-AMP, e.g., by 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR), which is metabolized to AICAR-PO₄ (ZMP), a 5'-AMP analogue; and anti-diabetic therapeutic agents, such as thiazolidinediones (TZDs) (9) and metformin (9,18,28). AMPK activation in turn increases fatty acid oxidation in various tissues, and stimulates glucose transport/uptake and glycolysis specifically in muscle, thereby increasing ATP generation (12). AMPK also diminishes expression and/or activation of sterol receptor element binding protein-1c (SREBP-1c) and other transcription factors in liver, thereby diminishing expression of mRNAs that produce enzymes that increase hepatic lipid synthesis and glucose production and release (8).

Agents that activate AMPK, such as AICAR and metformin, are important in the context
of obesity and type 2 diabetes mellitus. In these insulin-resistant states, insulin stimulation of
glucose transport in muscle and inhibition of gluconeogenesis in liver are understandably
impaired, but hepatic lipogenesis, which insulin normally stimulates, is paradoxically increased.
Activators of AMPK, on the other hand, increase glucose transport in muscle and diminish both
gluconeogenesis and lipogenesis in liver. The ability of AMPK activation to have stimulatory
effects on glucose transport in muscle and inhibitory effects on gluconeogenesis in liver similar to
those of insulin, and simultaneously have inhibitory effects on lipogenesis in liver opposite to
those of insulin is unexplained but nevertheless therapeutically fortuitous. Moreover, AMPK
appears to mediate many salutary gene-expression effects of exercise (19). Thus, agents that
activate AMPK can serve as important adjuncts for preventing and treating obesity and type 2
diabetes.

The mechanism whereby AMPK activators increase glucose transport in muscle is only
partly understood. Insulin stimulates glucose transport through activation of IRS-1-dependent
phosphatidylinositol (PI) 3-kinase (3K), which, via increases in membrane levels of the acidic
phospholipid, phosphatidylinositol-3,4,5-(PO4)3 (PIP3), activates both protein kinase B (PKB/Akt)
and atypical protein kinase (aPKC) isoforms (ζ,λ,ι) by increasing the ability of phosphoinositide-
dependent kinase-1 (PDK1) to interact with and phosphorylate threonine (thr) residues in the
activation loops (or "T-loops") of PKB/Akt (i.e., thr-308) and aPKCs (i.e., thr-410 in PKC-ζ, thr-411 in PKC-λ, and thr-403 in PKC-ι). In the case of aPKCs, in addition to activation loop
phosphorylation, PIP3 also facilitates subsequent steps, viz., auto(trans)phosphorylation and
allosteric alterations that are required for full enzyme activation (see 23). Together, PKB/Akt and
aPKC increase the translocation of glucose transporters, in particular, Glut4 transporters in
muscle cells and adipocytes, to the plasma membrane, thereby allowing glucose entry into the
cell. Note that PKC-ζ is the main aPKC in rat muscle, and PKC-λ is the main aPKC in mouse
muscle; also note that aPKC isoforms function interchangeably in support of glucose transport
(see 7,20).

In part different from insulin, the AMPK activator, AICAR, does not activate IRS-1, PI3K,
or PKB/Akt, but, nevertheless, activates aPKC in rodent muscle, by a mechanism that appeared
to involve the activation of the ERK pathway and phospholipase D(PLD)-dependent production of
the acidic phospholipid, phosphatic acid (PA), that appears to function like PIP3 (5). Similarly,
metformin increases aPKC activity in muscles of diabetic humans (16), and, like AICAR,
stimulates glucose transport in L6 myocytes (13). However, a number of uncertainties remain,
including: (a) whether AICAR and metformin activate aPKC via AMPK, or whether aPKC
activation precedes and leads to AMPK activation, as has been suggested to occur in endothelial
cells (27); (b) whether AMPK activates aPKC via mechanisms requiring PDK1 and ERK; and (c)
whether AMPK, PDK1, ERK and/or aPKC are required for glucose transport effects of AICAR and
metformin in muscle. Similarly, whereas muscle aPKC is activated during exercise (5), the
functional role of aPKC in exercise-induced glucose transport is uncertain, as exercise alters
signaling factors other than AMPK.

To evaluate these issues, we examined AMPK and aPKC requirements during actions of
AICAR and metformin in L6 myotubes by selective inactivation of AMPK and PKC-ζ, the major
aPKC in these rat-derived cells (20), and by muscle-specific inactivation of PKC-λ, the major
aPKC in mouse muscle (7), which was depleted by Cre-LoxP methodology. We also examined
the roles of PDK1 and ERK during activation of aPKC and glucose transport in L6 myotubes.
Through these studies, we demonstrate that both AICAR and metformin, but not exercise,
increase glucose transport in muscle through AMPK-dependent activation or actions of ERK,
PDK1 and aPKC. However, different from muscle, AICAR and metformin activate AMPK but not
aPKC in liver. These studies therefore define mechanisms that contribute importantly to the
clinical usefulness of AMPK activators as therapeutic agents for treating insulin-resistant forms of
diabetes and obesity.

**Materials and Methods**

L6 Myotube Studies. L6 myotubes were cultured as described (5,20), and, after
differentiation, where indicated, we added to the medium [α-modified Eagles medium (α-MEM)];
(a) 100nM small silencing/interfering RNA (RNAi) targeting PKC-ζ, or a control scrambled non-
targeting RNAi, each along with Oligofectamine (used as per instructions, Invitrogen) 72-96 hours
before experimental use to allow time to deplete aPKC, as described (20); (b) adenoviruses in
indicated multiplicity of infection titers (MOI, ratio of viral particles per cell) 48 hours before
experimental use to allow for expression of indicated proteins, as described (5,20); and/or (c)
2mM metformin over 16 hours, which elicits maximal increases in glucose transport (see 13). In
initial studies, metformin treatment for 1-3 hours elicited only modest increases in glucose
transport, and we therefore conducted subsequent studies using the longer 16-hour treatment
period.

On the day of experimental usage, the cells were incubated for 3 hours in fresh α-MEM
containing 1 mg/ml bovine serum albumin (BSA) and, where indicated, 2mM metformin. The cells
were finally equilibrated in glucose-free Krebs Ringer phosphate (KRP) medium containing
1mg/ml BSA, and then incubated with or without 2mM metformin or 50μM AICAR for 40 min, or
100nM insulin for 30 min prior to measurement of [3H]2-deoxyglucose uptake over 5 min. For
studies of AMPK or aPKC activation, similar treatment times were used for metformin and AICAR,
viz., 16 hours and 40 min, but, for insulin, an optimal treatment time of 15 min was used.

Rat Studies. Male rats weighing 250-300g were obtained either from Harlan Industries
(USA) and housed at the James A. Haley Veterans Hospital Vivarium in Tampa, Fl, USA, or from
Janvier (Le Genest-St. Isle, France) and housed in the Vivarium at the Institut Federatif de
Recherche in Nice, France. All experimental animal procedures in the USA were fully approved
by the Institutional Animal Care and Use Committee of the University of South Florida College of
Medicine and James A. Haley Veterans Administration Medical Center Research and
Development Committee, (Tampa, Fl). All animal procedures in Nice, France conformed to
current Guidelines for the Care and Use of Laboratory Animals of the National Institute of Health
and Medical Research of France (INSERM, France).

Muscle-Specific PKC-λ Knockout Mouse Studies. We previously reported (7) information
on: (a) introduction of loxP sites flanking the exon at nucleotides 110-233 in genomic mouse
PKC-λ; (b) insertion of this floxed PKC-λ into the genome of mouse embryonic stem (ES) cells;
and (c) effective deletion of this floxed PKC-λ by expression of Cre-recombinase in ES cells. ES
cells containing the floxed PKC-λ allele were then used to generate mice with germ-line
transmitted floxed PKC-λ, and these mice were crossed with mice harboring an MCK-regulated
Cre-recombinase transgene to generate homozygous muscle-specific PKC-λ knockout mice and
littermate wild type control mice. As reported (7), these knockout mice have diminished levels and
activity of total aPKC specifically in muscle (in accordance with the fact that PKC-λ is the major
aPKC in mouse muscle), and this deficiency of aPKC is attended by impaired ability of insulin to
stimulate muscle glucose transport both in vivo and in vitro. Of further note, muscle-specific
PKC-λ knockout mice have: (a) systemic insulin resistance and glucose intolerance, as
determined in standard insulin and glucose tolerance tests, but normal fasting glucose levels; (b)
diminished systemic glucose utilization specifically in muscle, as determined in hyperinsulinemic-euglycemic clamp studies; (c) other than for αPKC, no defects in insulin signaling to other factors,
most notably, IRS-1-dependent PI 3-kinase and PKB/Akt, in muscle; and (d) no defects in insulin
signaling or actions in either adipose or liver tissues.

Studies of glucose transport into isolated muscles, or muscles of intact mice, are
described below. Continuous standardized treadmill exercise studies in intact mice were
conducted as described (5), except that the uptake of glucose during the 20-min exercise period
was simultaneously measured as described below.

Adenoviruses. Adenoviruses encoding wild-type or kinase-inactive (KI) dominant-
negative forms of AMPKα1 and AMPKα2 have been described previously (25). Adenoviruses
encoding kinase-inactive (KI) PKC-ζ (5) and PDK1 (2) were described previously.

RNAi Studies. As reported (20), to deplete PKC-ζ in L6 myotubes, a SMART pool set of four 19nt
RNAi duplexes targeting four PKC-ζ mRNA sites and a scrambled control RNAi (Scr) were obtained from
Dharmacon and transfected with Oligofectamine (Invitrogen) into L6 myotubes over 96 hours. This
method depleted total aPKC levels [largely PKC-ζ with little or no PKC-λ, in rat-derived L6 myotubes (20)]
by approx 70-90%, and the specificity of the targeted mRNA for glucose transport studies was verified by
showing that: (a) insulin-stimulated PKB/Akt activation was intact, and (b) insulin-stimulated glucose transport was fully rescued by adenoviral-mediated expression of PKC-ζ, which was not affected by the RNAi that targeted PKC-ζ [note that PKC-ζ functions interchangeably with PKC-ζ in supporting glucose transport (20)].

**Lysate Preparations.** Samples from cultured cells or from rat or mouse tissues were homogenized as described (5,7,20). Homogenizing buffer contained 250mM sucrose, 20mM Tris/HCl (pH, 7.5), 1.2mM EGTA, 20 mM β-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride (PMSF), 10μg/ml aprotilin, 20μg/ml leupeptin, 1mM Na3VO4, 1mM NaF, 1mM Na4P2O7 and 1μM LR-microcystin. Homogenates were centrifuged for 10 minutes at 700xg to remove nuclei and cellular debris. Supernatants were then supplemented with 0.15M NaCl, 1% Triton X-100 and 0.5% Nonidet to disrupt membranes and then used for immunoprecipitation of PKC-ζ/λ or AMPK.

**aPKC Activation.** Combined PKC-ζ plus PKC-λ (i.e., total aPKC) enzyme activity was measured as described previously (5,7,20). In brief, aPKCs were immunoprecipitated from salt/detergent-treated cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies) that recognizes the C-termini of both PKC-ζ and PKC-λ; note- whereas rat-derived L6 myotubes, like rat muscle, contains primarily PKC-ζ, mouse muscle contains primarily PKC-λ (5,7,20). Precipitates were collected on Sepharose-AG beads (Santa Cruz Biotechnologies), and incubated for 8 min at 30°C in 100μl buffer containing 50mM Tris/HCl (pH,7.5), 100μM Na3VO4, 100μM Na4P2O7, 1mM NaF, 100μM PMSF, 4μg phosphatidylserine (Sigma), 50μM [γ-32P]ATP (NEN Life Science Products), 5mM MgCl2 and, as substrate, 40μM serine analogue of the PKC-ε pseudosubstrate (BioSource), a preferred substrate for aPKCs. After incubation, 32P-labeled substrate was trapped on P-81 filter paper and counted.

In some experiments, using the same assay system, but instead of immunoprecipitated aPKC, we measured the activity of recombinant glutathione S-transferase (GST) fusion protein forms of full-length human PKC-ζ incubated at 30°C for 30 min with or without full-length human PDK1 or constitutively-active human M1-C312 AMPKκ1 (all obtained from baculovirus/Sf9 insect expression systems and supplied by Cell Signaling Technology).

**AMPK Activation.** Immunoprecipitable AMPK (combined α1 and α2) activity was measured in lysates by the method of Wojtaszewski et al (26) using rabbit polyclonal anti-AMPK antiserum (Cell Signaling Technologies) and SAMS peptide (Upstate Cell Signaling) as substrate. As in other kinase assays, blank activities were determined in cell lysate precipitates obtained with non-immune serum.

**ERK Activation.** Immunoprecipitable ERK activity was measured using an enzymatic assay or by blotting for phospho-ERK as described (5).

**Glucose Transport Studies in L6 Myotubes.** Following the initial treatments with RNAi (72-96 hours), adenoviruses (48 hours) and/or metformin (16 hours) described above, unless otherwise indicated, myotubes were incubated in glucose-free KR0 medium with or without (a) 2mM metformin for 40 min, (b) 50μM AICAR for 40 min, or (c) 100nM insulin for 30 min, following which, uptake of [3H]2-deoxyglucose was measured over 5 min, as described (5,20).

**In Vivo Glucose Transport Studies in Mice.** As described (7), following an overnight 16-20 hr fast, 0.2 ml physiologic saline containing, each per gram body weight, 0.05μCi [3H]2-deoxyglucose (NEN/Life Science, Boston, MA, USA), 0.005μCi [14C] L-glucose (NEN); and, where indicated, 0.25 mg AICAR or metformin, was administered intraperitoneally 20 min (exercise studies) or 30 min (AICAR and metformin studies) before killing. Uptake of total hexose (mainly glucose plus tracer [3H]2-deoxyglucose) in vastus lateralis muscle was measured by dividing the tissue [3H]-cpm (corrected for non-specific uptake as per [14C]L-glucose radioactivity) by the specific radioactivity of serum glucose, i.e., [3H]-cpm per nmole glucose, as described (7).

**In Vitro Glucose Transport Studies in Mouse Muscle.** As described (7), uptake of [3H]2-deoxyglucose was determined over 10 min in isolated tension-maintained extensor digitorum longus muscles (EDL) incubated under 95%O2/5%CO2 in glucose-free Krebs Ringer bicarbonate (KRB) medium containing 0.5μM 2-deoxyglucose and tracer amounts of [3H]2-deoxyglucose, after treatment for 40 min with or without 2mM AICAR.

**Glut4/Glut1 Glucose Transporter Translocation Studies.** As described (5,7,20), plasma membrane glucose transporter 1 and 4 levels were measured by purification of plasma...
membranes on a discontinuous sucrose gradient and Western analysis, following incubation of
L6 myotubes with or without metformin, AICAR or insulin, or in gastrocnemius muscles obtained
from mice treated with or without AICAR or metformin.

AICAR Tolerance Test. Mice were subjected to AICAR tolerance testing, as described
(10). In brief, AICAR, 250mg/kg body weight, was administered intraperitoneally and tail vein
blood glucose levels were measured at 15-min intervals.

Western Analyses. As described (5,7,20), lysates or plasma membranes were
immunoblotted with: (a) PKC-rabbit polyclonal antiserum (Santa Cruz Biotechnologies) that
recognizes C-termini of both aPKCs, λ and ζ; (b) mouse monoclonal anti-Glut4 glucose
transporter antibody (AbDserotec, Ltd.); (c) rabbit polyclonal anti-Glut1 glucose transporter
antiserum (Santa Cruz Biotechnologies); (d) rabbit polyclonal anti-AMPK antiserum (Cell
Signaling Technologies); (e) rabbit polyclonal anti-phospho-thr-172-AMPK antiserum (Cell
Signaling Technologies); (f) rabbit polyclonal anti-PDK1 antiserum (Cell Signaling Technologies);
(g) rabbit polyclonal anti-phospho-thr-410-PKC-ζ antiserum (Cell Signaling Technologies); (h)
rabbit polyclonal anti-phospho-ERK antiserum (Cell Signaling Technologies); and (i) rabbit
polyclonal anti-phospho-ser-473-PKB/Akt (Cell Signaling Technologies).

Statistics. Means of 2 groups were analyzed by t-test. Means of 3 or more groups were
analyzed by ANOVA and the least significant difference multiple comparison method. All
experiments were conducted on at least 2 separate occasions.

Results

A. Studies in L6 Myotubes

Glucose Transport Effects of AICAR, Metformin and Insulin. As reported previously (5), in
L6 myotubes, 50μM AICAR, a maximally effective concentration, provoked 2-3-fold increases in
[^3H]2-deoxyglucose uptake within 40 min, i.e., comparable to those seen with maximal (100nM)
30 min insulin treatment (Fig 1a). As reported in previous studies of L6 myotubes (13),
maximally-effective 2 mM metformin treatment over 16 hours provoked approximately 4-6-fold
increases in[^3H]2-deoxyglucose uptake (Fig 1a).

In addition to increasing[^3H]2-deoxyglucose uptake, 2mM metformin treatment for 16
hours provoked the translocation of both Glut4 and Glut1 glucose transporters to the
plasma membrane that were at least comparable in magnitude to those seen with either 30 min
maximal insulin treatment (Figs 1b and c), or 40 min AICAR treatment (Figs 1d and e). Note that
total cellular contents of Glut4 and Glut1 glucose transporters in L6 myotubes were not altered by
the 16-hour 2mM metformin treatment [Glut4:1.01±0.11 (mean±SE; N=4) in metformin-treated vs
1±0.05 in controls (mean±SE; N=4); Glut1:1.02±0.14 (mean±SE; N=4) in metformin-treated vs
1±0.03 in controls (mean±SE; N=4)], or by the 40 min treatment with 50μM AICAR for 40 min
[Glut4: 0.98±0.06 (mean±SE; N=4) in AICAR-treated vs 1±0.05 (mean±SE; N=4) in controls; and
Glut1: 1.05±0.02 (mean±SE; N=4) in AICAR-treated vs 1±0.03 (mean±SE; N=4) in controls]. In
addition to these findings in L6 myotubes, as seen below in Fig 9 (Section C), AICAR and
metformin treatment in vivo over 30 min provoked acute increases in Glut4 and Glut1
translocation in gastrocnemius muscles of intact mice.

AMPK Activation by Metformin and AICAR. AMPK activity was comparably increased
approximately 2-fold in response to 40-min AICAR treatment and 16-hr metformin treatment (Figs
2a and 3a). Stimulatory effects of both agents on AMPK activity were diminished by expression
(see inset in Fig 2a) of dominant-negative AMPKα2, with maximal inhibition occurring at approx
100-200 viral MOI (Fig 2a). In contrast, stimulatory effects of both AICAR and metformin on
AMPK activity were significantly enhanced by expression of wild type AMPKα2 (Fig 3a). The
reason for the failure of expression of dominant-negative AMPKα2 to alter control, i.e.,
unstimulated, AMPK activity (Fig 2a) may reflect a spuriously high basal activity owing to non-
AMPK kinases co-precipitating with AMPK.

Although not shown, dominant-negative AMPKα1 was slightly less effective than
AMPKα2 for inhibiting AMPK activation, and combined addition of dominant-negative forms of
AMPKα1 and AMPKα2 was no more effective than AMPKα2 alone for inhibiting AICAR- and metformin-induced increases in either AMPK activity or, as seen below, glucose transport. Subsequent studies were therefore conducted only with adenoviruses encoding DN and WT forms of AMPKα2.

**AMPK is Required for Activation of Glucose Transport During Actions of Metformin and AICAR.** Both metformin and AICAR provoked increases in [³²]H]-deoxyglucose uptake, but, as noted above, metformin effects generally exceeded those of AICAR in L6 myotubes (Figs 1a, 2c and 3c). As with AMPK activity, expression of dominant-negative AMPKα2 inhibited effects of AICAR and metformin on [³²]H]-deoxyglucose uptake (Fig 2c), and, in contrast, expression of wild type AMPKα2 significantly enhanced the effects of both metformin and AICAR on [³²]H]-deoxyglucose uptake (Fig 3c). Of further note, insulin effects on [³²]H]-deoxyglucose uptake were not altered by expression of dominant-negative AMPKα2 (inset, Fig 2c); thus, the inhibition of effects of AICAR and metformin on glucose transport by dominant-negative AMPKα2 was not due to non-specific alterations in the transport process, and presumably reflected alterations in signaling pathways.

**AMPK is Required for Activation of aPKC During Actions of Metformin and AICAR.** It was previously shown that AICAR activates aPKC in rat-derived cultured L6 myotubes and isolated rat extensor digitorum longus (EDL) muscle (5). Presently, we found that metformin and AICAR provoked increases in aPKC activity in L6 myotubes that were inhibited by expression of dominant-negative AMPKα2 (Fig 2b), and enhanced by expression of wild type AMPKα2 (Fig 3b). Note that total aPKC levels were not altered by either AICAR and metformin treatments or by expression of dominant negative AMPKα2 treatments (Figs 2b and 3b). On the other hand, as discussed further below, the phosphorylation of thr-410 in the activation loop (or T-loop) of PKC-ζ was diminished by expression of dominant-negative AMPKα2 (Fig 2b). These changes in aPKC activity and/or phosphorylation occurring with expression of dominant-negative and/or wild type AMPK seemed to correlate reasonably well with changes in AMPK activity and glucose transport.

**aPKC Activation is Required For Metformin- and AICAR-Induced Increases in Glucose Transport.** Adenovirally-mediated expression of kinase-inactive PKC-ζ has been found to inhibit AICAR- as well as insulin-stimulated [³²]H]-deoxyglucose uptake in L6 myotubes (5). Presently, we found that comparable adenovirally-mediated expression of kinase-inactive PKC-ζ (see 2-3-fold increases in total aPKC levels in inset of Fig 4b) largely inhibited insulin effects, and partially inhibited metformin effects on [³²]H]-deoxyglucose uptake (Fig 4a) and aPKC activity (Fig 4b) in these cells. Similarly, the knockdown (i.e., causing approx 80%-depletion) of PKC-ζ or RNAi targeting PKC-ζ was diminished by expression of dominant-negative AMPKα2 (Fig 2b). These changes in aPKC activity and/or phosphorylation occurring with expression of dominant-negative and/or wild type AMPK seemed to correlate reasonably well with changes in AMPK activity and glucose transport.

**PDK1 is Required for Metformin- and AICAR-Induced Increases in aPKC Activity and Glucose Transport.** Studies of hypoxia-induced increases in AMPK activity in rat alveolar epithelial cells have shown that the activation of the PKC-ζ in these cells is attended by an increase in phosphorylation of the activation loop (T-loop) site, thr-410 (11); further, as it was also found that recombinant constitutively active AMPK provoked increases in [³²]P-incorporation into recombinant PKC-ζ in vitro, it was postulated that AMPK directly increases thr-410 phosphorylation (11). However, as discussed above, during insulin-induced aPKC activation, the phosphorylation of thr-410 in PKC-ζ is mediated by PDK1 (see 2,23), and this phosphorylation is facilitated by PI3K-derived PIP₃ (23), which most likely binds to basic residues in the regulatory domain of aPKC, thereby inducing: a dissociation of the pseudosubstrate sequence in the regulatory domain from the substrate-binding site in the catalytic domain; molecular unfolding; interaction of PDK1 with thr-410; auto(trans)phosphorylation of thr-560; and enhanced accessibility of substrate to the catalytic site (23). In this scheme, thr-410 phosphorylation is required, but alone is insufficient, for PKC-ζ activation by PIP₃ (23).

With respect to AICAR action, we previously proposed that the acidic phospholipid, PA, produced by ERK-dependent PLD activation, acted analogously to the PI3K-derived acidic phospholipid, PIP₃, which presumably increases accessibility of the activation- or T-loop sites of both PKC-ζ (i.e., thr-410), and PKB/Akt (i.e., thr-308) to PDK1 during insulin action (5). In keeping
with this idea, in L6 myotubes, we presently found that AICAR and metformin, like insulin,
increased phosphorylation of thr-410 in PKC-ζ (Figs 2b, 5a and 5c). Moreover, as previously
found in studies of insulin action in other cell types (2), adenovirally-mediated expression of
kinase-inactive PDK1 (see inset in Fig 5b) inhibited the increases in: (a) enzymatic activation of
aPKC (Fig 5c); thr-410 phosphorylation of PKC-ζ (Fig 5c); (b); and (c) increases in 2-
deoxyglucose uptake (Fig 5b) induced by AICAR and metformin, as well as insulin.

It may also be noted in Fig 5c, that insulin, but not AICAR or metformin, increased
phosphorylation of ser-473 in PKB/Akt, and, moreover, as expected, expression of kinase-
inactive PDK1 blocked this phosphorylation of PKB/Akt, as well as the phosphorylation of thr-410
in PKC-ζ, induced by insulin. These results are in keeping with the idea that AICAR and
metformin do not increases the enzyme activity, as such, of PDK1, since, if PDK1 had been
activated, it might be expected that PKB/Akt would have been phosphorylated at both thr-308
and ser-473, and thus activated, by these agents, which is clearly not the case. Accordingly, we
believe that, like insulin, AICAR and metformin increase the accessibility of the PKC-ζ T-loop site,
i.e., thr-410, to PDK; however, as discussed in more detail below, this increase in accessibility is
mediated via PA during AICAR and metformin action, and by PIP3 during insulin action. The
ability of insulin, but not AICAR and metformin, to activate PKB/Akt most likely reflects that PIP3,
but not PA, is able to interact with the PH domain in PKB/Akt that is required for T-loop
phosphorylation.

As the above-described findings suggested that PDK1 is required for effects of AICAR
and metformin on thr-410 phosphorylation and subsequent increases in PKC-ζ enzyme activity, it
was of interest to compare the effects of recombinant forms of PDK1 and AMPK on recombinant
PKC-ζ. As seen in Fig 6c, thr-410 in recombinant PKC-ζ was already partially phosphorylated,
but could be further phosphorylated by incubation with wild type PDK1, but not significantly by
incubation with constitutively active AMPK (Figs 6b and 6c). Similarly, PDK1, but not AMPK,
provoked increases in aPKC activity (Fig 6a). Also note that PDK1 provoked greater increases in
32P-incorporation into PKC-ζ than AMPK (Fig 6c).

**ERK Pathway Activation is Required for Metformin- and AICAR-Induced Increases in
aPKC Activity and Glucose Transport.** The above-described findings suggested that, in L6
myotubes, PDK1 is required for AMKP-induced increases in thr-410-PKC-ζ phosphorylation,
PKC-ζ enzyme activity and PKC-ζ-dependent glucose transport. As it was previously found that
inhibition of the ERK pathway and PLD blocked increases in aPKC activity and glucose transport
induced by AMPK activation by dinitrophenol in L6 myotubes (5), it was presently of interest to
find that two inhibitors of the ERK activator, MEK1, viz., UO126 and PD98050, but not an inhibitor
of the PI3K pathway, viz., wortmannin, inhibited effects of both AICAR and metformin on 3H-2-
deoxyglucose uptake (Fig 7a) and PKC-ζ thr-410 phosphorylation and enzymatic activation (Fig
7c), as well as ERK phosphorylation and enzymatic activation (Fig 7b), in L6 myotubes. Also
note that expression of dominant-negative AMPKζ inhibited the effects of AICAR and metformin
ton ERK phosphorylation and enzymatic activation (Fig 4d), indicating that AMPK is upstream of
ERK.

**B. Studies in Muscle and Liver of Intact Rats**

We questioned whether metformin and AICAR activate aPKC, as well as AMPK, in
tissues of intact rats. Comparison of the activation of these processes in muscle and liver seemed
important, as aPKC mediates stimulatory effects of insulin on both glucose transport in muscle
(7,20) and lipogenesis in liver (17,22,24), but, as discussed above, AMPK activators, like insulin,
stimulate glucose transport in muscle, but, unlike insulin, diminish lipogenesis in liver (8,12).
Thus, therapeutic usefulness of AMPK activators may be determined in part by differential effects
on aPKC in various tissues.

**Metformin Activates AMPK and aPKC in Rat Muscle.** Within 60 minutes after
subcutaneous injection of metformin (1g/kg body weight) into intact Sprague-Dawley rats, the
phosphorylation (Fig 8a) and activity of AMPK (Fig 8c), and the activity of aPKC (Fig 8e) were
increased approx 2-2.5-fold in the vastus lateralis muscle. Metformin-induced increases in muscle
aPKC activity were in fact comparable in magnitude to those observed with insulin treatment (Fig
8e). On the other hand, insulin, unlike metformin, did not increase muscle AMPK phosphorylation
AICAR and Metformin Activate AMPK, but not aPKC, in Rat Liver. As in muscle, metformin increased AMPK phosphorylation (Fig 8b) and AMPK activity (Fig 8d) approx 3-fold in rat liver, but, much differently from muscle, metformin failed to provoke an increase in hepatic aPKC activity (Fig 8f). Similar to findings with metformin treatment, 60 min after subcutaneous injection of AICAR (1g/kg body weight), AMPK phosphorylation (Fig 8b) and AMPK activity (Fig 8d) were increased approximately 2-fold in rat liver; but hepatic aPKC activity was not altered (Fig 8f). In contrast to both metformin and AICAR, insulin activated aPKC in rat liver (Fig 6f), presumably via PI3K (see 24).

C. Studies of AICAR and Metformin Action in Wild Type and Muscle-Specific PKC-λ Knockout Mice.

AICAR Tolerance Testing. As reported (10), administration of AICAR (250mg/kg body weight) caused a steady decline of blood glucose over 60 min in wild type mice (Fig 9a). However, in muscle-specific PKC-λ knockout mice, wherein the level of total aPKC in various muscles is diminished (Figs 9b, 10c and 11b), and wherein the ability of either AICAR or metformin to stimulate aPKC activity in muscle is largely abrogated (Fig 10c), the effects of AICAR on blood glucose lowering were significantly reduced by approximately 50% (Fig 9a). Whether remaining effects of AICAR on blood glucose lowering during the AICAR tolerance test in muscle-specific PKC-λ knockout mice reflected decreases in hepatic glucose output, continued uptake of glucose in muscle as mediated by residual aPKC, or operation of other factors is presently unclear.

Effects of AICAR on Glucose Transport in Vitro. Whereas AICAR provoked approximately 2.5-fold increases in [3H]2-deoxyglucose uptake in isolated extensor digitorum longus (EDL) muscles of wild type mice, this effect was diminished by 70-80% in EDL muscles of muscle-specific PKC-λ knockout mice (Fig 9b). These findings with AICAR are similar to those previously reported in studies of insulin-stimulated [3H]2-deoxyglucose uptake, i.e., 2-3-fold increases in wild type muscle, and marked impairment in muscles of muscle-specific PKC-λ knockout mice (7).

Effects of Treatment in vivo with AICAR and Metformin on Glucose Transport, Glut4/1 Glucose Transporter Translocation and aPKC Activity in Mouse Muscle. Acute treatment in vivo over 30 min with AICAR (250mg/kg body weight) or metformin (250mg/kg body weight) provoked increases in 2-deoxyglucose/glucose uptake (Fig 10a), Glut4 and Glut1 translocation (Fig 9c) and aPKC activity (Fig 10c) in hind limb muscles of wild type mice. However, in marked contrast, treatments with AICAR and metformin failed to significantly stimulate aPKC activity (Fig 10c) and 2-deoxyglucose/glucose uptake (Fig 10a) in muscles of muscle-specific PKC-λ knockout mice. Effects of AICAR and Metformin on AMPK and ERK Activation in Vivo. In contrast to the impairment in activating aPKC and glucose transport in muscles of muscle-specific PKC-λ knockout mice in response to treatment with AICAR and metformin, there was no impairment in either the phosphorylation of thr-172-AMPK (Fig 10b) or the enzymatic activation of AMPK (Fig 10d), in muscles of these knockout mice. Similarly, increases in the phosphorylation of ERK following AICAR and metformin treatments in these muscles were not diminished by depletion of aPKC (Fig 10e). Thus, the activation of skeletal muscle AMPK and ERK by these agents appeared to be independent of aPKC.

D. Studies of Treadmill Exercise in Wild Type and Muscle-Specific PKC-λ Knockout Mice.

It was previously shown that treadmill exercise provokes increases in aPKC activity in mouse muscle (5). Here, we found that comparable treadmill exercise provoked approx 2-3-fold increases in glucose uptake (Fig 11a) and AMPK activity (Fig 11c), as well as approx 2-fold increases in aPKC activity (Fig 11b) in muscles of wild type mice. In muscle-specific PKC-λ knockout mice, however, despite a nearly complete loss of ability to increase aPKC activity (Fig 11b), treadmill exercise continued to provoke increases in glucose transport (Fig 11a) and AMPK activity (Fig 11c) that were comparable in magnitude to those observed in wild type littermates.
The failure of aPKC depletion in muscle-specific PKC-λ knockout mice to diminish effects of exercise on glucose transport contrasts sharply with the inhibition of glucose transport effects of AICAR and metformin in muscle-specific PKC-λ knockout mice. On the other hand, the failure to find a requirement for aPKC during exercise-induced increases in AMPK phosphorylation and activity in muscles of muscle-specific PKC-λ knockout mice is similar to a comparable failure observed during AICAR and metformin treatment (cf Figs 10c and 10d). Thus, with each of the agonists presently used, AMPK activation in muscle was not dependent on aPKC.

Discussion

The present studies addressed several issues surrounding the mechanism of action of AMPK activators, including: first, whether chemical agents that activate AMPK and aPKC in muscle, such as AICAR and metformin, activate aPKC through AMPK activation, or oppositely, whether AMPK is activated through aPKC; and second, whether aPKC is required for glucose transport effects of these AMPK activators. Germane to the first question, findings in both L6 myotubes and muscle-specific PKC-λ knockout mice demonstrated that AICAR and metformin activate aPKC by a mechanism that is dependent on AMPK activation, and, conversely, AMPK activation by these agents is independent of aPKC. With respect to the second question, findings in both L6 myotubes and muscle-specific PKC-λ knockout mice demonstrated that aPKC is required for glucose transport effects of both AICAR and metformin. Taken together, these findings show that AICAR and metformin stimulate glucose transport in muscle through sequential activation of AMPK and aPKC. This conclusion takes on added significance as metformin therapy in humans provokes increases in AMPK and aPKC activities in vastus lateralis muscle that accompany increases in rates of whole body glucose disposal in euglycemic hyperinsulinemic clamp studies (16).

The present studies also addressed the question of whether exercise, which activates aPKC as well as AMPK in rodent (5) and human (3) muscle, requires aPKC for induction of increases in either AMPK activity or glucose transport. In this regard, it was particularly interesting to find in muscle-specific PKC-λ knockout mice that effects of treadmill exercise on muscle glucose transport and AMPK phosphorylation/activation were fully or largely intact, despite nearly complete loss of ability to provoke increases in muscle aPKC activity. It therefore seems clear that exercise-induced increases in both AMPK activity and glucose transport occur independently of aPKC. On the other hand, our studies leave open the question of whether exercise activates muscle aPKC by a mechanism that is dependent on AMPK; based upon our findings in studies of AICAR and metformin action, this possibility seems likely, but more definitive studies are needed.

The failure to find a requirement for aPKC during exercise-stimulated glucose uptake provided a sharp contrast for comparable studies of AICAR and metformin action wherein a requirement for aPKC was apparent from studies in muscle-specific PKC-λ knockout mice. Possible reasons for differences in aPKC requirements include: exercise, independently of AMPK, activates an aPKC pool that is not coupled to glucose transport; exercise, like AICAR and metformin, activates aPKC through AMPK, but coupling of AMPK-activated aPKC to glucose transport requires factors that are operative during actions of AICAR and metformin, but not exercise; and, perhaps most plausible, exercise, like AICAR and metformin, activates aPKC through AMPK, but this signaling mechanism is redundant for increasing glucose transport during exercise. In this regard, it is relevant to note that AMPKα2 is required for effects of AICAR-induced, but not electrical contraction-induced, increases in glucose transport in muscle (10,14). Thus, AMPK-induced increases in aPKC may serve as a primary mechanism for increasing glucose transport during simple AMPK activation, but these increases in aPKC activity may be but one amongst two or more exercise-stimulated signaling mechanisms that are capable of activating glucose transport.

Whereas prolonged metformin treatment had stronger effects on glucose transport than more acute AICAR treatment in L6 myotubes, effects of metformin and AICAR on AMPK activity were similar. It may therefore be questioned if the prolonged effects of metformin on glucose transport in L6 myotubes are mediated by AMPK-independent, as well as AMPK-dependent,
mechanisms, or if the prolonged activation of AMPK during 16-hr metformin treatment elicited increases in factors (? by gene expression) that favorably modulate glucose transport. Further studies are needed to answer these questions.

Although elicitation of full effects of metformin on glucose transport in L6 myotubes requires a relatively long treatment period (13), acute metformin treatment of both rats and mice in vivo for 30-60 min provoked increases in muscle aPKC activity and glucose uptake comparable to those seen with acute treatment with either AICAR or insulin (present results and ref 7). Thus, like AICAR and insulin, metformin can function as a rapid and potent activator of aPKC and glucose transport in muscles of intact rodents. However, here again, whether more prolonged treatment with metformin, or, for that matter, AICAR, in intact rodents provokes further increases in glucose transport beyond those seen in the acute studies, and the underlying mechanisms for such delayed increases, remain for future investigation.

In keeping with our previous report indicating that AICAR-induced activation of aPKC in L6 myotubes requires activation of the ERK pathway (5), we found that ERK activation by both AICAR and metformin is dependent on AMPK, and, furthermore, operation of the ERK pathway is required for AICAR- and metformin-induced increases in aPKC activity and glucose transport in L6 myotubes. Nevertheless, it remains uncertain how AMPK may activate the ERK pathway. In this regard, the non-receptor tyrosine kinase, PYK2, is activated by increases in intracellular Ca++ or activation of other non-receptor tyrosine kinases (1,4,15,21), and PYK2 activates ERK and other MAP kinases that activate PLD and thereby generate the acidic phospholipid, PA, which directly activates aPKC (5,21). In this regard, it is interesting that ERK activation is seen during AICAR (6) and metformin (unpublished observations – Luna V, Sajan MP, Farese RV) action in human muscle, but it is not clear if AMPK mediates these activations of ERK (see 6).

Similar to findings in studies of hypoxia-induced AMPK activation in rat alveolar epithelial cells (11), we found that the activation of PKC-ζ by AICAR and metformin in L6 myotubes is attended by an increase in phosphorylation of the activation loop site in PKC-ζ, viz., thr-410. However, our findings further suggested that, as with insulin, the phosphorylation of thr-410 is mediated by PDK1 during actions of AICAR and metformin in intact L6 myotubes. Moreover, in incubations of recombinant enzymes, we found that PDK1, but not constitutively active AMPK, provoked significant increases in both thr-410 phosphorylation and enzyme activity of PKC-ζ. Further studies are needed to define the specific amino acid residues in PKC-ζ that are directly phosphorylated by AMPK, and to determine if PDK1-induced thr-410 phosphorylation leads to an increase in phosphorylation of the auto(trans)phosphorylation site, i.e., thr-560, and/or other residues in PKC-ζ.

Although there are still many remaining uncertainties in how AMPK activators, such as AICAR and metformin activate aPKCs, we believe that AICAR/metformin-dependent aPKC activation is mechanistically similar to insulin-dependent aPKC activation, particularly in the later activation steps. Thus, as portrayed in Fig 12, in the case of insulin, it is most likely that the acidic phospholipid, PIP₃, as generated by PI3K action, binds to still uncertain basic residues in or near the pseudosubstrate site in the regulatory domain, thereby causing a dissociation of the pseudosubstrate site from the substrate-binding site, opening of the molecule, increased access of PDK1 to the activation loop site (at thr-410 in PKC-ζ), and auto(trans)phosphorylation (at thr-560 in PKC-ζ). In the case of AMPK activators, it seems clear that MEK1-dependent activation of ERK is required for all aspects of aPKC activation, and our previous finding indicating a requirement for PLD during AICAR action (5) leads us to believe that PA, as generated by PLD operating downstream of ERK, acts analogously to PIP₃ in activating aPKC.

Importantly, despite activating AMPK in both muscle and liver, metformin and AICAR activated aPKC in muscle, but not in the liver, of intact rats. [We have similarly observed that AICAR activates AMPK, but not aPKC, in liver and isolated adipocytes of mice – data not shown.] The reason for tissue-specific differences in aPKC activation during AMPK activation is uncertain. Nevertheless, this fortuitous difference is in all probability relevant to the clinical usefulness of metformin, and potentially of AICAR, as therapeutic agents, since aPKC activation in liver would be expected to increase SREBP-1c expression (17,22,24) and hepatic lipid synthesis, and thereby promote tendencies to hepatosteatosis, hypertriglyceridemia, obesity, and insulin resistance. In this regard, metformin is known to diminish SREBP-1c expression, and, we
Similarly found that 60-min metformin treatment diminished elevated hepatic SREBP-1c mRNA levels by 50-60% in type 2 diabetic Goto-Kakizaki rats (data not shown). Also note that selective inhibition of hepatic aPKC by administration of adenovirus encoding kinase-inactive aPKC elicits marked improvements in hepatosteatosis, hypertriglyceridemia and insulin resistance in murine obesity models (22).

Taken together, our findings show that, in skeletal muscle, AICAR and metformin activate aPKC via AMPK, ERK, and PDK1, and that aPKC is required for subsequent increases in glucose transport. Moreover, the ability of metformin and AICAR to activate aPKC selectively in muscle and thereby elicit increases in aPKC-dependent glucose transport, coupled with the ability to activate AMPK but not aPKC in liver, appear to be important determinants of the clinical usefulness of these and perhaps other AMPK activators for treating insulin-resistant forms of obesity and diabetes.

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References


**Figure Legends**

1. Effects of insulin (INS), AICAR and metformin (MET) on [³H]2-deoxyglucose (DOG) uptake (Panel a), and translocation of Glut4 (Panels b and d) and Glut1 (Panels c and e) glucose transporters to the plasma membrane in L6 myotubes. Myotubes were incubated with 2mM metformin for 16 hours, and finally incubated in glucose-free KRP medium with or without 2mM metformin for 40 min, 50μM AICAR for 40 min, or 100nM insulin for 30 min, prior to measuring [³H]2-DOG uptake and isolating plasma membranes for subsequent Western analyses of immunoreactive Glut4 and Glut1 levels. Values are mean ± SE of (N) determinations. Representative immunoblots of plasma membrane glucose transporter levels are also shown. P values indicate levels of significance of differences between treatment and control (CON) groups.

2. Dose-dependent effects of adenovirally-mediated expression of dominant negative (DN) AMPKα2 on AICAR-stimulated and metformin-stimulated AMPK activity (Panel a), aPKC enzyme activity (Panel b), phosphorylation of thr-410-PKC-ζ (Panel b) and [³H]2-deoxyglucose (DOG) uptake (Panel c) in L6 myotubes. Myotubes were incubated for 48 hours with indicated MOI of adenovirus encoding DN AMPKα2 (note increases in expression of total AMPK with increasing MOI) or adenovirus vector (total adenovirus was kept constant at 200 MOI by varying the amount of vector), and then, where indicated, incubated with 2mM metformin for 16 hours, and finally incubated in glucose-free KRP medium with or without 50μM AICAR or 2mM metformin for 40 min or insulin (100nM) for 30 min, prior to measuring AMPK activity, aPKC activity, p-thr-410-PKC-ζ immunoactivity, and [³H]2-DOG uptake. Values are mean ± SE of (N) determinations. Asterisk (*) indicates P<0.05 for DN AMPKα2-inhibited group versus the uninhibited (zero) group in AICAR and metformin series. Inset in Panel a shows increases in levels of total AMPK upon expression of DN AMPKα2 in various groups. Insets in Panel b show levels of immunoreactivity of total aPKC and p-thr-410-PKC-ζ. Inset in Panel c shows lack of effect of expression of DN AMPKα2 on insulin-stimulated [³H]2-DOG uptake.

3. Effects of adenovirally-mediated expression of wild type (WT) AMPKα2 on AICAR- and metformin-stimulated AMPK activity (Panel a), aPKC activity (Panel b) and [³H]2-deoxyglucose (DOG) uptake (Panel c) in L6 myotubes. Myotubes were incubated for 48 hours with 200 MOI of adenovirus vector or adenovirus encoding WT AMPKα2, and, where indicated, with 2mM metformin for 16 hours, and finally incubated in glucose-free KRP medium with or without 50μM AICAR or 2mM metformin for 40 min, prior to measuring AMPK activity, aPKC activity, and [³H]2-DOG uptake.
DOG uptake. Values are means \( \pm \) SE of (N) determinations. Asterisks indicates P<0.05 (*) and P<0.001 (**) for the WT AMPKα2 group versus the vector-treated group in AICAR and metformin series. Inset in Panel a shows increases in total AMPK levels upon expression of 200 MOI adenovirus encoding AMPKα2 in various groups. Inset in Panel b shows lack of change in total aPKC levels with AICAR and metformin treatments, and upon expression of WT A total AMPK levels upon expression of 200 MOI adenovirus encoding AMPKα2 in various groups (however, there appear to slight shifts in mobility due to phosphorylations elicited by treatments).

4. Effects of adenovirally-mediated expression of kinase-inactive (KI) PKC-ζ (KIζ; Panels a and b) and small interfering RNAi targeting PKC-ζ (α-ζ) (Panels c and d) on insulin- and metformin-stimulated glucose transport (Panels a and c) and aPKC activity (Panels b and d) in L6 myotubes. Myotubes were incubated for 96 hours with 100nM scrambled (Scr) control RNAi or RNAi targeting PKC-ζ, (α-ζ), or for 48 hours with 10 MOI of adenovirus vector (Vec) or adenovirus encoding KIζ, and then, where indicated, treated with 2mM metformin for 16 hours, and finally incubated in glucose-free KRP medium with or without 2mM metformin for 40 min or 100nM insulin for 30 min, prior to measuring uptake of \( [^{3}H] \)-2-deoxyglucose (DOG) and aPKC activity. Values are mean \( \pm \) SE of (N) determinations. Inset in Panel b shows increases in total aPKC content upon expression of KI-PKζ-ζ. Inset in Panel d shows representative immunoblots that portray the depletion of total aPKC in cells treated with RNAi targeting PKC-ζ.

5. Increases in phosphorylation of thr-410-PKC-ζ following treatment with insulin, AICAR and metformin (Panel a), and effects of adenovirally-mediated expression of kinase-inactive (KI) PDK1 on insulin-, AICAR- and metformin-stimulated glucose transport (Panel b) and aPKC phosphorylation and activity (Panel c) in L6 myotubes. Myotubes were incubated for 48 hours with 20 MOI of adenovirus vector (Vec) or adenovirus encoding KI-PDK1, and then, where indicated, treated with 2mM metformin for 16 hours, and finally incubated in glucose-free KRP medium with or without 2mM metformin or 50μM AICAR for 40 min, or 100nM insulin for 30 min, prior to measuring uptake of \( [^{3}H] \)-2-deoxyglucose (DOG) and aPKC activity. Values are mean \( \pm \) SE of (N) determinations. Inset in Panel a shows representative increases in immunoreactive levels of phospho-thr-410-PKC-ζ upon treatment with insulin, AICAR and metformin. Inset in Panel b shows increases in total cellular PDK1 content upon expression of KI-PDK1. Insets in Panel c show representative immunoblots that portray levels of p-thr-410-PKC-ζ and p-thr-473-PKB/Akt in cells treated with adenovirus vector or adenovirus encoding KI-PDK1. P values show levels of significance between treated and control groups in Panel a, and between indicated groups in Panels b and c.

6. Effects of incubation of recombinant forms of PDK1 and constitutively active AMPK with recombinant PKC-ζ on aPKC activity (Panel a), phosphorylation of thr-410 in PKC-ζ (Panels b and c) and increases in \( ^{32}P_{O_{4}} \) incorporation into PKC-ζ (Panel c). Panel c also shows levels of immunoreactive p-thr-410-PKC-ζ, PDK1, AMPK and PKC-ζ in the reaction mixtures, which contained 10ng of the indicated enzymes and all components of the aPKC assay system (see Methods). Values are mean \( \pm \) SE of (N) determinations. P values show levels of significance between PDK1- and AMPK-treated versus the control group in which PKC-ζ was incubated alone.

7. Effects of MEK1 inhibitors, UO126 (UO and PD98050 (PD), the PI3K inhibitor, wortmannin (W), (Panels a,b and c) and dominant negative (DN) AMPKα2 (Panel d) on \( [^{3}H] \)-2-deoxyglucose (DOG) uptake (Panel a), aPKC phosphorylation and enzyme activation (Panel c), and ERK phosphorylation and enzyme activation (Panels c and d) in L6 myotubes. Where indicated, myotubes were incubated for 48 hours with 200 MOI adenovirus vector (Vec) or adenovirus encoding DN AMPKα2, then for 16 hours without or with 2mM metformin, then for 30 min without or with 25μM UO126, 50μM PD98050 or 100nM wortmannin, and then for 40 min without (Control) or with 2mM metformin or 50μM AICAR, following which \( [^{3}H] \)-2-(DOG) uptake and
phosphorylation and enzyme activities of aPKC and ERK were assessed. Values are mean ± SE of (N) determinations. Asterisks (*) indicate P<0.05 for inhibitor-treated vs uninhibited AICAR- and metformin-treated groups. Insets show representative immunoblots of phospho-ERK in Panels b and d, and phospho-thr-410-PKC-ζ in Panel c.

8. Effects of insulin (INS) metformin (MET) and AICAR on phosphorylation of thr-172 in AMPK (Panels a and b), and enzyme activities of AMPK (Panels c and d) and aPKC (Panels e and f) in rat muscle (left) and liver (right). Rats were treated subcutaneously with 0.9% saline vehicle (CON), or insulin (1U/kg body weight) for 30 min, or metformin (250mg/kg body weight) or AICAR (1g/kg body weight) for 60 min. Values are mean ± SE of (N) determinations. P values indicate levels of significance between treatment and control (CON) groups.

9. Effects of muscle-specific knockout of PKC-λ (MλKO) in mice on AICAR-stimulated glucose disposal in vivo (AICAR tolerance test; Panel a) and [3H]2-deoxyglucose (DOG) uptake in vitro in isolated extensor digitorum longus muscle (EDL) (Panel b). AICAR tolerance tests were conducted in 6-hr-fasted mice with 10 males and 5 females in each group; initial blood glucose levels were 133 ± 4 and 134 ± 4 (mean ± SE; N = 15) in wild type and knockout mice, respectively. EDL studies were conducted with muscles of fed mice with 6 males and 5 females in each group. Result of males and females in both studies were indistinguishable and therefore pooled. Inset in Panel b shows total aPKC levels in EDL muscles of wild type (WT) and knockout (KO) mice treated with (+) or without (-) AICAR. Asterisks in Panels a and b indicate: *, P<0.05; and ***,P<0.001 for comparison of indicated functions of MλKO and WT groups. Shown in Panel c are representative immunoblots and levels of plasma membrane immunoreactive Glut4 and Glut1 glucose transporters in gastrocnemius muscles of wild type treated with 0.9% saline (CON), 250mg/kg body weight AICAR, or 250mg/kg body weight metformin (MET) 30 min before killing. Values indicate mean ± SE of (N) determinations. P values in Panel c indicate levels of significance of differences between treatment and control (CON) groups.

10. Effects of muscle-specific knockout of PKC-λ (MλKO) on AICAR- and metformin-induced increases in glucose uptake (Panel a), aPKC activity (Panel c), AMPK phosphorylation (Panel b), AMPK enzyme activity (Panel d) and phospho-ERK (Panel d) in vastus lateralis muscle. Wild type (WT) and MλKO mice were injected intraperitoneally with tracer amounts of labeled D-2-deoxyglucose/L-glucose (see Methods) in 0.9% saline vehicle, without (O) or with 250mg/kg body weight AICAR (A) or 250mg/kg body weight metformin (M) 30 min before killing. Values are mean ± SE of (N) determinations. Asterisks indicate: *, P<0.05; and **, P<0.01 for comparisons of AICAR or metformin (A or M) treatment groups versus the control (O) group. Insets in Panels b and c show representative immunoblots of p-thr-172-AMPK and total aPKC, respectively, in WT and MλKO muscles.

11. Effects of muscle-specific knockout of PKC-λ (MλKO) on treadmill exercise-induced increases in glucose uptake (Panel a), aPKC activity (Panel b) and AMPK activity (Panel c) in vastus lateralis muscle. Mice were subjected to continuous treadmill exercise for 20 min, during which glucose uptake was measured as described in Methods. Values are mean ± SE of (N) determinations. Asterisks indicate: *, P<0.05; **, P<0.01; and ***, P<0.001, for comparisons of exercise and control/non-exercising groups. Representative blots of total aPKC and p-thr-172-AMPK are shown in insets of Panels b and c, respectively.

12. Activation of PKC-ζ in L6 Myotubes by AICAR and Insulin. Increases in acidic phospholipids, PIP3 (phosphatidylinositol-(PO4)3) via Phosphatidylinositol 3-kinase (PI3K) action and phosphatidic acid (PA) via phospholipase D (PLD) action are thought to bind to basic residues in or near the pseudosubstrate (PS) site in the regulatory domain and thereby open the major cleft and facilitate PDK1 access to the activation loop site, thr-410, and subsequent auto(trans)phosphorylation at thr-560, as well as allowing substrate access to the catalytic site.
All alterations are needed to fully activate PKC-z. Note that AICAR is converted to ZMP, an analogue of 5'-AMP, and metformin, like AICAR, activates AMPK by an as-of-yet uncertain mechanism. See text for discussion of factors that couple AMPK to ERK (X1) and ERK to PLD (X2).
**Figure 1**

**Panel a:** Bar graph showing [3H]2-DOG uptake (cpm/mg protein) for Control, Insulin, AICAR, and Metformin. The bars indicate statistical significance with P-values: P<0.001, P<0.05.

**Panel b:** Bar graph showing Plasma Membrane Glut4 and Glut1 (relative values) for Control (CON), Insulin (INS), and Metformin (MET). The bars indicate statistical significance with P-values: P<0.001.

**Panel c:** Bar graph showing Glut1 (relative values) for CON, INS, and MET. The bars indicate statistical significance with P-values: P<0.001, P<0.001.

**Panel d:** Bar graph showing Glut4 (relative values) for CON, AICAR, and MET. The bars indicate statistical significance with P-values: P<0.001, P<0.001.

**Panel e:** Bar graph showing Glut1 (relative values) for CON, AICAR, and MET. The bars indicate statistical significance with P-values: P<0.011, P<0.05.
Figure 2
Figure 3

**Figure Description:**

**Panel a:**
- **Total AMPK Activity**
- Various groups are compared, each labeled with AMPK activity values per mg protein (cpm/immunoprecipitate).
- Statistical significance is indicated by asterisks (*).

**Panel b:**
- **Total aPKC Activity**
- Similar to Panel a, comparing different groups with aPKC activity levels.
- Statistical significance marked with asterisks (**and ***).

**Panel c:**
- **[3H]2-DOG Uptake**
- Groups include control, AICAR, and Metformin treatments.
- Statistical significance noted with an asterisk (*).

**Legend:**
- **Adenovirus Agonist**
- **Vec-tor** and **WT AMPK**
- **Control**, **AICAR**, and **Metformin** treatments.
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9

(a) AICAR Tolerance Test

Serum Glucose, % Basal

WT

MλKO

Minutes 0 15 30 45 60

(N=15)

(b) Extensor Digitorum Longus


(cpm x 10^3/mg protein)

WT KO WT KO

(11) (11) (11) (11)

(c) Gastrocnemius Muscle

Plasma Membrane Glut4 and Glut1

(relative values)

Treatment CON AICAR MET

Glut4

Glut1

P<0.001

P<0.005

P<0.01

P<0.001

Figure 9
Figure 10
Figure 11